Tissue Targets, Molecular Mechanisms and Health Effects of Bisphenolic Chemicals in Zebrafish

Submitted by
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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.

..... (John Moreman)

ABSTRACT

Bisphenol A (BPA) is a chemical incorporated in plastics and resins used for food and beverage containers that has been shown to have estrogenic activity. The fact that BPA possess this activity should not be surprising as it was originally explored for use as a pharmaceutical estrogen. Exposure to BPA has been associated with adverse reproductive and developmental effects in wildlife and laboratory animal models. There are also associations between exposure in humans and adverse health effects, although some of these findings are controversial. The mechanism(s) of action of BPA are well researched, however there is no definitive explanation for the frequently reported discrepancies between *in vitro* and *in vivo* studies. Metabolic activation of BPA *in vivo* has been suggested as a possible reason for this discrepancy in estrogenic potency. As public awareness of the possible health effects of BPA increases manufacturers have increasingly started to use replacement chemicals as monomers in materials that can be labelled as BPA-free. However there is still little information on the estrogenic potency of these structurally similar bisphenol chemicals or how they may affect health outcomes, as observed with BPA. The studies conducted in this thesis therefore aimed to investigate the tissue targets, molecular mechanisms and health effects of BPA, its related chemicals Bisphenol S (BPS), Bisphenol F (BPF) and Bisphenol AF (BPAF) and the BPA metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP). For this work, a novel ERE transgenic (ERE-TG) zebrafish, that expresses green fluorescent protein (GFP) in response to activation of ERE was employed. These fish can be applied to identify body targets of environmental estrogens in real time with high sensitivity and specificity.

BPA, BPF, BPS and BPAF were shown to all preferentially target the heart in ERE-TG zebrafish and GFP induction occurred first in the heart out of the different responding body tissues. The response to BPA was shown to be dependent on the classical estrogen receptor (ER) signalling pathway. However concentrations necessary to induce this response varied for the different bisphenols, with the rank order of potency of BPAF>BPA=BPF>BPS. Bioconcentration factors of the bisphenols were 4.5, 17.8, 5.3 and 0.067 for exposures to 1000 µg BPA/l, 1000 µg BPF/l, 100 µg BPAF/l and 50000 µg BPS/l respectively. These data indicate bioavailability is an important consideration in

the differing estrogenic potencies of the different bisphenols. The toxicities of the different bisphenols on early life stage zebrafish followed a similar rank potency order as for the estrogenic activity (BPAF>BPA>BPF>BPS). Specific morphological abnormalities were observed for the different bisphenolic chemical treatments in the toxicity assessments, possibly suggesting that they may act through different ways in inducing their toxic effects. It is recognised that the toxicities for the bisphenolic chemicals were observed at concentrations several orders of magnitude higher than those measured in most aquatic environments and thus the threat they pose to wildlife health might be considered as relatively low, except in circumstances where short but high exposures may occur from accidental release into the environment.

The BPA metabolite MBP was found to be up to 1000-fold more potent than the parent compound as an estrogen in ERE-TG fish. The heart was a key target tissue for MBP, as observed for the other bisphenolic compounds. The atrioventricular valves and bulbus arteriosus were identified as the primary targets within the heart. MBP was not measured in zebrafish embryos exposed to BPA and whether this is produced as a metabolite in zebrafish is still not known. Morpholino knockdown of specific ER subtypes indicated that esr1 is a major pathway for the estrogenic response to BPA in the heart during early life stages of zebrafish. Video capture and analysis was used to assess the cardiovascular health of zebrafish exposed to BPA and it was found that at very high exposure concentrations (2500 μg/l) BPA could induce an unstable atrial:ventricular beat ration in 5 dpf larvae and reduced heart beat rate in 14 dpf.

In the final study of this thesis transcriptomic profiling was conducted on hearts extracted from 96 hpf ERE-TG zebrafish larvae exposed to BPA. The findings demonstrated that BPA, at an exposure concentration of 150 μg/l caused a down-regulation of a number of genes associated with ion transport and cell-to-cell communication, functions that are essential in maintaining a regular and consistent heart rate. These effect mechanisms may help to explain the effects on the heart seen at the higher BPA exposure concentrations in the previous chapter, although this would need more extensive work to draw any such associations with good confidence

Overall, the findings presented in this thesis have provided a body of evidence to show that all of the bisphenolic chemicals tested possess estrogenic activity and as such have the potential for health effects in wildlife and also to humans. It is also the case however that currently in most ambient environments concentrations of these bisphenolic chemicals are far below those that could induce adverse health outcomes. The work in this thesis re-enforces the importance of understanding metabolic activation of chemicals *in vivo*. It furthermore illustrates the power of transgenic fish and an integrated approach for gaining greater insight into potential health effects of chemicals.

ACKNOWLEDGEMENTS

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LIST OF GENERAL FIGURES

RESEARCH PAPERS AND AUTHORS DECLARATION

Research Paper 1. John Moreman, Okhyun Lee, Maciej Trznadel, Arthur David, Elizabeth M. Hill, Tetsuhiro Kudoh and Charles R. Tyler (2016) Toxicity, Teratogenic, and Estrogenic Effects of Bisphenol A, and its Alternative Replacements Bisphenol S, Bisphenol F and Bisphenol AF, in Zebrafish. *Manuscript in preparation.*

Research Paper 2. John Moreman, Aya Takesono, Maciej Trznadel, Matthew J. Winter, Mark E. Wood, Tetsuhiro Kudoh and Charles R. Tyler (2016) Estrogenic mechanisms and effects of Bisphenol A and its metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) on the heart in zebrafish. *Manuscript in preparation.*

Research Paper 3. John Moreman, Tetsuhiro Kudoh and Charles R. Tyler (2016) Transcriptomic responses in the hearts of larval zebrafish in response to Bisphenol A exposure**.**

Statement: I, John Moreman made the following contributions to the papers presented in this thesis:

Paper 1: I planned and carried out all exposures, image capture and analysis and prepared all water samples for chemical analysis. I also wrote the manuscript. Concentrations of chemicals in test media were performed by Arthur David under the supervision of Elizabeth Hill at the University of Sussex. Uptake measurements were undertaken by Maciej Trznadel at the University of Exeter.

Paper 2: I planned and carried out all exposures, image capture and analysis. I performed all heart dissections, immunostaining and confocal microscopy for heart imaging. I performed all microinjection experiments using morpholinos to knock down estrogen receptor signalling, using morpholinos previously designed by Aya Takesono. I also performed all cardiovascular function analysis under the guidance of Matthew Winter. Uptake of chemical was performed by Maciej Trznadel. Mark Wood was responsible for the manufacture and purity checking of the MBP used in this paper. I wrote the manuscript.

Paper 3. I carried out all exposure work, image capture and analysis. I performed all steps in the heart removal procedure and in the subsequent RNA extractions.

Library preparation was carried out by the Exeter Sequencing Service of the first sequencing experiment in the paper, but I carried out all aspects of library preparation in the second sequencing experiment. Exeter Sequencing Service were responsible for performing the sequencing run on the Illumina HiSeq2500. I conducted all the bioinformatics analysis and wrote the paper.

Charles Tyler and Tetsuhiro Kudoh contributed to the study design on all research papers. Charles Tyler also contributed input into the editing of papers.

LIST OF GENERAL ABBREVIATIONS

ANOVA	Analysis of Variance
AR	Androgen receptor
BADGE	Bisphenol A diglycidy ether
BMI	Body Mass Index
BPA	Bisphenol A
BPAF	Bisphenol AF
BPAP	Bisphenol AP
BPB	Bisphenol B
BPF	Bisphenol F
BPP	Bisphenol P
BPS	Bisphenol S
BPZ	Bisphenol Z
BW	Bodyweight
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CV	Cardiovascular
DES	Diethylstilbestrol
DNA	Deoxyribose nucleic acid
dpf	Days post fertilisation
E1	Estrone
E2	17β-estradiol
E3	Estriol
EDC	Endocrine disrupting chemical
EE2	17α-ethinylestradiol
ER	Estrogen receptor
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinases
ERR	Estrogen related receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
GFP	Green Fluorescent Protein
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology

GPER	G protein-coupled estrogen receptor
GPR30	G protein-coupled receptor 30
hpf	Hours post fertilisation
HPLC	High pressure liquid chromatography
IVF	*In vitro* fertilisation
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LBD	Ligand binding domain
LOQ	Limit of quantitation
LC-MS	Liquid chromatography – Mass spectrometry
MBP	Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene
mRNA	Messenger RNA
NHANES	National Health and Nutrition Examination Survey
PCOS	Polycystic Ovarian Syndrome
PES	Polyethersulfone
PND	Postnatal day
ppt	Parts per thousand
RBA	Relative binding affinity
RNA	Ribose nucleic acid
SEM	Standard error of the mean
T3	triiodothyronine
T4	Thyroxine
TH	Thyroid Hormone
TR	Thyroid Receptor
UAS	Upstream activating sequence
VTG	Vitellogenin
WWTW	Waste water treatment works
YES	Yeast estrogen screen
ZRP	Zona radiata protein
µM	Micromolar

List of species Latin names

Aerobic bacteria	*Sphinogomonas sp.*
African clawed frog	*Xenopus laevis*
Asiatic grass frog	*Rana chensinensis*
Atlantic cod	*Gadus morhua*
Atlantic salmon	*Salmo salar*
Australian rainbowfish	*Melanotaenia fluviatilis*
Broad-snouted caiman	*Caiman latirostris*
Chicken	*Gallus gallus*
Common frog	*Rana temporaria*
European seabass	*Dicentrarchus labrax*
Fathead minnow	*Pimephales promelas*
Goldfish	*Carassius auratus*
Japanese quail	*Coturnix japonica*
Medaka	*Oryzias latipes*
Mummichog	*Fundulus heteroclitus*
Oriental fire-bellied toad	*Bombina orientalis*
Pond slider	*Trachemys scripta*
Rare minnow	*Gobiocypris rarus*
Soil bacteria	*Sphingobium fuliginis*
Tropical clawed frog	*Silurana tropicalis*
Turbot	*Psetta maxima*
Water flea	*Daphnia magna*
Wrinkled frog	*Rana rugosa*
Yellow perch	*Perca flavescens*
Zebrafish	*Danio rerio*

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: General Introduction

1.1 The endocrine system and estrogens

The endocrine system is a major signalling system in biological organisms that employs hormones to regulate and control the physiological processes of reproduction, growth, development and metabolism. In vertebrates the major tissues of the endocrine system include the various glands including the hypothalamus, pituitary, thyroid and adrenal glands and the gonads. The hormones produced by cells in these tissues are chemical messengers carried through the body via the circulatory system at very low concentrations, yet have the potential to elicit dramatic responses in target tissues. These effects are mediated through the binding of receptors in target cells, which may be present in the cell membrane or cytosol and these ligand receptor interactions in turn may induce changes by regulation of gene transcription. Nuclear receptors are a family of receptors that include the estrogen receptor (ER), and are ligand-activated regulators that directly interact with and control the expression of genomic DNA via specific response elements upstream of their target genes. In the case of steroid hormones, which are relatively small and lipid soluble allowing free diffusion into cells, hormone-receptor complexes form in the cytosol, dimerise with a second complex and other expressed co-factors and move into the nucleus. Here they bind DNA and stimulate the transcription of specific genes. In other cases, estrogens trigger their actions after binding to membrane estrogen receptors such as GPR30 (Revankar *et al.* 2005), or to ion channels (Valverde *et al.* 1999) capable of linking to intracellular signal transduction pathways that can generate rapid tissue responses (Hall *et al.* 2001).

Sex steroids are a group of steroid hormones that have traditionally been well documented for their effects on early sexual differentiation, regulation of gametogenesis and development of secondary sexual characteristics, and a wider range of pleiotropic effects. Traditionally the main sex steroids, estrogens and androgens, have been thought of as female and male respectively. However, it has been demonstrated that both play a role in growth, differentiation and homeostasis of reproductive organs in both sexes. Additional processes that depend on the actions of estrogens are varied and widespread, including functioning of the cardiovascular, central nervous, skeletal and immunological

systems. The three major natural occurring estrogens are estrone (E1), 17β-estradiol (E2) and estriol (E3), with E2 being the most prominent estrogen in terms of both levels detected and estrogenic activity.

1.2 Estrogen receptors

There are currently two ERs described in mammals, ERα and ERβ encoded by the genes *esr1* and *esr2* respectively. ERs can be found widely expressed in different tissue types, however distinct expression patterns have been shown in a range of vertebrate animals. High expression of ERα has been found in the uterus, mammary gland, placenta, central nervous system, cardiovascular system, bone and efferent ducts (Nilsson *et al.* 2001, Hess 2003). Whereas ERβ has been shown to be expressed at higher levels in the prostate, testis, ovary, pineal gland, thyroid gland, adrenals, gallbladder, skin, urinary tract, lymphoid tissues and muscle (Ciocca and Roig 1995, Taylor and Al-Azzawi 2000). ERs may bind as either homodimers or heterodimers to estrogen response elements (EREs) in the promoter region of responsive genes.

Both ERs are highly conserved (97% similarity) in their DNA-binding domains (DBDs), in mammals which is reflected in the similar affinities in binding to EREs (Klinge 2001). Although ligand binding domain (LBD) is less conserved (55% similarity) (Kuiper *et al.* 1998a) in mammals, E2 binds both ERs with similar affinity. However, this difference may account for observed differences in transcriptional responses to ligands in a promoter- and cell-dependent manner, indicating functional differences between the receptor subtypes.

In contrast with mammals there have been three receptor subtypes identified in many teleost fish (in some cases four have been identified), known as ERα, ERβ1 and ERβ2 encoded by the genes esr1, esr2b and esr2a respectively (Hawkins *et al.* 2000). Several studies have looked at the tissue distribution of ERs in different fish species, although results differ, common tissues expressing ERα and ERβ1 include the gonads and liver (Ma *et al.* 2000, Socorro *et al.* 2000, Menuet *et al.* 2002, Filby and Tyler 2005, Chandrasekar *et al.* 2010). Consistent with the role of the gonads in sexual development and the vital role the liver plays in the production of the proteins necessary for oocyte synthesis and maturation (including vitellogenin (VTG) and zona radiata protein (ZRP)). Other tissues that have been determined to express ERs in fish include the brain, heart, muscle,

neuromasts and pituitary, however frequency and subtype preference appears to differ between species (Socorro *et al.* 2000, Menuet *et al.* 2002, Choi and Habibi 2003, Tingaud-Sequeira *et al.* 2004, Filby and Tyler 2005). The ligand binding traits of different species appear to differ, which may help to explain the differences observed. Even within commonly used laboratory species both ER activation assays and VTG induction *in vivo* were found to differ considerably in responsiveness (Lange *et al.* 2012). Cloning of LBD into different ERs indicated the LBD plays a significant role in accounting for ligand sensitivity in different species (Miyagawa *et al.* 2014). Whole-mount *in situ* hybridization of ER genes in zebrafish found high levels of *esr2a* and *esr2b* mRNAs detected in the epidermis, pectoral fin buds, hatching gland and, to a lesser extent, the developing brain at 24 hours post fertilization (hpf), low levels of *esr1* were also detected (Tingaud-Sequeira *et al.* 2004). By 72 hpf all mature primary neuromasts in both the anterior and posterior lateral line systems express significant levels of *esr2a* and *esr2b* transcripts, whereas *esr1* remains weaker. In contrast (Gorelick *et al.* 2014) did not find ERs to be distributed in a similar pattern using *in situ* hybridization. At 120 hpf *esr2a* (reported as *esr2b*) is found expressed strongly in the liver whilst *esr1* is expressed specifically in the heart valves, *esr2b* (reported as *esr2a*) was not detected. The reasons for these differences are unclear as both studies were conducted at a similar life stage and found distinct expression patterns. It is unlikely that differences in methods or *in situ* probe synthesis would produce such differing results.

1.3 Disruption of Estrogen signalling

Exposure to both natural and anthropogenically derived chemicals is unavoidable for both humans and wildlife. A vast array of chemicals are produced and released into the environment and have been determined to have deleterious effects (reviewed in Kumar and Holt (2014) and Mathieu-Denoncourt *et al.* (2015). Due to the nature of the endocrine system, which relies on relatively small changes in expression of endogenous hormones to bring about sometimes large changes in physiology, it is possible that these chemicals, even at low concentrations may produce adverse developmental, reproductive and neurological effects. The chemicals capable of eliciting these changes are known as endocrine disrupting chemicals (EDCs), defined by the International Programme on Chemical Safety as "…*an exogenous substance or mixture that*

alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations".

A number of different chemicals have been described as endocrine disruptors including (anti)estrogens, (anti)androgens and thyroid disruptors. Chemicals now known to act as EDCs include natural steroidal estrogens (E2 and E1), phyto and myco estrogens (particularly genistein), synthetic steroidal estrogens (EE2 and DES), plasticisers (BPA and phalates), polyaromatic hydrocarbons (PAHs), androgens, surfactants (alkyl phenol ethoxylates, which break down into nonylphenol), dioxins and brominated flame retardants (reviewed in (Mills and Chichester 2005) and (Diamanti-Kandarakis *et al.* 2009)). These chemicals are diverse in structure and function and therefore can interact differently with the environment. For example, some have high bioconcentration factors and may result in high burdens even when exposure concentration is low, or others, such as BPA may have such ubiquitous exposure that effects may be evident despite having a low bioconcentration potential. Humans may be exposed to these chemicals through dietary or dermal absorption and inhalation from many everyday sources including medicines, detergents, flame retardants, plastic bottles, metal cans, pesticides and cosmetics. All of these substances may also enter the environment via waste water treatment works (WWTW) into rivers and estuaries or from agricultural runoff or landfill leachates. Our water bodies in particular tend to act as a sink for our chemical discharges.

A growing body of evidence from recent decades has provided a number of examples of reproductive and developmental abnormalities in a number of taxa as a result of exposure to EDCs (Tyler *et al.* 1998, Vos *et al.* 2000, Bernanke and Kohler 2009). Although the potency of different EDCs varies greatly, most tend to be weaker than natural hormones. Potency is directly related to both the affinity and efficacy of a typical ligand, where affinity refers to the interaction of the ligand and receptor and efficacy is measured by the relative ability to produce a downstream response. Whereas natural hormones tend to possess a high affinity and efficacy, some EDCs may have a high affinity but a low efficacy. Various other factors in cells, including receptor abundance and type can also have effects on efficacy complicating measurements. Nevertheless, many EDCs are found at high concentrations, are persistent or exist as complex mixtures which may have undescribed interactive effects, in regards to both affinity and efficacy.

It is generally accepted that these chemicals do pose some risk to humans and wildlife, though debate still exists over the potential to cause population level disruption at current exposure levels.

1.4 Mechanisms of disruption

Although it is possible for various EDCs to affect a number of cellular hormonal pathways, most research has investigated the interference of EDCs with nuclear receptors. Chemicals that bind receptors may do so in an agonistic way, stimulating a hormonal response. Others, though they may be structurally similar may not activate receptors when binding occurs, preventing the actions of endogenous hormones, therefore acting as an antagonist. Antagonists may appear to produce a similar phenotype as agonists, for example anti-androgenic chemicals and estrogenic chemicals have different modes of action but both result in apparent feminisation of fish (Filby *et al.* 2007). Chemicals that act as receptor agonists can have an additive effect, whereby low concentrations of various chemicals may elicit a larger effect than would be expect by individual chemical exposure (Rajapakse *et al.* 2001). EDCs can also act synergistically, increasing beyond an additive response, although it is unlikely that chemicals with the same mode of action will act this way (reviewed in Cedergreen (2014). Some EDCs have the ability to affect the metabolism or production of natural hormones either increasing or reducing existing levels, for example imidazole fungicides or organotins may modulate CYP19 activity post-transcriptionally disrupting estrogen biosynthesis and subsequently reproductive function (Cheshenko *et al.* 2008). These chemicals may exert their effects by interfering in other signalling systems, such as the thyroid, immune or nervous systems. Some chemicals may act through several different mechanisms, for example, BPA has been demonstrated to bind and activate ERs and estrogen related receptors (ERRs), act through non-genomic pathways, antagonise ARs and interact with thyroid receptors (Welshons *et al.* 2006). EDCs can also function more indirectly by interfering with receptor protein synthesis or degradation, modulating coactivators, affecting transport or excretion of hormones, interfering with feedback mechanisms or by disrupting endocrine tissues directly (Tabb and Blumberg 2006).

Of the vast array of EDCs, the most widely documented, due to effects and levels found in the environment are estrogenic. Chemicals that can mimic the effects of estrogen were identified more than 80 years ago (Dodds and Lawson 1936), however it wasn't for many decades that it was realised these chemicals could have widespread effects at the concentrations found in the environment. First alerted due to the observation of intersex (the presence of both male and female sex cells within the same gonad) in feminized male fish, this phenomenon has now been reported in various species. In more severely affected males a reduced capacity for fertilisation has been observed and adverse effects at the level of fish populations have also been suggested (Tyler and Jobling 2008, Hamilton *et al.* 2014). Although the effects of estrogens on the gonads in fish are well documented there is less research into the potential effects in other target tissues. As ERs are widely distributed and estrogens are known to have developmental and modulatory effects on many targets it is possible that these tissues are also targets for disruption.

This thesis largely concerns the effects and mechanisms of the environmental estrogen BPA and closely related chemicals, and the next sections of text provide a detailed background on these chemicals only, rather than on the wider known body of estrogenic chemicals.

1.5 Bisphenol A

BPA is a well-documented environmental estrogen. Largely employed in the production of plastics and epoxy resins, BPA based materials are versatile and widely used in a vast array of products. Originally synthesised in 1891 by Russian scientist A.P. Dianin by the condensation of acetone with two equivalents of phenol, BPA was not utilised until estrogenic activity was detected in 1936 by (Dodds and Lawson 1936). Dodds attempted to further develop BPA as a synthetic estrogen before abandoning it in favour of DES (Vogel 2009). It wasn't until the 1950s that the first epoxy resins were produced, shortly after US and Swiss scientists filed patents based on the production of a hard, clear plastic formed from the polymerisation of BPA called polycarbonate (Vogel 2009). Production of BPA has increased dramatically since the introduction of polycarbonate; world production capacity was approximately 1.1 million tonnes in the 1980s and more than 2.4 million tonnes in 2009. Over this period annual

production increased over five times. This occurred despite the fact that research in the 1990s identified BPA to be an endocrine disrupting chemical (EDC) (Krishnan *et al.* 1993, Steinmetz *et al.* 1997). Latest estimates (2012) on global production of BPA stands at 4.6 million tonnes with 70% being used in polycarbonates, 26% in epoxy resins and 4% in other applications (Ltd 2013).

Levels of BPA detected in media that has been in contact with polycarbonate plastics varies greatly, with differences often attributable to treatment conditions, including temperature and contact time. BPA may leach from plastics not only by diffusion of residual, unbound BPA remaining from the manufacturing process, but also by hydrolysis of the polymer (Geens *et al.* 2012). This causes observations in contrast to typical migration behaviour, whereby repeated use may see a reduction in leachate present; due to hydrolysis of the polycarbonate BPA migration has actually been observed to increase over time (Brede *et al.* 2003, Torres *et al.* 2014).

BPA leachate measured from polycarbonate baby bottles - a source of concern due to the vulnerability to EDCs during development – has been measured to be low under conditions which are most likely to occur under normal use (Biedermann-Brem *et al.* 2008, Simoneau *et al.* 2011). However after performing certain treatments based on changes in temperature pH, hardness and duration, levels of BPA were found to be much higher: (Biedermann-Brem and Grob 2009) detected BPA at 137µg/l in baby bottles that had only been sterilised for twice the recommended time, whilst (Cao and Corriveau 2008) found migration levels to range between 228 and 521 µg/l after being heated at 70 ^{o}C for 6 days (an unlikely scenario under normal use, however). BPA has also been detected in plastic bottles labelled as BPA free. In one study BPA was detected in 77% of polyamide bottles labelled BPA-free with levels measured ranging from 0.5 – 1005 µg/kg, much higher than some values found for polycarbonate plastic and over the European Union migration limit of 600 µg/kg (Simoneau *et al.* 2012). Although BPA has been banned by the European Union for use as an additive, or as a monomer in polycarbonate plastics, it is still possible that BPA can be used as a monomer in other materials such as polyamide. The authors carried out several confirmation studies including external testing which supported their previously observed results, concluding that the presence of BPA in this polymer is not only abnormal but illicit.

BPA Epoxy resins are the second most common use of BPA by weight. Although used in a variety of applications, it is their use in food contact materials that line food and drink cans or metal lids that is likely to be of most concern due to the potential dietary exposure to leached BPA; Goodson *et al.* (2004) determined that 80-100% of BPA present in the can coating migrated into the contents during the sterilisation process. The concentrations of BPA in the contents also remained stable across various temperature treatments for up to 9 months (Goodson *et al.* 2004).

Studies detecting the concentration of BPA in food from epoxy lined containers vary greatly. Even within the same study investigating the same contents, variation in internal concentration can be highly variable with an approximate 100-fold difference between peas (2.6-310 ng/g), refried beans (6.3-790 ng/g) (Noonan *et al.* 2011) and corn (1.2-8.2 ng/g) (Geens *et al.* 2010) . It is possible that some variation can be attributed to the handling of containers after processing. BPA concentration has been shown to be increased by can damage across several brands of tomato (Errico *et al.* 2014). However, it is possible that these inconsistencies are a result of different can styles or coatings which may differ by supplier. These differences are poorly investigated and not currently subject to regulation. There is evidence that indicates BPA has an affinity to partition into the solid portion of the contents, with concentrations in the solid phase measured to be 10-fold higher than the liquid phase (Yoshida *et al.* 2001, Geens *et al.* 2010, Noonan *et al.* 2011). It was suggested these results are due to surface adsorption or migration due to fat content and the lipophilic nature of BPA.

Although polycarbonate plastics are the most common use of BPA by weight the importance of epoxy lined food cans has been demonstrated as the dominant route of dietary exposure (Christensen and Lorber 2014, Lorber *et al.* 2015). Using data from Canada and the USA (Cao and Corriveau 2008, Noonan *et al.* 2011) (Christensen and Lorber 2014) estimates that average daily exposure to BPA via canned food in individuals under the age of 20 is 92.3 ng/kg-day, whereas exposure via non-canned food items is predicted to be just 0.3 ng/kg-day. A study investigating the effect of eating canned or fresh soup once a day for five days without restricting any other lifestyle factors found urinary BPA concentrations to be on average 22.5 µg/l higher when consuming canned food

(representing a 12-fold increase) (Carwile *et al.* 2011). However, it should be noted that in this study there was no attempt to measure the BPA concentration of the food consumed. Furthermore, with only one time point, it is likely to be a transient peak of unknown duration.

BPA is commonly found in the monomers used for resins in dental sealants. Although these resins do not strictly incorporate BPA itself as a component, it may be present as an impurity or through degradation of the resins themselves (Rathee *et al.* 2012). BPA has been detected in saliva of specimens collected an hour and three hours after sealant placement between 5.8 and 105.6 µg/l; however there was no measured increase of BPA serum concentrations up to five days after placement (Fung *et al.* 2000). Another study investigating salivary BPA in Korean children fitted with dental sealants found lower concentrations of salivary BPA (0.002 to 8.305 µg/l), though concentrations in treated individuals were still significantly higher than those without any sealant (Han *et al.* 2012). (Joskow *et al.* 2006) determined that the amount of BPA leached from dental sealant may depend on the sealant type, with one brand increasing salivary BPA significantly more than another. Although there do not appear to be any findings where BPA has been significantly elevated in the blood serum following sealant placement, one study observed an increase in urinary BPA in children with a number of sealants from the NHANES cohort. However upon adjusted analysis, this increase was not found to be significant (McKinney *et al.* 2014). Although it is possible that dental sealants may increase the load of BPA exposure, especially in the short term, it appears unlikely that it contributes significantly when compared to other sources.

BPA has also been detected in paper and paper products, particularly thermal papers where it is used as a colour developer. The ubiquitous use of thermal paper, for example in till receipts and card printers often results in daily contact where BPA may be absorbed dermally. Studies have reported high concentrations of BPA in thermal paper from cash registers, between 3 and 17 mg/g (Biedermann *et al.* 2010, Mendum *et al.* 2010). However, it is thought that actual dermal transfer may be relatively low, with one study estimating daily intake values of BPA through dermal absorption being 17.5 and 1300 ng/kg bw/day for the general population and occupationally exposed individuals respectively (Liao and Kannan 2011). Although much higher levels were

calculated by (Biedermann *et al.* 2010) they also concluded low levels of absorption, where even with exaggerated contact (repeated contact for 10hrs/day) estimated concentrations were still 42 times lower than the present recommended tolerable daily intake (71µg/day).

Although absorption may be argued to be low from thermal paper, the large amount produced may result in the release of BPA into other products or the environment. It has been estimated that approximately 30% of thermal paper enters recycling facilities, where it may potentially end up in other paper products, toilet roll in particular (Liao and Kannan 2011). (Fukazawa *et al.* 2001) investigated the presence of BPA in the final effluent of eight plants manufacturing paper from recycled sources and BPA was detected in all samples ranging at concentrations between 8 and 370 µg/l. Any paper products not recycled can be assumed to end up being released into the environment either via landfill or loose disposal. Using conservative estimates (Liao and Kannan 2011) calculated up to 30.4 tonnes of BPA could be released into the environment through discharge of thermal paper alone in the USA and Canada every year.

Despite the fact the BPA does not occur naturally its widespread and varied use and continuous release have led to it being ubiquitous in the environment. Release of the raw chemical can furthermore occur during the manufacture and subsequent transport and processing. Release from BPA containing products may then commonly occur via effluent discharge, landfill leachates, combustion and the environmental degradation of materials. In 2012 in the United States alone, total release of BPA was reported to be 745 metric tonnes (an increase of 238 tonnes from 2007). Total transfers off site or for further waste management, including incineration or wastewater treatment accounted for an additional 1129 metric tonnes (USEPA 2014). Similar release information is not available for Europe and Asia which hold larger market shares in overall production volume (North America accounts for 18% of the world BPA production volume (Ltd 2013), therefore global release is likely to be many times higher. Although there is limited information on the specific amounts of BPA released from production sites, several studies have attempted to quantify the levels of BPA in environmental samples. Due to the ubiquitous nature of BPA and its methods of release/disposal it has been commonly detected in aqueous, sediment and aerial samples.

BPA concentrations measured in surface waters vary greatly with regards to location, time of year and sampling method. In a review by (Flint *et al.* 2012) of 22 studies investing BPA concentrations in surface waters, 17 found maximum BPA concentrations to be 1µg/l or less, with minimum measurements at low ng/l concentrations, or not detected. However some studies have determined concentrations of BPA in environmental waters to be very high including measurements at 21, 19 and 12µg/l in the Netherlands, Japan and USA respectively (Flint *et al.* 2012). However, the Netherlands river where 21µg/l was detected was measured to be <0.011µg/l several months later (Belfroid *et al.* 2002). If there were no unreported errors in the methodology these values may be from single high discharges. Short term high exposures to BPA, particularly during critical stages of development can have lasting alterations, therefore these brief but high measurements should not be overlooked (Saili *et al.* 2012, Tse *et al.* 2013).

BPA detected in industrial or waste water treatment effluent displays a similar wide range of reported levels, with most measurements being lower than 1µg/l (Flint *et al.* 2012). However maximum concentrations have been found to be several orders of magnitude higher than those found in surface waters. A Japanese study found BPA present in paper-mill effluent at 370µg/l, with a mean of 59µg/l, and an Australian study found a mean concentration of 23µg/l in a waste water treatment effluent (Fukazawa *et al.* 2002, Al-Rifai *et al.* 2007). Similarly, BPA has been measured at very high concentrations in a small number of landfill leachates. In Japan, for example concentrations as high as 5,400 (Yamada *et al.* 1999) and 17,200 µg/l (Yamamoto *et al.* 2001) have been reported.

1.6 Observations on the effects of BPA in Mammals/humans

1.6.1 Male fertility and reproductive function

1.6.1.1 Mammalian models

Exposure to BPA has been reported to decrease sperm production and fertility in male mice and rats, possibly through the disruption of meiotic progression during spermatogenesis. (Liu *et al.* 2013) found that adult male rats dosed orally with 200µg/kg bodyweight (BW)/day had significantly reduced sperm counts,

associated with meiotic disruption. By using an estrogen receptor (ER) antagonist, it was also determined that ER signalling was responsible for mediating the BPA-induced reproductive impairment. However (Qiu *et al.* 2013) found that BPA induced spermatogenesis disorders in adult rats mainly through decreasing androgen receptor expression. This illustrates that the mechanisms by which BPA may be acting in the testis are likely complex and probably result from multi pathways effects. Estrogens can act to modulate androgen signalling without directly binding to androgen receptors (ARs). Another study also found that sperm production was reduced in rats orally exposed to BPA at concentrations of only 20µg/kg BW/day (Sakaue *et al.* 2001). (Tiwari and Vanage 2013) found significant reductions in sperm production at 10 µg/kg BW/day and an increase in sperm DNA damage and decreased motility, but only at a very high dose of 5.0 mg/kg BW/day. Studies in mice have resulted in similar findings, with oral exposure to BPA as low as 25 µg/kg BW/day causing a significant reduction in sperm counts and sperm production efficiency, which in turn was associated with a significant reduction in pregnancy rates (Al-Hiyasat *et al.* 2002). Quality and quantity of spermatozoa were decreased in mice orally exposed for 5 weeks, and this was associated with a slowing of meiotic progression of germ cells. Offspring of these mice were classed as abnormal as they were smaller at 35 days compared with controls (Zhang *et al.* 2013). Subcutaneous injection of 20 µg/kg BW/day BPA into adult rats and mice has been shown to cause abnormalities in spermatids, including deformations of acrosomes and nuclei after 6 days. 2 months after dosing cessation adverse effects were shown to be reversed (Toyama *et al.* 2004). (Chitra *et al.* 2003) Also found that orally dosing rats at between 0.2 and 20 µg/kg BW/day caused a reduction in epididymal sperm motility and sperm count in a dose-dependent manner.

Alterations in male reproductive tissue weights have also been recorded, notably reduction in prostate and seminal vesicle weights in mice and rats exposed to a high dose of BPA (20 mg/kg BW/day) (Takao *et al.* 1999, Takahashi and Oishi 2003). In contrast with these findings however in rats exposed to 20µg/kg BW/day BPA orally reductions in the testis and epididymis weights were observed, but there was no significant change in the weight of the seminal vesicles, prostate weight was reported to increase (Sakaue *et al.* 2001, Chitra *et al.* 2003). The concentration needed in this study to produce detectable effects was below the

current EPA exposure limit of 50 µg/kg BW/day, it was however above estimates of human exposure in the NHANES cohort, as estimated by urinary BPA concentrations (0.017-0.274 µg/kg BW/day) (Lakind & Naiman 2008, 2011). The differences observed between the different studies (decreasing/increasing prostate weights etc.) may be due to the very high dose inhibiting prostate growth. However, the lower dose (which reflects more accurately environmental exposures) is possibly more likely to indicate a hormonal response, which typically only requires concentrations at ppt.

1.6.1.2 Humans

There have been few human studies that have made associations between urinary BPA concentrations and sperm concentration/quality. Notably (Li *et al.* 2011) studied the effect of occupational exposure to BPA from males across four regions in China. Comparisons between these men and men who had not been exposed to BPA showed increasing urine BPA level was associated with many factors pertaining to sperm quality, namely decreased sperm concentration, decreased total sperm count, decreased sperm vitality, and decreased sperm motility. There was no significant change in semen volume or abnormal sperm morphology. Males, who were not occupationally exposed to BPA were still found to have measurable concentrations of urinary BPA, but at lower levels, and these males also demonstrated a significant negative correlation between BPA and sperm concentration. Adjustments were also made for potential confounders, for example, exposure to other chemicals/metals. A study recruiting US males through an infertility clinic found that urinary BPA concentrations measured in spot urine samples collected on the same day as a semen sample were associated with declines in semen quality, including concentration, motility and morphology and increased sperm DNA damage (comet assay)(Meeker *et al.* 2010). However, some cohort studies have reported finding no significant association between male fertility and urinary BPA action (Mendiola *et al.* 2010). Embryo quality, in particular reduced embryo cell number and increased embryo fragmentation score, were associated with increased serum BPA concentration in men (Bloom *et al.* 2011a).

In addition to looking at semen quality, Li et al published two further studies investigating associations between urinary BPA concentrations and sexual

function in Chinese male populations, exposed through factory work where BPA or epoxy resin is manufactured (Li *et al.* 2010a, Li *et al.* 2010b). Again potential confounders, such as chemical/metal exposure were statistically controlled for. Increased urinary BPA concentrations was found to be associated with a number of factors pertaining to sexual function including decreased libido, erectile dysfunction, ejaculation strength and satisfaction with sex life (Li *et al.* 2010a). Similar results were found in both studies for different populations. BPA-exposed workers also reported significantly reduced sexual function within 1 year of employment within the exposed environment (Li *et al.* 2010b).

1.6.2 Female fertility and reproductive function

1.6.2.1 Mammalian models

Few studies have reported direct links between BPA exposure in mammals and reduced female fertility; those that do exist do not provide definitive conclusions. A decline in the reproductive capacity of female mice perinatally exposed to BPA has been observed and in a dose dependant manner. A decline in fertility and fecundity has been observed over time for concentrations as low as 25ng/kg bodyweight (BW)/day (Cabaton *et al.* 2011). (Markey *et al.* 2005) also report that female mice exposed in a similar way, *in utero*, had genital tract alterations during adulthood, though there was no attempt to observe the effect of these abnormalities on reproductive output. However mice exposed orally for 2 generations to BPA at concentrations between 0.05-5 mg/kg BW/day found no adverse effects on female reproduction (Kobayashi *et al.* 2010). Exposure of female mice to 20-100 µg/kg BW/day of BPA resulted in a significant increase in meiotic abnormalities, including aneuploidy in the oocytes; this abnormality was initially observed in mice that were housed in polycarbonate cages and provided water in polycarbonate bottles that had been damaged by washing with a harsh detergent (Hunt *et al.* 2003). Oocytes of females exposed during foetal development displayed similar meiotic abnormalities, including synaptic defects and increased levels of recombination (Susiarjo *et al.* 2007). In the mature female, these aberrations were translated into an increase in aneuploid eggs and embryos. Neonatal subcutaneous injection of BPA at doses between 10-1000µg/kg BW/day resulted in severe pathologies of the uterus including adenomyosis, leiomyomas, atypical hyperplasia, and stromal polyps and a

statistically significant increase in cystic ovaries (Newbold *et al.* 2007). There are a number of other studies that have found a link between BPA exposure and the occurrence of cystic ovaries (Kato *et al.* 2003, Signorile *et al.* 2010, Vo *et al.* 2010).

1.6.2.2 Humans

There have been several cohort studies on individuals receiving *in vitro* fertilisation (IVF) treatment, where BPA concentrations have been measured in relation to multiple reproductive endpoints, such as ovarian response, fertilisation and implantation success and embryo quality. Ovarian response is measured by number of retrieved oocytes and peak E2 serum concentrations after hyperstimulation with human chorionic gonadotropin (hCG). Ovarian response has been observed to be reduced in US cohort studies in women found to possess higher total urinary BPA (Mok-Lin *et al.* 2010, Ehrlich *et al.* 2012). (Bloom *et al.* 2011b) also found BPA concentrations were associated with lower serum E2, however no reduction in the number of oocytes retried was observed. Fertilisation success of oocytes was also found to be lower in the study by Ehrlich *et al*. This finding was also observed in a separate cohort study by Fujimoto et al. 2011 where a 55% decrease in the probability of fertilisation was found with increased female serum BPA. Women with higher urinary BPA were also found to have a higher chance of embryo implant failure in a separate study (Ehrlich *et al.* 2012).

There have been several studies that relate BPA concentrations to the prevalence of Polycystic Ovarian Syndrome (PCOS), one of the leading causes of female sub-fertility (occurring in populations at an incidence of 4-8%(Knochenhauer *et al.* 1998, Michelmore *et al.* 1999, Azziz *et al.* 2004). In a study by Takeuchi and Tsutsumi 2002, total serum BPA was found to be significantly higher in women with PCOS compared with those with no evidence of PCOS. These results were supported by further studies that investigated associations between serum BPA concentrations and the occurrence of PCOS in women (Takeuchi *et al.* 2004, Kandaraki *et al.* 2011, Tarantino *et al.* 2013). Other features commonly associated with PCOS include obesity, insulin resistance, type 2 diabetes and cardiovascular, symptoms also often linked to BPA exposure in human populations.

1.6.3 Obesity/metabolism

1.6.3.1 Mammalian models

Reported over a decade ago, pregnant mice treated with 2.4 µg BPA/kg BW/day, during days 11-17 of gestation, produced male and female offspring that were significantly heavier than control females on postnatal day 22, despite having a similar body weight at birth (Howdeshell *et al.* 1999). Many subsequent studies in rats and mice have observed an association between increase in body weight and perinatal exposure to BPA in males and females (Rubin *et al.* 2001, Takai *et al.* 2001, Miyawaki *et al.* 2007), only in females (Markey *et al.* 2003, Nikaido *et al.* 2004, Somm *et al.* 2009) and in males alone (Akingbemi *et al.* 2004, Patisaul and Bateman 2008). However, significant differences appeared to vary between life stage, with some studies appearing to show statistically significant alterations in body weight almost immediately after birth (Rubin *et al.* 2001, Somm *et al.* 2009), while others showing little difference until weeks or months after birth (Howdeshell *et al.* 1999, Takai *et al.* 2001, Akingbemi *et al.* 2004, Nikaido *et al.* 2004, Markey *et al.* 2005).

In contrast to these findings, a separate study found no difference in body weights between mice prenatally exposed to BPA at 10-1000 µg/kg BW/day and control mice (Newbold *et al.* 2007). A possible reason for this is that mice were only weighed at 18 months, a much more advanced age than adopted in most other studies. Although puberty was accelerated in female mice exposed prenatally to 2 and 20 µg BPA/kg BW/day subcutaneously, body weight decreased in relation to unexposed females (Honma *et al.* 2002). A decrease in weight was suggested to be a result of the mice being raised by their own mothers, a factor not shared with studies that observed an increase in weight. Exposure to BPA during pregnancy and lactation may cause a detrimental effect on nursing behaviour and efficiency possibly leading to a reduction in weight (Palanza *et al.* 2002, Della Seta *et al.* 2005).

Body weight is not necessarily an accurate measure of obesity in rodents and only a few studies have assessed the impact of BPA exposure on more accurate measurements of obesity such as fat mass, fat pad weight, and adipose tissue cell composition (Thayer *et al.* 2012). (Miyawaki *et al.* 2007) found dosing pregnant/lactating mice with 1 and 10 µg/ml and continuing with exposure

through to PND 31, resulted in a mean adipose tissue weight increase (132% at 1µg/ml in females; 59% at 10 µg/ml in males) and the mean total cholesterol level also increased (33% at 1µg/ml, 17% at 10 µg/ml in females). (Ronn *et al.* 2013) dosing mice with 25-2500µg/l BPA, found no increase in fat pad, but the mice had a significantly higher liver fat content when compared to untreated individuals. Exposure of rat Fa hepatoma cells, a liver cell line, at both 30 and 300 ng BPA /ml significantly increased intracellular triglyceride content and lipid accumulation, without any effect on cell viability (Grasselli *et al.* 2013).

Male and female mice express ERs in adipose tissue and there is evidence that E2 acts via ERα to have an inhibitory effect on adipocyte number and lipogenesis. Elimination of ERα via knockdown models also causes impaired glucose tolerance and insulin resistance in addition to increased fat mass, adipocyte area and number (Heine *et al.* 2000, Cooke *et al.* 2001). Bisphenol A has been shown to imitate the action of E2 *in vivo* to affect blood glucose homeostasis through genomic and non-genomic pathways. At the same concentrations and the action of both BPA and E2 on insulin content was blocked by the anti-estrogen fulvestrant (Alonso-Magdalena *et al.* 2006). The role of E2 on glucose homeostasis is not straightforward. High E2 levels have been noted to be associated with the development of insulin resistance since the 1960s (Wynn and Doar 1969), however, women with low estrogen levels post-menopause are also at risk of developing type 2 diabetes (Godsland 2005, Morimoto *et al.* 2011). ERα knockout models have demonstrated glucose intolerance and insulin sensitivity, suggesting estrogen signalling is necessary to prevent these symptoms (Heine *et al.* 2000).

Male mice exposed to BPA at a dose of 100 µg/kg per day via injection for 4 days demonstrated elevated levels of circulating insulin, leading to insulin resistance and glucose intolerance (Alonso-Magdalena *et al.* 2006). In the same study Pancreatic β cells in primary culture exposed to low doses of BPA demonstrated rapid glucose-induced insulin secretion and increased insulin content, reflecting the *in vivo* observations. It is possible that the increased insulin production of the pancreas is in response to the developing insulin resistance, or a direct effect of action by BPA or E2 on the pancreatic β cells, or both. Isolated rat pancreatic islets exposed to 10 µg/l BPA demonstrated a significant increase in insulin

secretion after 24hrs incubation, via cytosolic/nuclear ER dependant pathway (Adachi *et al.* 2005).

1.6.3.2 Human

The idea that dramatic global rises in the incidence of obesity and associated metabolic factors may not be solely as a result of increased caloric intake and decreased physical activity, but may involve the interference of xenobiotic chemicals dubbed environmental obesogens, is a relatively new field. In this context the term obesogen was first adopted by Felix Grun and Bruce Blumberg in 2006 (Grun and Blumberg 2006). Since then obesity and metabolic disorders have been a frequently studied endpoint with regard to human health and BPA. Several studies using data obtained from the United States NHANES surveys found associations between the occurrence of BPA in urine samples and BMI/waist circumference in both adults (Carwile and Michels 2011, Shankar *et al.* 2012a) and children/adolescents (Trasande *et al.* 2012, Bhandari *et al.* 2013, Eng *et al.* 2013). A further study included analysis of hypertension, elevated serum triglycerides, glucose intolerance and reduced high-density lipoprotein in addition to assessing obesity. Participants that measured positive for three of these endpoints were considered to have metabolic syndrome, and levels of BPA were positively associated with the metabolic syndrome, independent of additional factors including age, gender and ethnicity (Teppala *et al.* 2012).

A study investigating the association between pre and postnatal BPA urinary concentrations and BMI found that BPA concentrations at 9 years were positively correlated with BMI, waist circumference and fat mass in both boys and girls (Harley *et al.* 2013). Prenatal urinary BPA concentrations from mothers were also measured and determined to be associated with a decreased BMI in girls at 9 years old, but not boys (Harley *et al.* 2013). This study is unique in its measurements of prenatal urinary BPA and obesity in childhood. As observed in the rodent studies mentioned previously, results can vary depending on life-stage at sampling, the authors note it is possible that the association my change as the subjects enter adolescence and additional analyses at older ages will help clarify the role of puberty in these observations (Harley *et al.* 2013). Two further studies investigated the association between urinary BPA and BMI/weight in Chinese school children. Findings support the evidence that BPA exposure may contribute

to an increase in BMI of children, though one study only found the association in girls (Li *et al.* 2013), whilst the other also found older children had significantly higher levels of BPA than younger ones (Wang *et al.* 2012a). Increased urinary BPA in adults was also found to be significantly associated with increased weight factors in European (Galloway *et al.* 2010) and Chinese (Zhao *et al.* 2012, Wang *et al.* 2012b) cohorts.

As with many epidemiological studies positive correlations between BPA concentration and BMI/obesity need to be treated with some caution. It appears that several factors may contribute to the observed effects and results vary across studies when comparing, age, sex, ethnicity etc. It is possible that BPA may not be leading to an increase in BMI, but is present at higher concentrations due to the larger amount of body fat present. It has been suggested that BPA accumulates in body tissues, fat in particular, therefore excess lipid-rich tissue may lead to storage and continuous release of BPA (Stahlhut *et al.* 2009). It is also possible that a diet high in packaged and processed foods may possess a higher concentration of BPA, whilst independently leading to an increased BMI. However, interestingly one of the studies investigating the NHANES cohort found that higher urinary BPA concentrations, but not concentrations of other environmental phenols, were associated with an increase in obesity arguing for specificity of the association (Trasande *et al.* 2012).

One study investigated the effect of BPA on preadipocytes from donors with a healthy body mass index. Treatment of preadipocytes with 1-50 µM BPA induced a dose dependent increase in differentiation and lipid accumulation and induced expression of adipogenic markers (Boucher *et al.* 2014). Although co-treatment with ICI 182 780 inhibited the observed effects of BPA, treatment with 17β-estradiol alone had no effect suggesting action may be mediated through a non-classic ER pathway (Boucher *et al.* 2014).

Several studies investigating association between BPA and various endpoints in cohort studies have reported that an increase in urinary BPA is associated with type 2 diabetes diagnosis and glucose concentrations (Lang *et al.* 2008, Melzer *et al.* 2010, Shankar and Teppala 2011, Silver *et al.* 2011). Although there are existing links between obesity and the onset of type 2 diabetes in the study by

(Shankar and Teppala 2011), the positive association between BPA and diabetes was present regardless of the weight group of the subject.

However, there was no clear association between BPA levels and type 2 diabetes in a survey of Chinese adults, though it should be noted that occurrence of diabetes was based on serum glucose and insulin concentrations, whereas subjects with diagnosed diabetes with successful treatment may obscure apparent associations. A study on Korean subjects also found no association between urinary BPA and prevalence of type 2 diabetes, though results were based purely on participant survey, there is a possibility that non-professional diagnosis may lead to an under-representation of actual prevalence (Kim and Park 2013).

1.6.4 Cardiovascular

1.6.4.1 Mammalian models

Estrogen has been considered to play a role in the cardiovascular system for some time, often linked to a possible protective effect due to pre-menopausal women experiencing less cases of coronary heart disease than men or post-menopausal women (Stampfer *et al.* 1991, Pelzer *et al.* 1996, Mendelsohn and Karas 1999). Because of this it has been suggested that BPA and other xenoestrogens will likely exert some effects on the cardiovascular system, however there are still only a small number of studies that have tried to actively determine the role of BPA in relation to this vital system.

BPA was found to rapidly (<7 min) induce arrhythmia in isolated ventricular myocytes from adult females, but not males at the same concentration as E2, with the effects of BPA being particularly pronounced when exposed in combination with E2 (Yan *et al.* 2011). In the same study there was little effect of BPA on arrhythmia *in vivo* under normal conditions, however with the addition of catecholamine-induced stress BPA promoted ventricular arrhythmias, again only in female rats. Exposure to E2 and/or BPA rapidly altered Ca2+ handling, in particular increasing sarcoplasmic reticulum Ca2+ leak; the rapid response of female myocytes to estrogens was not seen in ERβ knockout mice, suggesting the observed effects are mediated specifically by Erβ-signalling. A further study investigating the effect of BPA on contractility and arrhythmia in ventricular

myocytes found effects in the pM range, only in female myocytes, although this sensitivity was abolished in ovariectomised females (Belcher *et al.* 2012). Results suggested the rapid actions were induced by membrane-associated receptors, with Erα and Erβ having opposing actions in myocytes. Rat hearts subjected to ischemia followed by reperfusions were found to have a marked increase in sustained ventricular arrhythmia when exposed to BPA at 1nM, however infarction size was also reduced agreeing with data that suggests a possible protective effect against cardiac infarction (Yan *et al.* 2013)

BPA effects on cardiac structure/function from gestation through to 16 weeks (dosing at 0.5 µg/kg/day and 5.0 µg/kg/day) were found to include male mice having increased relative heart wall thickness, that was not a result of a pathological increase in blood pressure (suggesting that BPA acts directly upon the cardiac tissue) (Patel *et al.* 2013). Results from this study also implied that the impact of BPA on heart structure was long lasting and unaffected by BPA removal, possibly due to the completion of cardiac structure prior to birth. This could be of particular concern as it has been suggested that BPA may accumulate in the placenta, increasing exposure to developing foetuses (Poidatz *et al.* 2012). Developmental exposure to BPA through to adulthood has also been found to induce a raised systolic blood pressure (Cagampang *et al.* 2012).

It is apparent that BPA has an effect on the heart both during development and in adults, being capable of inducing rapid responses at very low concentrations. It would appear that sex in particular plays a role in determining the severity of the disruption. However, the number of studies investigating the specific effects on cardiac signalling and function are still severely lacking and more data is necessary before real conclusions may be drawn. It is also necessary to determine the specific gene pathways activated by BPA and E2 to better understand the mechanism of disruption.

1.6.4.2 Human

Data from several epidemiological studies indicates that BPA may be a key factor in cardiovascular risk. Using NHANES survey data (Lang *et al.* 2008) found that higher urinary BPA concentrations were associated with cardiovascular diagnoses (including coronary heart disease, heart attack, and angina) in models adjusted for factors such as age, sex etc. To corroborate these finding another

NHANES survey from 2005/06 found higher BPA concentrations were associated with coronary heart disease (Melzer *et al.* 2010). To strengthen the evidence for causal interference Melzer et al. 2012a conducted a longitudinal study using the European Prospective Investigation into Cancer and Nutrition –Norfolk cohort. Urinary BPA was measured from samples given at the study commencement and found to be lower than both previous NHANES studies at 1.3 ng/ml, however concentrations were still positively associated with the incident of coronary artery disease up to 10.8 years after the study commencement (Melzer *et al.* 2012a). Another study by Melzer *et al.* again compared urinary BPA with grades of severity of coronary artery disease from patients participating in a UK study, urinary BPA concentration was significantly higher in patients with severe coronary artery disease when compared to the controls and nearly significant for those with intermediate disease (Melzer *et al.* 2012b).

In a study using a Korean cohort of participants over 60 years with multiple examinations of its subjects, a positive association was found between urinary BPA and high blood pressure (Bae *et al.* 2012). There was a negative correlation between BPA concentration and heart rate viability (a measurement of the beat-to-beat variation of heart rate) (Bae *et al.* 2012). Decreased heart rate variability and high blood pressure are risk factors for cardiovascular disease. A similar result was found in the NHANES group by (Shankar and Teppala 2012b), in that a positive association was observed between increasing levels of urinary BPA and high blood pressure independent of confounding factors.

All the results in the above sections indicate that BPA does induced health effects in humans. In many cases there is strong experimental evidence to support this. However further experimental work is needed, particularly to determine the mechanisms of action of BPA in these different tissues/cell types.

1.7 Effects of BPA on other model vertebrates

Although mammalian models are often used in an attempt to determine the impact of chemicals on human health and development, there are a wide number of alternative model vertebrates that provide advantages over some mammalian models. For environmentally important pollutants such as BPA it is also essential to determine the impacts on key wildlife species necessitating the

need to determine possible effects across a wide range of taxa. The following sections describe the effects of BPA on key vertebrate wildlife taxa.

1.7.1 Amphibians

Bisphenol A has been investigated in amphibian studies and it appears to have effects on both estrogen and thyroid signalling. BPA has been shown to induce the egg precursor protein vitellogenin *in vitro* and *in vivo*, supporting its role as an estrogen. Examples of this include VTG production in cultured hepatocytes of *X. laevis* (Mitsui *et al.* 2007) and *R. chensinensis* (Bai *et al.* 2011). Intraperitoneal injection of adult *Bombina orientalis* with BPA at a concentration of 100 mg/kg BW also induced hepatic VTG mRNA in male frogs to levels similar to those found in female livers (Gye and Kim 2005). Similar results were found for *X. laevis* (Kloas *et al.* 1999). However, a study on *Rana temporaria* hepatocytes did not find any effect of BPA up to a concentration of 100µM on vitellogenin concentration (Rouhani Rankouhi *et al.* 2005) .

Amphibian metamorphosis is controlled by thyroid hormones, therefore disruption of metamorphosis by BPA has been linked to its role as a thyroid disrupter. BPA supressed T3-induced tail regression in *Rana rugosa* and also inhibited *Silurana tropicalis* spontaneous metamorphosis (Goto *et al.* 2006). When using a transgenic *X. laevis* model capable of producing GFP in tissues with TH response element binding activity, t3 was found to produce a clear GFP signal in hind limbs. This expression was suppressed with the addition of BPA, suggesting BPA inhibits TR-mediated gene expression (Goto *et al.* 2006). However a study using *X. laevis* found that even up to a concentration of 497µg/l BPA had no effect on growth, sex ratio or metamorphosis (Pickford *et al.* 2003). Timing of exposure and species sensitivity may possibly contribute to differences in observed results, however for these studies no information is given on the state the larval stage at the time of exposure.

X. laevis exposed to BPA (at concentrations of 20µM and above) were found to have marked developmental abnormalities of the head and abdomen (Oka *et al.* 2003). In that study, apoptosis was also found to occur specifically in the central nervous tissue cells of the brain and spinal cord. When exposed to 10µM 17β-estradiol abnormalities were also observed, however these differed from those seen for BPA suggesting the observed effects are a result of non-estrogenic

activity (Oka *et al.* 2003). Sone *et al.* also determined that exposure to 20µM BPA induced developmental abnormalities including short body length, microcephaly, oedema and abnormal gut coiling (Sone *et al.* 2004). Apoptosis of spermatogenic cells in *Rana chensinensis* has been observed with the apoptotic index increasing with increasing BPA concentration and treatment time (Lin *et al.* 2008). *X. Laevis* exposed to BPA at concentrations from 10^{-8} to 10^{-4} M from the first developmental day resulted in dose-dependent defects in otolith formation. Treatment with TH, 17β-estradiol and ICI 182, 780 had no observed effect on otolith formation, suggesting the observed effects of BPA are independent of ERs or THRs (Gibert *et al.* 2011). BPA at 10^{-7} M has also been found to produce a dose dependant skew in sex ratio towards females in *X. laevis*(Levy *et al.* 2004).

When fertilised *R. rugosa* eggs were exposed to BPA at a concentration of 500nM for two days to determine the accumulation and pharmacokinetics it was found to have concentrated in the embryo body with a bioconcentration factor of 382.3 (Takase *et al.* 2012). Interestingly BPA had a higher bioconcentration factor than the other chemicals tested, 17β-estradiol, 17α-ethinylestradiol and nonylphenol (bioconcentration factors of 217.9, 170.2 and 289.1 respectively) (Takase *et al.* 2012). It has been suggested that temperature could have an effect on BPA uptake in *R. temporaria*, with uptake increasing at higher temperatures (Honkanen and Kukkonen 2006).

1.7.2 Fish

BPA has been detected in samples from freshwater and marine environments, occasionally at high concentrations up to 12-21 µg/l, although most measurements are usually reported lower than this in the ng/l range (Crain *et al.* 2007). This has led to a significant number of studies on the effect of BPA on aquatic organisms, fish in particular.

BPA has been determined to be lethal to fish, however only at concentrations many times higher than typically found in the environment. 96hr LC50 for zebrafish and fathead minnows are 8.04 and 4.6 mg/l, respectively (Alexander *et al.* 1988, Chow *et al.* 2013). Various fish species have exhibited morphological deformities as a result of BPA exposure including zebrafish (Lam *et al.* 2011) salmon *Salmo salar* (Honkanen *et al.* 2004), Medaka *Oryzias latipes* (Pastva *et al.* 2001) and Fathead minnow *Pimephales promelas* (Warner and Jenkins 2007).

Developing zebrafish exposed to BPA above 1500 µg/l had a significant increase in incidence of cardiac oedema, and treated fish also exhibited cranio-facial abnormality, retarded swim bladder development and partial yolk-sac reabsorption (Lam *et al.* 2011). The authors note that these abnormalities have been described previously in studies investigating the effect of thyroid hormone on developing zebrafish. The effect of BPA on transgenic zebrafish line that allowed clearer visualisation of circulatory and developing neural systems found BPA caused deformities to neuromast cells, suppressed vascularisation in the abdominal region, and inhibited branching of axons from the spinal cord (from 500 µg/l) (Lam *et al.* 2011).

A variety of reproductive effects have been reported in various fish species resulting from BPA exposure. Most of these effects again occur for exposure to BPA concentrations much higher than those measured in the environment. Intersex has been found to occur in both medaka after three weeks and carp after two weeks for exposures to 837 and 1000 µg BPA/l respectively (Kang *et al.* 2002, Mandich *et al.* 2007). At 274 µg BPA /l male guppies were found to have a reduced (40-75%) total sperm count, which was indicated to be due to interference with Sertoli-cell function (Haubruge *et al.* 2000). A diverse range of fish species have been shown to produce vitellogenin and/or zona radiata proteins in plasma upon exposure to various concentrations of BPA, some may be deemed to be environmentally relevant, *Gobiocypris rarus* – 5 µg/l, sea bass (Dicentrarchus labrax) – 10 µg/l; however, many are higher, cod – 50 µg/l (Larsen *et al.* 2006), fathead minnow – 64 µg/l (Mihaich *et al.* 2012), carp – 100 µg/l (Mandich *et al.* 2007), zebrafish – 500 µg/l (Song *et al.* 2014), and medaka – 1000 µg/l (Ishibashi *et al.* 2005). BPA exposure at environmental concentrations has also been found to reduce several parameters relating to sperm and testis quality in several species (Lahnsteiner *et al.* 2005, Mandich *et al.* 2007, Hatef *et al.* 2012). These data indicate, that despite BPA being reported as weakly estrogenic *in vitro* it may have reproductive consequences at concentrations currently measured in the environment. BPA's actions *in vivo* may differ due to reasons discussed later in this chapter.

Estrogens are known to act in the brain and the central nervous system via a variety of signalling pathways, therefore if BPA is present in brain tissue it may disrupt signalling necessary for important brain functioning. Fish from BPA

contaminated waters in Pittsburgh were found to have BPA in their brain tissue (Renz *et al.* 2013). In fact, it was suggested that due to the high lipid content of brain tissue it is likely that lipophilic chemicals such as BPA are likely to bioconcentrate here, possibly increasing the risk of exposure (Renz *et al.* 2013). BPA has been found to induce a strong brain-specific overexpression of cyp19a1b RNA in fish, specifically in the same sub-regions of the developing brain (mediobasal hypothalamus, preoptic area and telencephalon) as 17β-estradiol (Chung *et al.* 2011, Shanthanagouda *et al.* 2014). This is thought to be largely coordinated through nuclear ERs. Female *Gobiocypris rarus* exposed to 15µg/l BPA for 35 days were found to have significantly unregulated levels of gonadotropin-releasing hormone (GnRH) and GnRH receptor in their brain (Qin *et al.* 2013).

Some studies have determined BPA may have an effect on neurodevelopment and behaviour. Hyperactivity has been induced in zebrafish larvae when exposed to BPA (Selderslaghs *et al.* 2013). Saili *et al.* 2012 also found that a similar response was observed when exposed to E2 and an ERRγ agonist, indicating that it is possible this response could be mediated through either traditional ER or ERRγ signalling, or a combination of the two. In mature zebrafish BPA exposure was found to significantly increase the time to learn a maze compared with control fish (Saili *et al.* 2012). Behavioural effects seen in adults were sex specific, having no significant effect on females (Weber *et al.* 2014).

There is limited evidence that BPA may also have an effect on the fish immune system, *Perca flavescens* and *Carassius auratus* exposed to BPA from 4 µg/l and 5 µg/l respectively, showed leukocyte blood counts significantly higher than in the controls (Yin *et al.* 2007, Rogers and Mirza 2013). (Rogers and Mirza 2013) also noted that the immune response to BPA was comparable to that of a pathogen exposure. A significant increase in the expression of genes related to immune response was observed in 168 hpf zebrafish larvae, specifically cytokines interferon γ, *interleukin-1β*, *interleukin 10*, *Myxovirus resistance*, and *tumour necrosis factor α* and chemokines *CC-chemokine* and *CXCL-clc* (Xu *et al.* 2013). The proinflammatory mediator NO was also found to be increased by BPA from 100 µg/l accompanied by increased NOS activity (Xu *et al.* 2013).

BPA has been noted to have some effect on steroid synthesis and metabolism in fish including for androgens and estrogens. BPA has been determined to alter E2 and testosterone plasma levels in carp (Mandich *et al.* 2007). Disruption of sex steroid ratio has been reported in turbot (*Psetta maxima*) exposed to 59 µg/l BPA where a reduction in androgen to estrogen ratio was observed in male fish (Labadie and Budzinski 2006). Sex steroids govern a wide range of biological processes at very low concentrations; as a result even small alterations can result in disruption of multiple pathways.

Species sensitivity to the effects of BPA is evidenced through the varying degrees of response or the concentration necessary to elicit a similar response. Although these differences may sometimes be attributed to differences in experimental setup in one case where a study (Larsen *et al.* 2006) directly compared the response of two fish species, Atlantic cod (*Gadus morhua*) and turbot (*S. maximus*). At 59 µg/l both VTG and ZRP levels were significantly higher in the cod, interestingly when exposed to the estogenic chemical nonylphenol, the two species did not exhibit any species sensitivity in their response (Larsen *et al.* 2006). One suggestion for this sensitivity is the rate at which BPA may be metabolised and/or excreted, (Lindholst *et al.* 2003) compared the toxicokinetics between zebrafish and rainbow trout, data indicated zebrafish metabolised BPA more rapidly than the trout leading to weaker estrogenic sensitivity. A further explanation is the specific binding affinities of ERs across different species, *in vitro* assays have demonstrated that estrogenic chemicals can have different binding affinities depending on the specific species ER used; specifically with the construction of chimeras (swapping LBDs between species), it has been shown LBD plays a significant role in accounting for BPA sensitivity in different species (Miyagawa *et al.* 2014).

Although several studies indicate that the concentrations of BPA needed to induce effects are often higher than those found in the environment, it is important to remember that wildlife and indeed humans, are seldom exposed to chemicals individually but as complex mixtures, that can often have interactive or possibly synergistic effects. Mixtures of estrogenic chemicals, including BPA, have been shown to induce estrogenic effects *in vivo* (Vtg production) and *in vitro* (YES assay), even when individual concentrations produced no detectable effect (Peterson *et al* 2013). Empirical data gained from single chemical exposures

remains important, as elucidating specific chemical effects and mechanisms is difficult when testing in combination. However when endpoints are not observed to be significantly altered at concentrations close to environmental levels mechanistically important pathways may be altered at higher concentrations. This could indicate that even at lower concentrations, especially when exposed as part of a complex mixture of chemicals effects could still occur. In addition to possible effects of mixtures, it should also be noted that timing of exposure plays a key role in the potential toxicity of a chemical. The endocrine system in adults largely serves the purpose of maintaining optimum cellular and organ function, whereas in developing embryos, and prematurity the endocrine system also serves the purpose of correct development. Therefore, even slight immeasurable changes in function during critical windows of development can lead to much wider effects later in life, in humans for example, pesticide exposure during gestation has been seen as an influencing factor in leukaemia development later in life (Brown 2005).

These data indicate that BPA can have a large number of effects in fish, including at environmental concentrations. These effects appear to be pleiotrophic, targeting multiple mechanisms and signalling pathways. This is concerning for wildlife as fish are one of the most commonly exposed vertebrates due to the largely aquatic nature of chemical disposal, for example, through WWTW. However as vertebrates fish possess many conserved molecular pathways with humans, indicating this data should also be of concern to human health.

1.7.3 Reptiles

Reptiles rely on temperature for sexual determination, however endocrine disruptors may also affect sexual phenotype regardless of developmental temperature. The numbers of current studies investigating the disruptive effect of BPA on reptilian development or physiology are still sparse. When exposed to 9mg BPA/egg *in ovo* the caiman *Caiman latirostris* exhibited complete male to female sex reversal when incubated at the male-determining temperature (33^{o}C), however unlike control females, oocytes in different developmental stages were not observed, suggesting gonad development was delayed (Stoker *et al.* 2003).The same study also exposed a lower concentration (90µg/egg), where no sex reversal was observed, however seminiferous tubule development was identified as abnormal (Stoker *et al.* 2003).

BPA could possibly act *in ovo* to disrupt estrogen metabolism in the turtle *Trachemys scripta* (Clairardin et al. 2013). When administered BPA at 40µg/egg, levels of both 17β-estradiol and estrone were elevated, whereas levels of estrone sulphate were lower, possibly indicating estrogen sulfotransferase activity is altered in the presence of BPA, though this was not specifically tested and several metabolic conversions needed to produce estrone sulphate from 17β-estradiol could be potential targets. This supports the suggestion that non-ER related mechanisms could be responsible for certain measured endpoints.

1.7.4 Birds

There have been a relatively small number of studies published investigating the impact of BPA on birds. In studies on Japanese Quail (*Coturnix japonica*) ERα and ERβ using a competitive binding assay, the relative binding affinities for BPA were found to be very low (0.0035 and 0.011 for ERα and ERβ respectively when compared to 17β-estradiol)(Hanafy *et al.* 2004, Hanafy *et al.* 2005). An earlier study comparing the relative binding affinities (RBAs) of estrogenic chemicals to ERs across species also found that BPA had a weak RBA to the chicken (*Gallus gallus*) ERα (0.044 when compared to 17β-estradiol), however this was stronger than both human and mouse ERα binding (0.0080 and 0.0086 respectively) (Matthews *et al.* 2000).

Ovotestis has been reported to occur in both *C. japonica* (Oshima *et al.* 2012) and *G. gallus* (Berg *et al.* 2001) when exposed during development. However in a subsequent study on *C. japonica* by the same group no statistical differences in testis morphology between control groups and BPA treated animals were observed (Halldin *et al.* 2001). In a long term study where male *C. japonica* were fed 2mg BPA/day BPA treatment delayed time taken to perform sexual interactions and was associated with a decrease in cloacal gland size, there was also a significant decrease in seminiferous tubule diameter (El-Gawish *et al.* 2008). It should be noted that only a single, particularly high dose of BPA was tested, so definitive conclusions are unclear. (Furuya *et al.* 2003) found that orally dosing male *G. gallus* chicks at a high dose of BPA (200mg/week) over a period of 16 weeks had no effect on body growth, however comb and testes weight were lower in BPA-treated birds. Histology of testes and seminiferous tubuli demonstrated an immature appearance with limited spermatogenesis in BPA-

treated birds compared to controls. These results were supported by a further study with a more robust methodology (Furuya *et al.* 2006).

A study investigating the effect of BPA on immune system development in *G. gallus* chicks found that at a concentration of 250ug administered via direct injection the weight index of the bursae of Fabricus (a specialised organ necessary for B cell development in birds), number of lymphatic follicles in the bursae of Fabricus and the thickness of the thymus cortex and medulla were all significantly reduced (Tian *et al.* 2014). Microscopic analysis also showed that lymphatic follicles and epithelial cells of the bursae of Fabricus had irregular development in BPA exposed chicks, suggesting an involvement of BPA on immune system disruption in developing chicks. However only one dose was tested, although this was described as a very low-dose level there is no indication as to its environmental relevance. A single injection does also not take into account how the chemical may be handled and metabolised *in vivo.*

In chicken cerebellar granule neurons isolated from BPA injected eggs Pax6 level was increased following 6 days of *in vitro* culture, Pax6 is critical in the correct development of granule neurons in the developing cerebellum (Mathisen *et al.* 2013). The authors suggest BPA may contribute to incorrect development of the cerebellum, which is essential for motor control, sensory discrimination and complex cognitive tasks (Mathisen *et al.* 2013).

The uptake of radiolabelled BPA remained low in chick embryos in comparison to the yolk, however pronounced labelling was observed in the bile and allantoic fluid, indicating BPA is readily metabolised and excreted (Halldin *et al.* 2001). When administered to laying adults BPA was largely excreted via the bile, with only small amounts of BPA remaining within the body 9 days after dosing, maternal transfer of BPA to eggs was also low (Halldin *et al.* 2001).

1.8 Mechanism of BPA action

Although largely regarded as an estrogenic chemical research has shown that the mechanisms of action of BPA are diverse. In addition to the ability to bind to and activate ERs (though apparently weakly so compared to E2), BPA binds strongly to the estrogen related receptor ERRγ, it can antagonise androgen and thyroid signalling by binding to the relevant receptors and has other non-genomic

effects (Fig 1.1). It is also possible that differing potencies observed between *in vitro* and *in vivo* studies could be related to the possibility of metabolic activation of BPA into a more potent metabolite. Understanding on the mechanisms of BPA action is now discussed.

1.8.1 BPA as an estrogen

As BPA was originally designed to be used for its estrogenic properties it is not surprising that the majority of research concerning its action is based on its estrogenic activity. The classical view of estrogen signalling has been that an estrogenic ligand, typically 17β-estradiol, acts by binding to an estrogen receptor (ER) located in the cytosol triggering migration to the nucleus. Dimerisation with another ER then occurs subsequently functioning as a transcription factor by binding to specific DNA sequences known as estrogen response elements (EREs). In mammals there are two known ERs: ERα and ERβ, with a third distinct receptor being described in teleost fish (resulting in ERα, ERβ1 and ERβ2). This nuclear signalling of the ER is now known to include the indirect activation of other transcription factors such as activator protein-1 (AP-1) and Sp1 (Kushner *et al.* 2000, Levin 2005). In addition to their nuclear function, membrane bound ERs have been shown to exert effects outside the nucleus, resulting from interactions with specific kinase domains to create cascades both in the cytosol and at the plasma membrane, these effects are characterised by a rapid response and are not exerted through regulation of gene expression (Fu and Simoncini 2008). Secondary messengers such as cAMP, cGMP and intracellular Ca^{2+} can in incorporated into rapid estrogen signalling, which in turn can also regulate gene expression (Alonso-Magdalena *et al.* 2012). Estrogen has also been found to bind to and activate other membrane sites including the orphan receptor GPER (formally GPR30) (Thomas *et al.* 2005, Funakoshi *et al.* 2006, Filardo *et al.* 2007) and ion channels (Valverde *et al.* 1999), activity which has been attributed to be responsible for certain non-genomic actions.

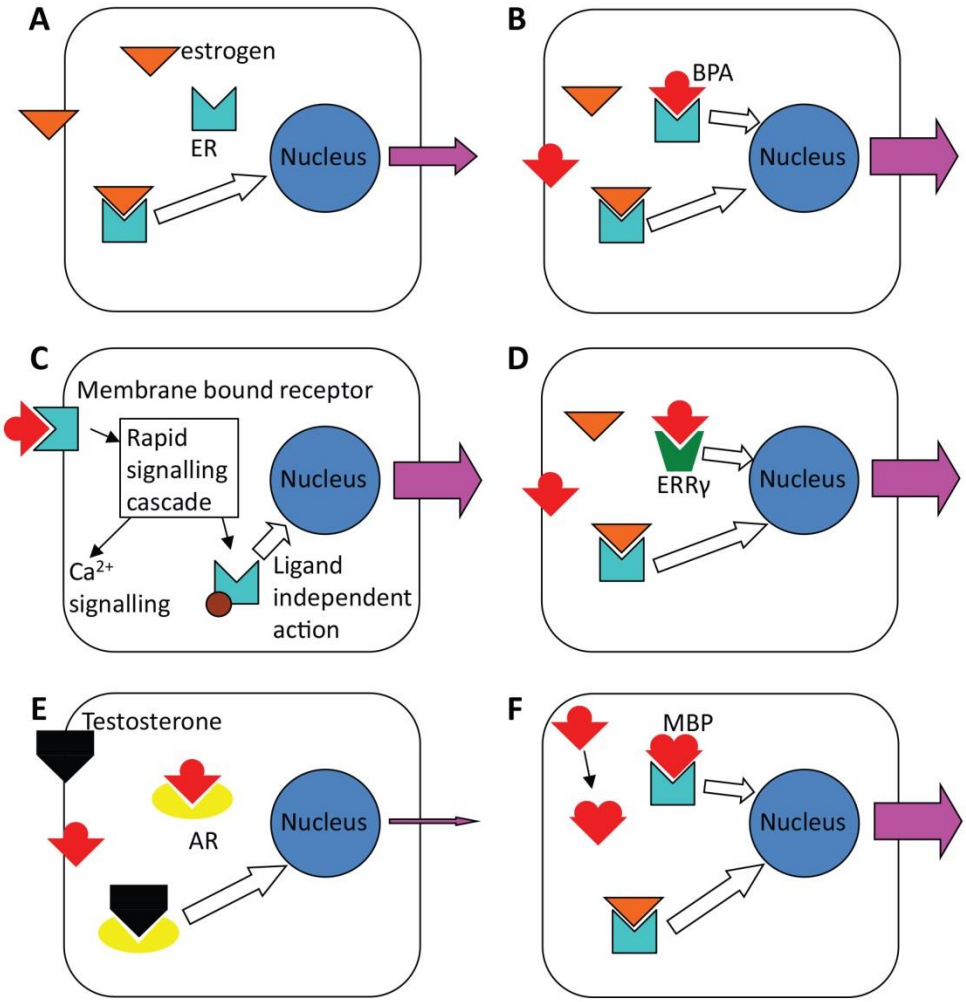

Figure 1.1 Possible mechanisms of toxicity for Bisphenol A (BPA). A: Normal hormonal and cellular response, B: BPA elicits an agonistic response by binding and activating the estrogen receptor (ER), C: BPA can act through non-genomic pathways via membrane bound receptors which can trigger rapid intracellular signalling cascades, affecting calcium signalling and ligand independent transcription through the recruitment of co-factors and other transcription factors, D: BPA elicits an agonistic response by binding and activating the estrogen related receptor γ (ERRγ), E: BPA binds and inhibits the androgen receptor (AR) affecting the normal signalling of androgens (antagonistic response), F: BPA is metabolically activated into a more potent estrogen e.g. 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) which may elicit an agonistic response by binding to and activating the ER.

1.8.1.1 BPA acting through nuclear receptor signalling

Many effects of BPA have been discussed in context of its ability to induce responses by interacting with classic nuclear ERs. Indeed, it is well established that BPA has the ability to bind to the ERs and modify gene expression. However, it has long been considered a weak estrogen as binding affinities to these ERs have been estimated to be several orders of magnitude lower than those found for 17β-estradiol. BPA binds both mammalian receptor subtypes, ERα and ERβ, however studies differ concerning their estimated binding affinities. Some studies have found very little difference in the ability of BPA to bind to the different subtypes (Kuiper *et al.* 1998b, Paris *et al.* 2002, Takayanagi *et al.* 2006, Baker and Chandsawangbhuwana 2012). Whereas several other studies have reported that BPA has a higher affinity for ERβ (Kuiper *et al.* 1997, Matthews *et al.* 2001, Yoshihara *et al.* 2004, Takemura *et al.* 2005).

It has also been suggested that BPA may interact with the ERs in a different manner compared with 17β-estradiol. *In vitro* activity of BPA has been shown to possess actions distinct from other ERα agonists/antagonists, possibly due to a unique conformation of the activated ERα (Gould *et al.* 1998). 3D modelling of BPA interacting with ERs has shown that BPA lacks some contacts with amino acids in the ER found with E2, possibly explaining its lower affinity (Baker and Chandsawangbhuwana 2012). However novel contacts may be made at other points in the ER resulting in a distinct mode of activity. BPA could therefore theoretically induce a unique set of ER responsive genes, resulting in a biological response which differs from the classic E2-ER interaction (Baker and Chandsawangbhuwana 2012).

Transcriptional activation may also be affected by co-activator recruitment. EC50 potencies for the transcriptional activation of ERα by BPA was $1.6x10^{-4}$ M in the yeast estrogen screen (YES), whereas in the Yeast Two-hybrid with rat ERα and the co-activator TIF2 EC50 was $1.4x10^{-5}$ M (Yoshihara *et al.* 2004). Thus, mimicking more the conditions in mammalian cells with the inclusion of co-activators resulted in an approximately 10-fold increase in transcriptional activation. Recruitment of co-activators and the effects of BPA also differ with respect to ER subtype. When bound to ERβ, BPA has an enhanced ability to recruit the co-activator TIF2 but not SRC-1e, consistent with a greater ability of

ERβ to potentiate reporter gene activity in transfected HeLa cells expressing TIF2 (Routledge *et al.* 2000). These results indicate that ligand-dependent differences in the ability of the ERs to recruit co-activators may alter, in part, the receptors' ability to potentiate gene expression in whole cells.

BPA has also been shown to bind to ERs in other non-mammalian vertebrates. In the amphibian *Xenopus laevis,* BPA has been shown to bind to the ER in hepatocytes *in vitro* (Lutz *et al.* 2005). BPA has also been found to cause the synthesis of VTG through ER up-regulation in *Rana chensinensis,* albeit weakly active in doing so compared with E2 (Bai *et al.* 2011). BPA has also been shown to bind to fish ERs and promote gene transcription of several different species, including in carp, trout, medaka and zebrafish (Segner *et al.* 2003, Terasaki *et al.* 2005, Torres-Duarte *et al.* 2012). In contrast to many studies on mammals that report BPA has a greater affinity for ERβ, in a zebrafish luciferase induction assay, BPA was found to display a stronger relative estrogenic potency, compared to E2 when binding to ERα than either ERβ subtype (Cosnefroy *et al.* 2012). In that work no significant estrogenic effect was produced by cells transfected with ERβ1. It should be noted that the binding affinity for a receptor is not always predicative of the magnitude of

response and xenoestrogen action alone and this can lead to an underestimate of chemical potency (Routledge *et al.* 2000). Estrogen responsive transgenic organisms are a relatively new tool in assessing the effects of estrogenic chemicals, particularly in fish. In an Estrogen responsive element transgenic (ERE-TG) zebrafish (Lee *et al.* 2012) demonstrated that BPA promoted transcription in many previously unreported tissues, including the liver, heart, brain, somite muscle, neuromast and fin. Treatment with the estrogen inhibitor ICI 182 780 demonstrated that this transcription was dependant on the classic estrogen receptor pathway.

1.8.1.2 BPA actions through non-genomic pathways

Although studies that involve BPA promoting transcription through binding with ERs often describe BPA as a weak estrogen, there are studies that indicate that BPA may exert a response at very low concentrations and in some cases at a similar concentration to 17β-estradiol, though through rapid signalling pathways (Alonso-Magdalena *et al.* 2005, Bouskine *et al.* 2009, Yan *et al.* 2011).

Considering the non-genomic pathways of action, BPA was discovered to induce rapid Ca^{2+} signals in pancreatic beta cells at an equally potent dose to E2 (Nadal *et al.* 2000, Quesada *et al.* 2002). BPA and E2-induced calcium signals were initiated at the plasma membrane and activated the transcription factor cAMP-responsive element binding protein (CREB). Phosphorylated CREB (the activated form of CREB) was then increased after just 5 minutes of exposure (Quesada *et al.* 2002). Molecular activation by both E2 and BPA was unaffected by the addition of the anti-estrogen ICI182, 780, indicating that the classical ERs do not play a role in this pathway. In alpha cells of the pancreas it has been demonstrated that 1 nM of BPA can suppress intracellular calcium ion oscillations induced by low-glucose, the signal that triggers glucagon secretion, through a membrane estrogen receptor using a cGMP/PKG-mediated pathway (Alonso-Magdalena *et al.* 2005). However, data obtained from ERβ(-/-) mice indicates that removal of this ER subtype prevents the BPA induced rapid decrease in K-ATP channel activity, increased glucose-induced calcium signals and insulin release in beta cells, indicating receptor crosstalk may still play a pivotal role in raid signal responses (Soriano *et al.* 2012). In addition, *in vivo* exposure of adult mice to a single low dose of 10 µg/kg of E2 and BPA correlates with a rapid rise in plasma insulin, in a ER independent manner, whereas longer exposure to either chemical, at the same low dose induces an insulin increase in an ER dependant manner (Alonso-Magdalena *et al.* 2006). (Adachi *et al.* 2005) also determined that long term exposure of rats to the same dose of BPA promotes insulin secretion via nuclear estrogen receptors.

Studies assessing the effects of estrogen on cerebellar signalling indicate the rapid effects on ERK1/2 signalling in cerebellar granular cells are induced through a G protein-coupled receptor, requiring PKA and Src-kinase activity (Belcher *et al.* 2005). Interestingly, short term and long term exposure of cerebellar neurons to 10 pM E2 induced differing results. In the short term, exposure induced mitogen-activated protein kinase (MAPK) dependent decreases in viable granule cell numbers, whereas in contrast continuous exposure significantly increased granule cell numbers (Wong *et al.* 2003). Exposure to BPA at the same dose as E2 has also been shown to result in mimicking of the neurotoxic response in granule cells (Zsarnovszky *et al.* 2005, Le and Belcher 2010). Additionally, the ability of DPN (an ERβ agonist), but not PPT (an ERα agonist), to selectively

induce estrogen-like affects in the cerebellum supports the notion that rapid signalling effects of estrogens are initiated through ERβ-like receptor systems (Le and Belcher 2010). Further, co-administration of E2 and BPA highlighted the complexity of the rapid signalling mechanism in developing neurons as BPA demonstrated the ability to inhibit rapid E2 induced ERK1/2 activation in developing cerebellar neurons (Zsarnovszky *et al.* 2005). These results highlight the ability of BPA to not only act as a potent E_2 mimic at low doses, but to also disrupt the rapid actions of E_2 during cerebellar development indicating a potential impact on the developing brain.

1.8.2 Interactions with Estrogen Related Receptors

Within the family of NRs there are orphan receptors, for which there is no known ligand. Despite the fact that Estrogen Related Receptors (ERRs) were the first orphan receptors to be identified there is still no known endogenous ligand (Horard and Vanacker 2003). ERRα and β were originally discovered in 1988 (Giguere *et al.* 1988), with ERRγ being discovered later in 1999 (Hong *et al.* 1999). ERRs share a similar structure to ERs, particularly in their DBD regions; ERRα and ERα, for example, share over 60% sequence identity within their DBD (Horard and Vanacker 2003). 17β-estradiol (E2), does not bind to any of the ERR family, despite similarities in LBDs, however ERRs can still functionally bind to EREs in ER target genes, inducing translational responses, and suggesting a possible overlap in the action of ERs and ERRs (Huppunen and Aarnisalo 2004).

There is evidence that a possible mechanism of action for BPA may be mediated by ERRγ. BPA has been shown to bind to ERRγ with high specificity (binding affinity IC_{50} = 9.78-13.1 nM) whereas natural estrogens E2 and E3 were inactive, showing no binding affinity up to 10μM (Takayanagi *et al.* 2006, Okada *et al.* 2008). Interestingly, BPA also completely prevented the inhibitory action of the selective estrogen receptor modulator 4-hydroxytamoxifen (4-OHT) on ERRγ (Takayanagi *et al.* 2006). Analysis of human tissues has found ERRγ to be preferentially expressed in the placenta; ERRγ mRNA was also detected at lower levels in other reproductive tissues, the ovary, uterus, prostate and testis (Takeda *et al.* 2009). The presence of ERRγ in reproductive and placental tissue indicates it may be responsible for some of BPA's attributed actions during foetal development. It has also been suggested that since ERRγ is highly expressed in

the placenta, it may facilitate the accumulation of BPA, thus increasing exposure of the developing foetus (Poidatz *et al.* 2012).

Zebrafish also have an ERRγ, that is structurally similar to its mammalian counterpart and is expressed in the brain during early development (Bardet *et al.* 2004). The killifish *Fundulus heteroclitus* also possess an ERR gene, indicated to be a form of ERRγ. In adult killifish this was found to be largely expressed in the gill, heart and gut (Tarrant *et al.* 2006). Developmental exposure of zebrafish to either 0.1 µM BPA or 0.1 µM GSK4716 (a synthetic ERRγ ligand) resulted in an equally significant increase in aggressive behaviour in a behavioural assay in adults (Weber *et al.* 2014). The fact that exposure to a higher BPA concentration (1µM) or 0.1 µM E2, resulted in a decrease in this response possibly suggests that at the lower concentration BPA could be acting as an ERRγ agonist.

1.8.3 Interactions with Androgen Receptor

BPA has also been reported to act as an androgen receptor (AR) antagonist, and this could serve to increase 'estrogenic effects'. However, compared with estrogen receptors there is substantially less data concerning BPA interactions with androgen receptors and the molecular mechanisms for this antagonism are unclear. Several *in vitro* ligand competition assays have demonstrated the ability of BPA to displace synthetic androgen from recombinant ARs (Paris *et al.* 2002, Fang *et al.* 2003, Kim *et al.* 2010). Using several different assays, including yeast two-hybrid system, ligand competition and transient transfection assays results suggest BPA affects multiple steps of the activation and function of AR. These effects include inhibiting the binding of native androgens to AR, inhibiting AR nuclear translocation, and inhibiting AR interaction with its co-regulator, and its subsequent transactivation (Lee *et al.* 2003, Sun *et al.* 2006, Teng *et al.* 2013). *In vivo* exposure of fathead minnows has demonstrated clear evidence through observation of changes in liver metabolite profiles for the ability of BPA to mitigate the effects of 17-β-trenbolone (a strong AR agonist) which is consistent with an anti-androgenic mechanism of action. AR antagonistic activity of BPA was confirmed *in vitro* (Ekman *et al.* 2012).

1.8.4 Interactions with Thyroid Receptors

Thyroid hormones (THs) are responsible for regulating a number of processes in a large number of taxa. In vertebrates they are responsible for normal brain and somatic development and in adults play a key role in metabolism (Zoeller 2010). There is evidence that BPA binds to the TH receptor (TR), acting as an antagonist. Specifically, *in vitro* studies have demonstrated that BPA antagonises the activation of TR by triiodothyronine (T3) (Moriyama *et al.* 2002, Seiwa *et al.* 2004, Zoeller *et al.* 2005). BPA has been shown to displace T3 from rat liver thyroid receptor TR (with an inhibition constant (K_i) of 200 μM, being described as a weak ligand(Moriyama *et al.* 2002). It was suggested that the ability of low levels of BPA to inhibit thyroid hormone receptor-mediated gene expression may be made possible by enhancing the recruitment of the co-repressor N-CoR to the TR.

A small number of *in vivo* studies in rats have shown that pups exposed to BPA, from a concentration of 0.1 mg/l (in drinking water), during gestation and lactation had an increase in circulating levels of thyroxine (T4) (Zoeller *et al.* 2005, Xu *et al.* 2007). An increased expression of a thyroid responsive gene in the brain was also detected (Zoeller *et al.* 2005, Xu *et al.* 2007). The thyroid system is complex and mechanistic interactions are poorly understood, it is therefore currently difficult to determine how BPA and other thyroid disruptors exert their effects on circulating thyroid hormone. Contrary to previously mentioned studies it has also been indicated that *in utero* and lactational exposure of BPA to mice does not affect thyroid functions, with BPA groups demonstrating no significant change in plasma (T4) concentration and thyroid gland function (Kobayashi *et al.* 2005). The experimental design in these different studies was similar and it is possible therefore that these contrasting results may be due to different strains of mouse used; Sprague-Dawley were used in the former mentioned studies and Crl:CD(SD) in the latter. Additionally parental exposure of gestating pups found no effect on TR expression indicating that the possible observed effects on thyroid function (transient hypothyroidism and behaviour) may not be mediated by BPA directly targeting the TR, but instead may operate via an alternative mechanism (Xu *et al.* 2007).

The thyroid system is well conserved across vertebrates and it is possible therefore that BPA may interact with TRs from other taxa in a similar manner to mammals. In *Xenopus laevis* embryonic and larval exposure to BPA blocked the actions of T3 and suppressed TR gene expression both *in vivo* and *in vitro* (Iwamuro *et al.* 2003). Microarray analysis has revealed that BPA antagonises the regulation of 33% of T3-induced genes and 36% of T3-repressed genes, including the majority of the most dramatically T3-regulated genes, and this may explain the observed inhibitory effect on metamorphosis (Heimeier *et al.* 2009). In zebrafish, BPA at environmentally relevant exposure concentrations (as low as 10(-9) M via the water) altered expression of genes involved in thyroid hormone synthesis and thyroid specific transcription factors in a concentration and time dependant manner (Gentilcore *et al.* 2013). A study investigating the effect of BPA on T3 binding in the Japanese quail found that BPA was more likely to disrupt TH signalling by targeting transthyretin, a major TH-binding protein in plasma rather than the TR directly (Ishihara *et al.* 2003).

1.8.5 Activity of Bisphenol A metabolites

There have been multiple studies carried out on the metabolism and toxicokenetics of BPA, largely in mammalian systems, and these have shown a large number of possible metabolites produced *in vitro* (Nakagawa and Tayama 2000, Nakagawa and Suzuki 2001, Pritchett *et al.* 2002, Inoue *et al.* 2003, Jaeg *et al.* 2004)and *in vivo* (Knaak and Sullivan 1966, Snyder *et al.* 2000, Volkel *et al.* 2002). The majority of BPA is rapidly metabolised through glucuronidation to BPA glucuronide and subsequently excreted in the faeces and urine. *In vitro* studies on BPA glucuronide have found that it does not exhibit any estrogenic activity (Matthews *et al.* 2001). Although some metabolites have been found to possess estrogenic activity, for example BPA catechol and BPA *o*-quinone, this is largely weaker than the activity exhibited by the parent compound (Yoshihara *et al.* 2001). As BPA is largely metabolised to BPA glucuronide it is also likely that the concentrations of these metabolites would be very low *in vivo*. Their weak activity and low concentration therefore mean it is unlikely their activity should be a concern. However there is increasing evidence that in mammalian livers, BPA may be metabolised to the much more potent estrogenic chemical 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) (Yoshihara *et al.* 2004).

The possibility of a potent metabolite was originally discovered when the estrogenic activity of BPA was increased after incubation with rat S9 liver fraction proportionately to the time of incubation (Yoshihara *et al.* 2001). In contrast incubation of rat S9 liver fraction with other estrogenic chemicals including 17β-estradiol resulted in their inactivation (Yoshihara *et al.* 2001). Further investigation discovered two potent metabolites from S9 incubation of BPA, one being MBP the other was designated 4-methyl-2,4-bis(4-hydroxyphenyl)pent-2-ene. Despite the much lower concentration of MBP compared with BPA the estrogenic activity of MBP, determined using the YES assay was higher (Yoshihara *et al.* 2004). S9 fractions from mouse, monkey and humans also produced similar metabolism profiles (Yoshihara *et al.* 2004). *In vivo* studies in rats and medaka (*Oryzias latipes*) have found that MBP exerts a strong estrogenic effect up to 400-1000 times stronger than that of its parent compound (Ishibashi *et al.* 2005, Nagae *et al.* 2005, Yamaguchi *et al.* 2005, Okuda *et al.* 2010). 3D modelling of human ERs, BPA, MBP and 17β-estradiol suggest that the increased spacing between the phenolic rings in MBP from BPA allows key amino acid contacts with the ERs that are important in receptor binding of E2 (Baker and Chandsawangbhuwana 2012).

Discrepancies seen between the estrogenic potency of BPA *in vitro* and *in vivo* suggest a complex mechanism of action that may involve multiple pathways. *In vitro* studies demonstrating the estrogenic activity of BPA in cell lines and yeast screens will likely not include interactions chemicals may experience *in vivo*, in particular metabolic transformation. However, it should be noted that under normal circumstances metabolic activation of BPA may not be significant as the predominant metabolite is BPA glucuronide, and MBP is likely to be present at very low concentrations. In fact, MBP has not yet been measured as an *in vivo* metabolite. Nevertheless, under certain circumstances glucuronidation activity may be reduced. For example the developing livers of rats and humans, have been found to lack the necessary enzymes for glucuronidation, developing this capability only postnatally (Coughtrie *et al.* 1988). Additionally, rat foetuses exposed via parental exposure were found to contain BPA but not BPA glucuronide (which does not easily pass through the placental barrier), supporting the poor capacity for glucuronide conjugation in the foetus (Takahashi and Oishi 2000). The developing foetus is identified as a key target of EDCs and research

on BPA in particular has often focused on the effects exerted at this crucial life stage. It is possible that combined with a poor ability to glucuronidate BPA, metabolic activation to MBP may be significant, however further research on the ability of the foetal liver to produce MBP is necessary.

There is little information on the *in vivo* metabolism of BPA in fish. In brown trout exposure to 50 µg/l BPA did not result in the production of the conjugated metabolite bisphenol - a glucuronic acid BPAGA, during the early stages of development, but did from around 28 dpf, shortly before hatching (Bjerregaard *et al.* 2008).

1.9 Alternative Bisphenol Analogues

As the scientific evidence for biological effects of BPA increases, several organisations have called for legislation to reduce the manufacture/use of BPA for use in food contact materials and thermal paper. The European Union passed a directive to prohibit the manufacture of baby bottles containing BPA in 2011 (European_Commission 2011). However, this movement has led to increased use of alternative bisphenols in certain products that may then be labelled BPA-free. However, there is little information concerning the biological effects of these alternatives when compared to BPA, and current evidence does not indicate these as necessarily being safer alternatives. Alternative analogues to BPA possess the same basic structure of two phenol groups, joined by a carbon (or in the case of bisphenol S (BPS) sulphur) bridge, with the hydroxyl groups at the para positions (see Fig 1.2). Differences in the chemical groups attached to the phenolic rings and/or bridge can vary, including alkyl groups or halogens. Some of these bisphenols are already commonly used in various applications, for example in the use of food/beverage containers and thermal paper.

1.9.1 Bisphenol S

1.9.1.1 Occurrence

The US EPA predicted a national production volume of 1 to 10 million lbs/year for BPS (U.S. EPA, 2012). BPS is commonly used as a developer in thermal paper due to the reduction in BPA papers, removed from the market due to fears of dermal absorption (Biedermann *et al.* 2010). (Liao *et al.* 2012a) analysed the occurrence of BPS in 16 types of paper and paper products, samples were

collected from USA, Japan, Korea and Vietnam. 100% of thermal receipt paper samples were found to contain BPS at concentrations ranging from 13.8 ng/g to 22.0 mg/g, similar mean concentrations were found for BPA and BPS, however there was a negative correlation between both chemicals, possibly implying that as BPA is used less, BPS is used more frequently. BPS was also detected in 87% of currency bills from 21 countries and 14 other paper products at concentrations ranging from <LOQ to 8.38 µg/g (Liao *et al.* 2012a).

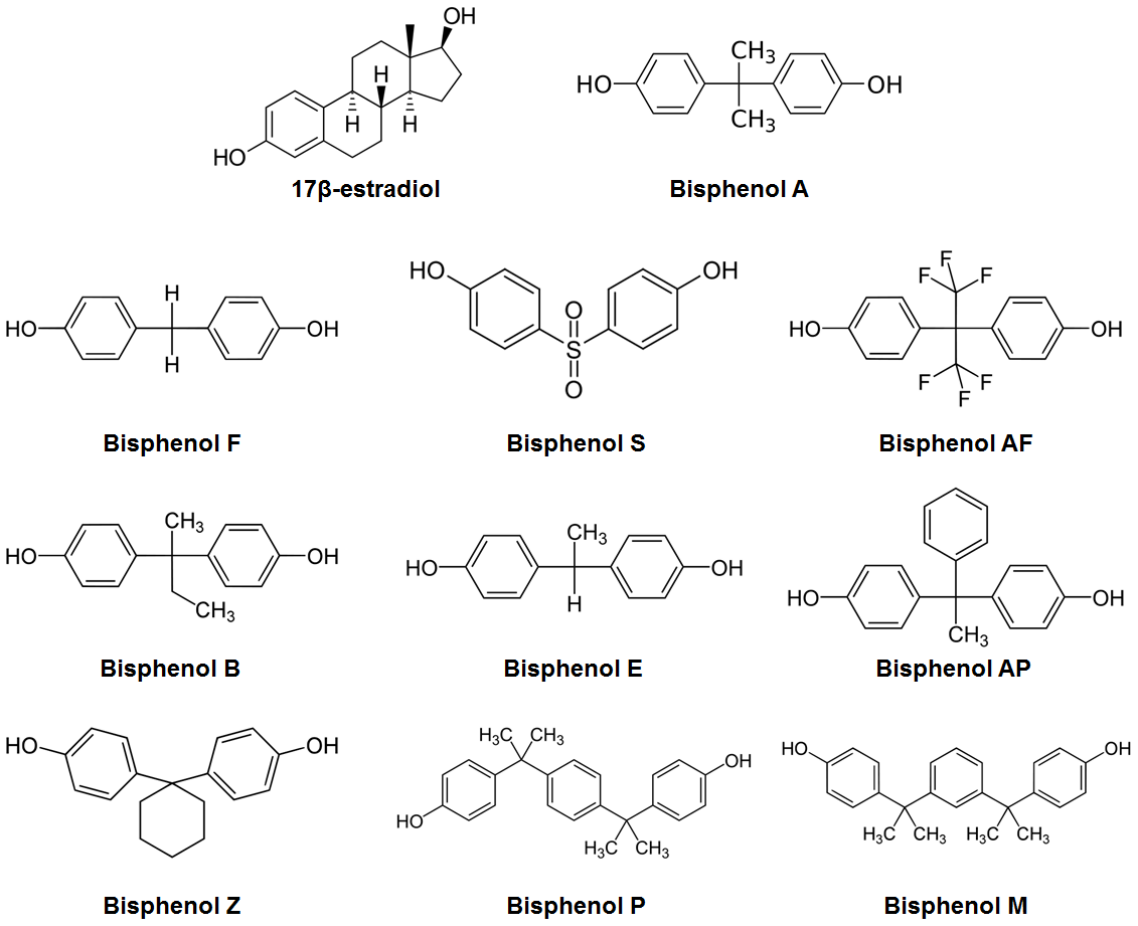

Figure 1.2 Chemical structures for the endogenous steroidal estrogen 17β-estradiol (E2), Bisphenol A and the alternative analogues discussed in this chapter, Bisphenol F, Bisphenol S Bisphenol AF, Bisphenol B, Bisphenol E, Bisphenol AP, Bisphenol Z, Bisphenol P and Bisphenol M.

Polycarbonate incorporating BPA may also be substituted for BPS incorporating polyethersulfone (PES), used to make plastics and epoxy resins. In one study BPS was found to be present in 20.9% of food and beverage products in the USA at a relatively low average concentration of 0.13 ng/g.(Liao and Kannan 2013). BPS was also found in seven of nine canned food samples analysed by (Viñas *et al.* 2010), however in six of these samples BPS was only found in the liquid supernatant as opposed to the food itself, concentration ranged from 11.5 ng/g to 175 ng/g. However BPS was not detected in eleven different canned beverage samples in Spain (Gallart-Ayala *et al.* 2011)

The concentration of eight bisphenol analogues, including BPS was analysed in indoor dust samples from four countries; BPS was found in 100% of samples at a mean concentration of 0.34 µg/g (Liao *et al.* 2012b) Analysis of aqueous sediment from USA, Japan and Korea found BPS to be present in 28.5% of samples at a mean concentration of 12.4 ng/g dry weight (dw), but as high as 1970 ng/g dw (Liao *et al.* 2012c). Interestingly, when analysing vertical concentration in sediment cores, BPS was found to increase in concentration from 2001, whereas BPA was found to decline, these results correspond with legislation passed against the use of BPA in thermal paper in Japan in the last decade. In a recent study measuring the concentrations of BPS in river and sediment samples from several industrialised sites in China BPS was found to be present in all river samples and three out of five sediment samples (Yang *et al.* 2014). Though sediment concentrations were relatively low, 0.12ng/g dw on average compared to 16ng/g dw for BPA, concentration in river samples was found to be much higher, highest measured concentration was 18.99 ng/l, compared to 74.58 ng/l for BPA. As BPS is incorporated into many products that frequently come into contact with consumers and appears frequently in the environment, it is not surprising that BPS has been detected in 81% of urine samples across eight different countries at concentrations up to 1.18 ng/ml (Liao *et al.* 2012d).

BPS has also shown some resistance to degradation when compared to alternative bisphenols, which could lead to accumulation in the environment. Although degradation under anaerobic conditions was observed to be similar to BPA, in one study BPS was found to degrade less than the other seven compounds tested (Ike *et al.* 2006). When looking at biodegradation in seawater,

no notable degradation of BPS was detected after 60 days (Danzl *et al.* 2009). An isolated strain of bacteria, *Sphinogomonas* sp. Strain BP-7, was found to degrade a variety of bisphenols, however it could not degrade BPS (Sakai *et al.* 2007), a recent study used the bacterium *Sphingobium fuliginis* OMI and found that aerobic degradation of BPS could be achieved, the first study to demonstrate this finding (Ogata *et al.* 2013).

1.9.1.2 Health Effects

There have been several *in vitro* assay studies to determine the potential of BPS to bind hormone receptors, therefore indicating the potential to elicit endocrine activity. Several studies have shown that BPS may bind and activate ERs, using a variety of reporter assays including, E-screen in MCF-7 cells (Hashimoto and Nakamura 2000), GFP expression system in MCF-7 cells (Kuruto-Niwa *et al.* 2005), luciferase reporter assay (Kitamura *et al.* 2005), and MELN transactivation assay (Grignard *et al.* 2012). However, some studies have found significant differences in ER activation between BPS and BPA, with BPS always acting as the weaker estrogen (Hashimoto and Nakamura 2000, Molina-Molina *et al.* 2013) (Chen *et al.* 2002). *In silico* predictions of binding to hERα found that there was a 37-fold higher probability of BPA exhibiting stronger binding compared with for BPS (Grignard *et al.* 2012). There is also evidence that BPS can bind to the AR (Fang *et al.* 2003), however there is conflicting evidence whether it does so in an agonistic or antagonistic manner (Kitamura *et al.* 2005, Molina-Molina *et al.* 2013, Teng *et al.* 2013).

BPS has also been indicated to affect adipogenesis in 3T3-L1 cell line. This investigation found that BPS increased the triacylglycerol content and adipocyte-specific fatty acid binding protein mRNA of the cultures, markers of adipogenic agents, with a very similar potency to BPA (92%) (Masuno *et al.* 2005). Potential genotoxicity was also determined for BPS in the HepG2 cell line at concentrations between 0.1 µM to 10 µM, after 24hrs; BPS induced significant DNA damage as determined by the comet assay (Fic *et al.* 2013). Analysing chromosomal aberration in chicken DT40 cells (Lee *et al.* 2013) also showed that BPS has genotoxic potential, though weaker than that of BPA.

Although there are several *in vitro* studies indicating the ability of BPS to induce biological effects, there are still few *in vivo* studies on BPS. Female rats

subcutaneously injected with 20 and 500 mg BPS/kg/day were found to have a significantly higher uterine weight than controls, though there was no significant increase found at 100 mg/kg/day (Yamasaki *et al.* 2004). A 48hr EC_{50} of BPS was measured at 55 mg/l in the water flea *Daphnia magna* compared with 10 mg/l for BPA (Chen *et al.* 2002). In zebrafish (Ji *et al.* 2013) found that egg production and gonadosomatic index were significantly reduced in female zebrafish from an exposure concentration of 0.5 µg BPS/l when exposed for 21 days. In that study plasma concentration of E2 was found to be significantly increased in both male and female fish. Furthermore, in males there was a significant reduction in plasma testosterone concentration. Parental exposure to BPS resulted in a both a delay and reduction in hatching rate in the F1 generation, even when the offspring were reared in untreated water. When raised in BPS dosed water hatching rate was further reduced and an increase in malformation rates was observed (Ji *et al.* 2013). Zebrafish exposed to a range of concentrations of BPS for 75 days confirmed these findings, including a significant elevation in E2 concentrations for exposure to 1 µg BPS/l and postponed/decreased hatching in fertilised eggs from exposed fish (Naderi *et al.* 2014).

1.9.2 Bisphenol F

1.9.2.1 Occurrence

BPF is an important monomer in the production of epoxy resins which may be used for coatings, floorings, linings mouldings and laminates. BPF may also be used to produce polycarbonates used for e.g. in food packaging (Gardziella 2000). There does not appear to be any public data on the import/production values of Bisphenol F in the EU, USA or elsewhere. In the USA BPF was found in 10.1% of selected food and beverage products, around half the number found for BPS, however at a higher mean concentration of 0.929 ng/g (Liao and Kannan 2013). Out of thirteen canned food and beverage products tested BPF was only found to be present in two, at concentrations of 218 and 141 ng/l (Gallart-Ayala *et al.* 2011). However, in an analysis of BPA and BPF in food-contact recycled paper materials, BPF was found to be present in two out of eight samples, however concentrations were too low to accurately quantify. BPA was found in all samples from <LOQ to 25.4 µg/g in a paper tablecloth.(Pérez-Palacios *et al.* 2012). BPF was not detected in any canned food products in British or Iranian

markets (Goodson *et al.* 2002, Rastkari *et al.* 2011), or in honey samples from glass or plastic containers from various global locations (Inoue *et al.* 2003).

Where indoor dust samples were analysed in four different countries, BPF was found to be present in 74.4% of samples, however at a relatively lower average concentration of 0.054 µg/g (1.33 and 0.34 µg/g for BPA and BPS respectively) (Liao *et al.* 2012b). Occurrence of BPF in the environment has been reasonably well documented. In sediments from USA, Japan and Korea. Here BPF was found to be present in 62.2% of samples at a concentration of 69.7 ng/g dw, making BPF the second most abundant bisphenol present after BPA. In two separate studies investigating the occurrence of BPF in Germany, 75% and 58% of sediment samples contained BPA up to approximately 7ug/kg dw (Fromme *et al.* 2002, Stachel *et al.* 2003). Fromme et al also found BPF in 77% of water samples and 72% of sewage water samples. BPF was found in two out of five of the river samples in China and three out of five sediment samples (Yang *et al.* 2014). The highest concentration measured in the sediment was 30.16 ng/g dw, only slightly lower than the 42.76 ng/g dw found for BPA. Finally, BPF has been detected in surface and groundwater samples in Spain (Herrero-Hernández *et al.* 2013), river samples in China (Ballesteros-Gómez *et al.* 2007) and in raw and treated sewage in Spain (Ruiz *et al.* 2007).

In several studies assessing the biodegradation of BPF, it was found to be more biodegradable than BPA and BPS, (Ike *et al.* 2006, Danzl *et al.* 2009, Toyama *et al.* 2009). However, degradation by the bacteria *Sphinogomonas* sp. Strain BP-7, had no effect on BPF, despite demonstrating the ability to degrade several alternative bisphenols including BPA(Sakai *et al.* 2007). Using radio-labelled BPF (Cabaton *et al.* 2006) concluded that female rats orally dosed with BPF for four days mainly excreted BPF residues in the urine (43-54%) and faeces (15-20%), with the major metabolite being a sulphate conjugate. Immediately after the exposure, BPF was detectable in all tissues, with the largest amount present in the liver. Pregnant rats were also found to have large amounts of BPF in the uterus, placenta, amniotic fluid and foetuses (Cabaton *et al.* 2006). Incubation of BPF with human and rat S9 liver fractions demonstrated the production of several metabolites (Cabaton *et al.* 2008). However, estrogenic potential of metabolites was not assessed

1.9.2.2 Health effects

Several *in vitro* studies have attempted to determine the potential estrogenic activity of BPF. In a reporter gene assay in HepG2 cells, BPF significantly increased luciferase activity from 10^{-7}M, and the estrogenic response was approximately three times stronger in cells transfected with ERα (Cabaton *et al.* 2009). In a yeast two-hybrid assay, BPF was found to induce significant levels of β-galactosidase activity and the estrogenic activity measured was similar to that of BPA in one study (Chen *et al.* 2002) and higher in another *(Zhang et al. 2011)*. However in another similar study, BPF was found to be significantly more estrogenic than BPA in a fluorescence polarisation system, but not when using MCF-7 proliferation as an endpoint (Hashimoto and Nakamura 2000). Another MCF-7 proliferation study found BPF to be significantly more potent than BPA, having an EC_{50} (concentration at which half maximal activity of E2 was observed) of 84.8 nM compared to 410 nM (Stroheker *et al.* 2004). As with many other estrogenic chemicals there are conflicting results between some reported studies and some studies have demonstrated the estrogenic potency of BPF may actually be much lower than that of BPA. Again using the MCF-7 proliferation test, BPF was determined to have the lowest estrogenic activity of the five bisphenols tested. BPF was also found to be the weakest estrogen when comparing mRNA levels of the estrogen responsive Progesterone Receptor (Perez *et al.* 1998, Kanai *et al.* 2001). Activity of BPF in a MCF-7 estrogen luciferase reporter assay was found to be weaker than BPA and similar to that of BPS (Kitamura *et al.* 2005). In addition BPF was found to be capable of binding to the orphan receptor ERRγ but was approximately thirteen times weaker than BPA (Okada *et al.* 2008).

BPF increased the triacylglycerol content and adipocyte-specific fatty acid binding protein mRNA of the cultures in the 3T3-L1 cell line (used to study adipogenesis). The relative potency of BPF for inducing triacylglycerol content was 89% of that of BPA (Masuno *et al.* 2005). No genotoxicity was found for BPF in the HepG2 cell line, at concentrations ranging from 0.1 µM to 10 µM, BPF (DNA damage over 24 hrs) (Fic *et al.* 2013). As with BPS, BPF showed genotoxic potential weaker than BPA measured by chromosomal aberration in chicken DT40 cells (Lee *et al.* 2013). However in another study the genotoxicity of BPA and BPF was compared in HepG2 cells, where BPA was not found to be genotoxic and BPF was (Audebert *et al.* 2011). (Cabaton *et al.* 2009) also found

that using the comet assay on HepG2 cells BPF was effective at fragmenting cell DNA from 78.7 µM.

As found with BPS, there are few *in vivo* studies on the effects of BPF. In immature rats BPF induced a significant increase in wet (from 100 mg/kg/day) and dry (200 mg/kg/day) uterine weight, whereas BPA did not induce any increase in wet or dry uterine weight (Stroheker *et al.* 2003). (Yamasaki *et al.* 2004) also found female rats subcutaneously injected with 100 to 1000 mg/kg/day were found to have a significantly higher uterine weight compared with in controls, supporting a previous study that showed 200 mg/kg BPF increased uterine blotted weight in immature rats (Yamasaki *et al.* 2002). In contrast oral dosing of rats with BPF at 20 to 500 mg/kg/day for 28 days showed no estrogen-mediated effects, based on sperm analysis, estrous cycle, organ analysis and histology (Higashihara *et al.* 2007). There are very little ecotoxicological data concerning BPF though (Chen *et al.* 2002) did report the 48hr EC_{50} of BPS at 56 mg/l compared to 10 mg/l for BPA in the water flea *D. magna*.

1.9.3 Bisphenol AF

1.9.3.1 Occurrence

BPAF is structurally similar to BPA, where the two methyl groups are replaced with trifluromethyl groups, mainly used in fluroelastomers and heat resistant adhesives and also in the fabrication of electronic materials, gas-permeable membranes, and plastic optical fibres (Delfosse *et al.* 2012). Because of the potential for occupational exposure and possible exposure in the general population ,the USA National Institute of Environmental Health Sciences nominated BPAF as one of only six chemicals for comprehensive toxicological characterisation based on the potential for endocrine disruption and lack of toxicity data (NTP 2008). Production volume was reported to be <500 000 lbs by the US EPA in 2006 (U.S. EPA, 2006)

In environmental samples collected around a manufacturing plant in China BPAF was found in river water at <LOQ to 15.3 µg/l, in sediment from 0.52 to 2000 ng/g dw and soil from <LOQ to 331 ng/g dw (Song *et al.* 2012). Contamination of river and sediment samples in a recent study by found BPAF at much higher levels

than other bisphenol chemicals present, including BPA (Yang *et al.* 2014). Average concentrations of BPAF in river and sediment samples were 74.8 ng/l and 663 ng/g dw respectively, compared to 26.0 ng/l and 16.1 ng/g dw for BPA (Yang *et al.* 2014). Occurrence of BPAF in indoor dust and well water was also assessed in this study, BPAF was found to be present ranging from 7.82 to 739 ng/g dw in dust and <LOQ to 300ng/l in well water samples. (Liao *et al.* 2012b) determined BPAF did not occur in any dust samples from USA and China and only 9% of samples in Japan, however it was detected in 76% of samples in Korea. Mean concentration (0.0039 µg/g) was however much lower than observed for BPA, BPF and BPS (4.07, 0.50 and 0.43 µg/g respectively). Similarly BPAF was only discovered in 20.6% of river sediment samples in Korea, being absent in USA and Japan samples (Liao *et al.* 2012c). Finally BPAF was discovered in 10.5% of selected food products in the USA, however at the lowest mean concentration measured for all bisphenols analysed, this may be due to the main uses of BPAF not bring in food packaging materials (Liao and Kannan 2013).

In rats orally dosed with BPAF, the highest concentrations were found in the faeces, suggesting the main route of elimination is in an non-conjugated form (Yang *et al.* 2012). It is not clear if the compound is poorly absorbed, or if some metabolites are deconjugated by micro fauna *in situ*. The highest levels of BPAF in tissues were found in the liver, kidney and serum samples (Yang *et al.* 2012). Several metabolites of BPAF in human liver microsomes have been detected (Schmidt *et al.* 2013). These findings also support those by (Nakamura *et al.* 2011) and (Yoshihara *et al.* 2004) where BPAF could be degraded in the same manner as BPA and BPF possibly creating a more potent estrogenic metabolite.

1.9.3.2 Health effects

BPAF has been shown to be estrogenic in a range of reporter systems, spanning MCF-7 based estrogen luciferase reporter, vitellogenin production in CARP-HEP cells and transcriptional activation assays and results vary. Most findings indicate that BPAF has the potential to be a stronger estrogen than BPA (Kitamura *et al.* 2005, Bermudez *et al.* 2010). Using an MCF-7 cells based estrogen luciferase reporter assay, BPAF was found to be much more potent than BPA (EC50 of 0.05 µM compared to 0.63 µM) (Kitamura *et al.* 2005). A transcriptional activation

assay using cells expressing ERα and ERβ transfected with and ERE promoter found BPAF to be a significantly stronger estrogen (Bermudez *et al.* 2010). Vitellogenin production measured in CARP-HEP cells found BPAF to induce a significant increase at 10 μM, BPA did not at this concentration, although a significant increase was observed for both chemicals at 100 μM. In a competitive receptor-binding assay BPAF was twenty times more potent than BPA as a ligand for ERα and forty-eight times more potent as a ligand for ERβ, suggesting the binding site of, particularly ERβ, possess specific structural elements that interact much more favourably with the trifluromethyl groups of BPAF than the methyl groups of BPA. However there are some studies that show that BPAF has an estrogenic potency comparable to that seen for BPA (Perez *et al.* 1998, Rivas *et al.* 2002)and in one study was actually weaker than BPA, though still stronger than the other alternative bisphenols included in the study (Kanai *et al.* 2001). Although it would appear to be a potent estrogen, at least when compared to BPA, BPAF interacts inconsistently with the ERs. It is reported to be a strong full agonist for ERα, however when interacting with ERβ, BPAF has been reported to be both a strong agonist and antagonist (Matsushima *et al.* 2010, Li *et al.* 2012, Li *et al.* 2013). Therefore, it would appear that BPAF has the potential to be a more potent estrogenic chemical than BPA, though it may act as an agonist or antagonist to perturb physiological processes mediated through the ERs.

In addition to binding to the ERs BPAF has also been assessed for its potential to bind to other nuclear receptors. Out of six bisphenols tested, BPAF was the weakest at binding to the orphan receptor ERRγ being much weaker than BPA and BPF (Okada *et al.* 2008, Matsushima *et al.* 2010). Using a competitive inhibition assay with the synthetic androgen R1881, BPAF was also found to be an AR antagonist (Teng *et al.* 2013). Thyroid hormone disruption may be mediated by impacts on deiodinase activity, which regulates the levels of active hormones available to bind to nuclear receptors, BPAF has demonstrated the ability to inhibit deiodinase activity in a dose dependant manner, similarly to tetrabromobisphenol A, a known thyroid disruptor, though activity is less potent (Butt *et al.* 2011).

BPAF produced no effect on DNA damage over 24 hrs when testing for potential genotoxicity in the HepG2 cell line at concentrations from 0.1 μM to 10 μM (Fic *et al.* 2013).

Again as with other alternative bisphenols *in vivo* data on the effects of BPAF are lacking. Uterine weight of immature rats exposed to 100 mg/kg of BPAF were increased significantly compared with controls, demonstrating estrogenic properties of the chemical *in vivo* (Yamasaki *et al.* 2003). Other estrogenic effects include reduction in serum testosterone, increases in LH and FSH and disruption of estrous cycle (Feng *et al.* 2012, Umano *et al.* 2012) Other reported *in vivo* effects relate to cholesterol and steroid biosynthesis, with reduced serum cholesterol reported from 50 mg BPAF/kg/day

1.9.4 Other Bisphenols

1.9.4.1 Occurrence

Many more bisphenols exist with differing substitutions attached to either the phenolic rings or bridge of the basic structure. Though these often occur at lower concentrations than the bisphenols previously discussed, several have shown endocrine activity similar to those above. These bisphenols are used in similar processes, such as production of polycarbonate plastics and epoxy resins. As more countries introduce legislation banning the use of BPA in these products it is possible their use and exposure could increase. Although not detected in Japan and the USA, sediments in Korea were found to contain BPAP, BPB, and BPZ (detection rate 38.2, 2.9 and 2.9% respectively, mean concentration 8.63, 0.31 and 1.86 ng/g dw respectively) (Liao *et al.* 2012c). Indoor dust samples analysed for the same group of chemicals determined BPAP was found in only 2.63% of samples in the USA at a mean concentration of 0.35 ng/g dw. BPB and BPP were only found in dust samples from China at a rate of 10.9 and 1.82%, at mean concentrations of 0.99 and 0.80 ng/g dw respectively (Liao *et al.* 2012b).

BPAP, BPB, BPP and BPZ were present at an average concentration of 0.059, 0.013, 0.207 and 0.034 ng/g fresh weight respectively (detection rate 11.2, 2.62, 3.37 and 2.25% respectively) in a selection of foodstuffs (n=267), compared to BPA at 3.0 ng/g fresh weight (56.9% detection rate) (Liao and Kannan 2013). In a study analysing the bisphenol content of canned soft-drinks BPE and BPB were not detected in any of the eleven samples, whereas BPA was only absent from one sample (Gallart-Ayala *et al.* 2011). BPB was also only detected in two samples of canned fruit and vegetables from thirty-nine samples analysed from a wide number of Asian and European countries, the concentration found was

also lower than the levels of BPA in the same samples (3.4 and 3.0 µg/kg for BPB and 6.2 and 55.7 µg/kg for BPA) (Cunha and Fernandes 2013). Conversely in a study by (Grumetto *et al.* 2008) it was discovered that although BPB was found in fewer samples than BPA (21% and 52% respectively) their mean concentrations were comparable. In cans with a epoxyphenolic lining mean concentrations were 33.4 µg/kg for BPB and 38.7 µg/kg for BPA and in cans with a low Bisphenol A diglycidyl ether (BADGE) lining mean concentrations were 37.7 and 42.3 µg/kg for BPB and BPA respectively. These results are reflected in another study that found BPB to be detected in the majority of samples that also detected BPA, however often at a largely reduced concentration (Cunha *et al.* 2011).

When subjected to aerobic degradation testing, it was found that both BPP and BPB were both less susceptible to degradation than both BPA and BPF, though neither were as stable as BPS, under anaerobic degradation however BPB had less potential to degrade than BPA, BPF and BPS (Ike *et al.* 2006). When BPB was incubated with rat liver S9 fraction to assess the metabolic activation compared to BPA, similar HPLC peaks and a similar estrogenicity pattern was obtained, producing two metabolites less polar than the parent compound, displaying particularly potent estrogenicity (Yoshihara *et al.* 2004). Though the metabolites were not specifically identified, the active metabolite was thought to be very similar to the BPA metabolite MBP, in fact incubation of S9 with both BPA and BPB formed intermediary metabolites likely formed from the dimerisation of products from both parent chemicals (Yoshihara *et al.* 2004). A further study investigated if incubation with rat liver S9 fraction metabolically activated a range of bisphenols, BPB and DMBPA both increased their estrogenic potency when incubated with S9, whereas BPAP and BPZ both experienced a reduction in potency, investigation into the structure of metabolites found the potent estrogenic fractions to consist of analogues structurally similar to MBP.(Okuda *et al.* 2011).

1.9.4.2 Health Effects

The numbers of studies investigating the ability of these bisphenols to activate nuclear receptors are few, despite the wide range measured in environmental samples, even when compared to those that exist for the alternatives already

discussed. In an ERE-luciferase reporter assay in MCF-7 cells to assess estrogenic activity, BPB was found to be of a similar potency to BPAF, activating the ERE at a much lower concentration than BPA (EC50 was 0.07 μM for BPB, compared to 0.63 μM for BPA) (Kitamura *et al.* 2005). BPB was found to bind the AR with a stronger potency than BPA and the anti-androgenic activity of BPB, investigated by means of an ARE-luciferase reporter assay using NIH3T3 cells activity was determined to be in the same order as that of potent anti-androgen flutamide (Fang *et al.* 2003, Kitamura *et al.* 2005). BPB was also found to activate the hPXR in a dose-dependent manner and was one of only two chemicals tested that were more potent than BPA (Sui *et al.* 2012).

Both BPB and BPE increased the triacylglycerol content and adipocyte-specific fatty acid binding protein mRNA of the cultures, the potency of BPB to increase triacylglycerol content was comparable to that of BPA, with BPE activity also being 93% of that of BPA (Masuno *et al.* 2005), indicating both are adipogenic agents, likely acting in similar fashion to BPA. BPP, BPM and BPAP exhibited greater genotoxicity compared to BPA based on chromosomal aberrations, with BPP demonstrating the strongest potency (Lee *et al.* 2013).

In vivo assessment of the estrogenic potential of BPB and BPZ has been performed in a small number of studies using the rat uterotrophic assay, uterine blotted weight was found to increase in rats subcutaneously administered 20 and 200 mg/kg BPB, watery uterine contents were also discovered in rats exposed to 200 mg/kg (Yamasaki *et al.* 2002). Watery uterine fluid was also detected in rats similarly dosed to 150 mg/kg BPZ, uterine weight was significantly higher in rats exposed to 30 and 150 mg/kg.

The acute toxicity of several bisphenols to *D. magna* was determined by (Chen *et al.* 2002) this study found that based on the 48-hr EC50 BPA was more toxic than BPE (10.0 and 18 mg/l respectively), whereas BPB and BPP (5.5 and 1.6 mg/l respectively) were both found to be more toxic.

All the results in the above sections indicate that it is likely that these alternatives to BPA may have similar estrogenic effects, though potency may vary. It would also appear that some are capable of disrupting signalling pathways other than estrogenic, as has been reported for BPA. There are many discrepancies in the *in vitro* estrogenic potential with potencies varying for individual chemicals

between cell systems and methodologies. Further experimental data, particularly *in vivo* work is necessary to gain a greater understanding of the possible risks these chemicals may have to wildlife and human health should exposure increase. In addition a greater understanding of how they interact with different ERs and exert their observed effects is also necessary.

1.10 Methods to assess endocrine disruption

As described in previous sections there are a wide variety of experimental methods and models that have been developed to determine the various effects of EDCs, with a large number of these employed to investigate the actions of BPA and its related chemicals. *In vitro* approaches have several advantages over i*n vivo* experiments, including ethical, economical and technical. They also tend to be cheaper and easier to set up, especially where adequate *in vivo* facilities do not already exist. Turnaround and throughput can also be much higher in many cases. However there are also several limitations to using *in vitro* systems in that they may ignore compounding biological factors that could affect activity of the tested chemical, including bioavailability, bioconcentration, certain expressed co factors or metabolism, which may serve to alter chemical activity (Folmar *et al.* 2002, Van den Belt *et al.* 2004).

A common *in vitro* assay routinely used to screen estrogenic chemicals is the yeast estrogen screen (YES) which reports estrogenic activity of a chemical as an E2 equivalent. This may be used to assess either estrogenic or anti-estrogenic activity. Other systems include the luciferase gene-expression (ER-CALUX) assay, reported to be more sensitive than YES; or ER reporter assays which may be carried out in a variety of cell lines increasing their relevance within specific studies (Cabaton *et al.* 2009, Bermudez *et al.* 2010). Further to reporter lines there are cells that specifically respond to estrogens, for example MCF7 cells which have a proliferative response to estrogens (Hashimoto and Nakamura 2000). Although *in vitro* methods may be useful indicators of estrogenic activity, in particular with regards to receptor binding, they may lack key pathways that could occur *in vivo*.

Due to these limitations it has been necessary to still utilize a range of *in vivo* models and exposures to help further elucidate the activity of these chemicals, indeed with regards to BPA a great number of model species across different

taxa have been utilised. Various short and long term outcomes can be measured including reproductive, physiological and molecular endpoints. Fish have emerged as leading taxa in *in vivo* assays, due to both their environmental relevance, suitability as a model vertebrate and ease of use. In particular the zebrafish (*Danio rerio*) have become an increasingly used model species in recent decades. Attributes leading to its increased use include ease of breeding and developmental observation, similarities with mammalian physiology and development and more recently the advent of powerful tools allowing genetic manipulation. These properties have led to zebrafish being used in a wide range of studies including those to determine vertebrate embryonic development, as a model for studies on human disease, including cardiovascular, metabolic, immunological, tumour development or disorders of various tissues/organs (Yoder *et al.* 2002, Chico *et al.* 2008, Oka *et al.* 2010, Liu and Leach 2011, Swanhart *et al.* 2011, Xi *et al.* 2011). Zebrafish have also been widely used in ecotoxicology, in studies of estrogenic chemicals and others to determine the effects that may occur in the environment and the molecular mechanisms by which these effects may occur (Örn *et al.* 2003, Van den Belt *et al.* 2003, Vosges *et al.* 2010).

A further development in recent years has been the generation of transgenic organisms, furthering the effectiveness and insight gained by fish in research. Many fish lines have emerged as new biosensor systems for ecotoxicology research or to assist in the modelling of human disease. It is now possible to visualize expression of a target gene of interest, which may be used to label specific cells, tissues or response pathways using reporters such as green fluorescent protein (GFP). This allows incorporation of both *in vivo* and *in vitro* advantages, throughput analysis can be rapid and highly sensitive, with fairly low running costs (although line establishment requires significant investment), whilst also taking into account the real-time action of the chemical in a live organism.

There have been a number of transgenic zebrafish lines developed to detect estrogenic activity, reviewed by (Lee *et al.* 2015). The most effective lines general employ an estrogen response element (ERE) promoter, which binds the estrogen receptor-ligand complex initiating transcription of estrogen responsive genes.(Legler *et al.* 2000) developed the first of these models incorporating 3 x EREs inserted upstream of TATA minimal reporter with luciferase as the reporter.

Although responses were titrated down to low concentrations (3 ng/l EE2) fish needed to be terminated and dissected, removing one of the key benefits of transgenic fish – that changes may be detected in real time *in vivo (Bogers et al. 2006)*. Another line was created by (Gorelick and Halpern 2011) containing 5 x ERE upstream of a mouse *c-fos* minimal promoter with GFP as the reporter. This fish was effective in regards to tissue specificity, with responses seen in various tissues and to different environmental estrogens; however the sensitivity was less effective than other models, with E2 concentrations of 1-100 µg/l employed to induce responses. More recent work claimed that a response could be detected from environmental samples, however it should be noted that these samples were filtered and eluted at a much higher concentration than originally sampled (Gorelick *et al.* 2014).

Currently the most sensitive estrogen responsive transgenic zebrafish was developed by (Lee *et al.* 2012) and incorporates a 3 x ERE and a *Tol2*-mediated GAL4FF-UAS system. This model is capable of detecting responses down to 1 ng/l EE2 and 5 ng/l EE2. Also responses were detected in a wide variety of tissues, including the liver, heart, skeletal muscle, neuronal cells and the brain. There was also evidence of tissue specific expression patterns for different environmental estrogens, indicating different specific tissue toxicities (Lee *et al.* 2012).

1.11 Aims of this thesis

Despite the fact that BPA is a chemical of concern for both human health and the environment and various adverse effects have been linked to its exposure, there is still a lack of evidence on specific tissue activity and the mechanisms by which these effects are mediated. As concern grows and public awareness of these effects increases the demand for products free of BPA is becoming increasingly more common. There is still very little research on the *in vivo* effects these structurally similar chemicals may have.

The overall aim of the work presented was to use the zebrafish including the novel estrogen responsive transgenic zebrafish as a model organism to assess the tissue specificities and responsiveness for BPA and its most commonly used alternatives. Whilst also attempting to further determine the mechanisms of action for BPA with particular respect to a key target organ, using an integrated

physiological and molecular approach.

In order to achieve these aims the following experimental work was conducted with the following specific aims:

Aim 1 (Chapter 2): To compare the toxicity, teratogenic effects, estrogenic tissue targets and uptake of Bisphenol A, and its alternative replacements Bisphenol S, Bisphenol F and Bisphenol AF, in zebrafish.Also to determine if observed estrogenic effects are a result of signaling via the classical Estrogen Receptor pathway.To achieve this aim, initially, zebrafish were exposed for 96 hours to different chemicals to assess the comparative acute toxicities including effects on hatching rate and morphological abnormalities. Further investigations employed the use of ERE-Transgenic zebrafish to determine tissue responsiveness for all chemicals and thresholds necessary to induce responses. Exposures were also conducted with estrogen receptor antagonist to assess pathway(s) of action. To determine if observed responses could be linked to bioavailability, analyses to quantify the uptake of compounds into zebrafish larvae were also carried out.

Aim 2 (Chapter 3): Once it was determined that the heart was a key tissue target for BPA and other related biphenolic chemicals, Chapter 3 aimed to further investigate the specific estrogenic mechanisms and functional health effects of Bisphenol A on the heart in zebrafish. The action of BPA in zebrafish was also compared with its metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) to determine if MBP was more potent as an estrogen, as observed in mammalian cells. Finally we aimed to determine if this metabolite is produced *in vivo* in zebrafish.

ERE-Transgenic zebrafish were used to achieve these aims to determine specific targets and mechanisms of action of BPA and the potent BPA metabolite 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene on early zebrafish heart development. Immunohistochemistry was conducted to more accurately determine specific regions of fluorescence detected in the heart. To assess pathways of ER mediated action exposures were also conducted in conjunction with estrogen receptor morpholinos. A final element of this chapter sought to determine if transcriptional activation via estrogen receptors in the heart resulted in any detectable functional differences in 5 and 14 dpf larvae, this was achieved using video capture and analysis of heart and blood vessels *in vivo*.

Aim 3 (Chapter 4): The final aim of this thesis was to determine how the response seen in the heart of ERE-TG zebrafish, which indicated estrogen dependent transcription, related to transcriptomic responses in the hearts of larval zebrafish. This transcriptomic approach aimed to produce an in depth understanding of the mechanisms of toxicity of these chemicals in the heart and characterise possible toxicological effects.

RNA-seq using Illumina next generation sequencing was applied to determine gene expression profiles of ERE-TG zebrafish hearts from fish exposed to Bisphenol A. Measuring responses to Bisphenol A exposure was the primary aim, however a further aim was to determine if these responses differed from those observed in fish exposed to the classic steroidal estrogen 17α-ethinylestradiol (EE2) and the potent BPA metabolite 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), following on from work carried out in Chapter 3.

The final thesis chapter (chapter 5) aims to provide a critical overview on the key findings of the research chapters, the challenges and limitations encountered and possible future directions for study.

CHAPTER 2

Toxicity, Teratogenic, and Estrogenic Effects of Bisphenol A, and its Alternative Replacements Bisphenol S, Bisphenol F and Bisphenol AF, in Zebrafish

Manuscript in preparation

CHAPTER 2: Toxicity, Teratogenic, and Estrogenic Effects of Bisphenol A, and its Alternative Replacements Bisphenol S, Bisphenol F and Bisphenol AF, in Zebrafish.

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Abstract

Bisphenol A (BPA) is a chemical incorporated into plastics and resins that has estrogenic activity and is widely reported to be associated with adverse health effects in humans and wildlife. These reported effects have resulted in the regulation of BPA use in many countries and a growing market for BPA-free products and use of similarly structured BPA analogue replacements. Increased industrial use of BPA analogues has inevitably led to increases in their measured levels in both humans and environmental samples, however, little is known about the potential toxicity and estrogenic mode of action of these BPA replacements in *in vivo* systems.

We compared the uptake, toxicity, and teratogenic effects of the bisphenol chemicals BPA, BPS, BPF and BPAF in zebrafish and assessed their estrogenic effect mechanisms in an estrogen responsive transgenic fish Tg(ERE:Gal4ff)(UAS:GFP). The rank order for toxicity was BPAF>BPA>BPF>BPS. Common developmental deformities observed for early life stage exposures included the presence of cardiac edema, craniofacial abnormality, spinal/tail malformations, cranial haemorrhaging, pigment reduction and yolk sac malformations. These effects occurred at concentrations between 1.0 and 200 mg/l depending on compound, far exceeding most environmental concentrations. All bisphenol compounds induced estrogenic responses in Tg(ERE:Gal4ff)(UAS:GFP) zebrafish that were inhibited by co-exposure with ICI 182,780 showing an ER dependent mechanism. The tissues affected included the heart, liver, somite muscle, fins and corpuscles of stannius and the rank order for estrogenicity for the bisphenols tested was BPAF>BPA=BPF>BPS. Bioconcentration factors were determined to be 4.5, 17.8, 5.3 and 0.067 when

external concentrations measured 1000, 1000, 100 and 50000 µg/l for BPA, BPF, BPAF and BPS respectively.

We show that some Bisphenol A replacement products induce similar toxic and estrogenic, effects as for BPA and BPAF was more potent than BPA. We illustrate that bioavailability of the bisphenol products, as well as established differences in their binding affinities for ER(s) may play a role in the estrogenic potential for some analogues *in vivo*.

Introduction

Endocrine disrupting chemicals (EDCs) possess structural similarities with endogenous hormones, and/or alter hormone biosynthesis, biodegradation or excretion, and exposure to them can alter biological homeostasis, in some cases at environmentally relevant exposure concentrations. Many groups of chemicals have been identified with endocrine disrupting properties and this, together with their widespread presence in the environment, has led to health concerns for both wildlife and humans. One of the best known groups of EDCs mimic and/or disrupt the activity of estrogen (Diamanti-Kandarakis *et al.* 2009). Disruption of sexual development in wildlife is a proven exposure consequence (reviewed in Segner *et al.* (2013) and Bhandari *et al.* (2015)) and in fish exposure to environmental estrogens causes feminisation of males and alters sexual behaviour characteristics (Tyler *et al.* 1998, Vos *et al.* 2000, Segner *et al.* 2003, Kang *et al.* 2007). Estrogens, however, play much wider roles in many other developmental and homeostatic processes and therefore chemicals capable of disrupting normal estrogen signalling may have wider health effects. In humans exposure to estrogenic chemicals has been associated with increased incidences of breast (Russo and Russo 2006) and testicular cancer (McGlynn and Cook 2009), urogenital tract malformation (Boisen *et al.* 2004), decrease in immune function (Rogers *et al.* 2013), metabolic disease and heart disease (Alonso-Magdalena *et al.* 2006, Newbold *et al.* 2007).

Bisphenol A (BPA) is a chemical used in a variety of industrial materials, particularly polycarbonate plastics and epoxy resins. Due to the ever increasing popularity of these durable, lightweight materials, the production of BPA has steadily increased, with a production of more than 2.4 million tonnes in 2009 (Vogel 2009). BPA has been described as slightly to moderately toxic to aquatic organisms (Alexander *et al.* 1988) and has been identified also as an environmental estrogen. It has been measured in the urine of most humans (Calafat *et al.* 2008, Zhang *et al.* 2011) and although BPA is relatively easily conjugated and excreted, there is an almost continuous exposure for both humans and wildlife.

In human epidemiological studies, BPA exposure (measured predominantly via urinary concentrations) has been linked to a variety of health symptoms including

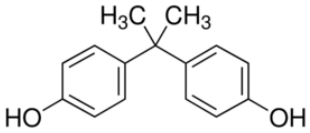
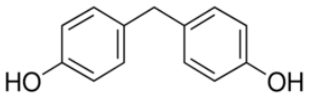
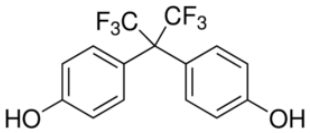
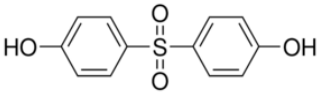
reduced sperm quality (Li *et al.* 2011) and reduced fertilisation success (Fujimoto *et al.* 2011), Polycystic Ovarian Syndrome (Takeuchi and Tsutsumi 2002), obesity (Shankar *et al.* 2012), cardiovascular disease (Melzer *et al.* 2012, Melzer *et al.* 2012), and type 2 diabetes (Shankar and Teppala 2011). However, it should be noted that these reported effects are statistical associations only and the findings have been treated with some caution (LaKind *et al.* 2012, Lakind *et al.* 2014). Many of the associated health effects are based on a spot analysis of BPA and the studies have not necessarily considered historical exposures to BPA or exposure simultaneously to other chemicals. Controlled exposures to BPA, in rodents however, have shown effects similar to those identified in human epidemiological studies, and they include developmental defects in reproductive tissues, immune system effects, and neuro-developmental effects (reviewed in (Richter *et al.* 2007). Some biological effects have also been reported in studies for environmentally relevant exposure doses and below the European Food Safety Authority currently recommended dose of 4µg/kg body weight/day (Newbold *et al.* 2009).

The aquatic environment is a major route for the disposal of industrial and domestic chemicals, including bisphenols, and health impacts of BPA exposure are documented for a range of aquatic animal species. The majority of studies into the effects of bisphenol exposure, have focused on fish (Oehlmann *et al.* 2009, Flint *et al.* 2012) and effects predominantly relate to sexual development and function, and include the induction of the egg precursor protein vitellogenin (VTG) (Larsen *et al.* 2006, Mandich *et al.* 2007), intersex (the presence of female oocytes in male gonads) (Mandich *et al.* 2007), inhibitory effects on sperm maturation and numbers (Sohoni *et al.* 2001), oocyte atresia (Mandich *et al.* 2007), alterations in sex steroid level (Labadie and Budzinski 2006) and modified behaviour (Weber *et al.* 2015). Despite the possible wide range of health effects associated with exposure to BPA proposed in mammals and fish, studies have been restricted largely to effects on sexual development/reproduction. Effects of BPA exposure on development in fish for the most part, have been reported only for concentrations far exceeding those found in most natural environments (Lam *et al.* 2011, Chow *et al.* 2013).

In response to the significant data sets published supporting adverse health effects associated with BPA exposure, several authorities have taken steps to

reduce human exposure. As an example, use of BPA in food contact materials has now been banned in Japan and Canada and in 2011 the European Union prohibited the manufacture and import of baby bottles containing BPA (European_Commission 2011). This action has increased public awareness about BPA, resulting in a demand for BPA-free products and the manufacture and use of bisphenol chemicals as alternatives to BPA (enabling those products to be classed as BPA-free). These alternatives (the most commonly used being Bisphenol AF -BPAF, Bisphenol F -BPF and Bisphenol S -BPS) possess the same basic structure as BPA with two phenol groups with the hydroxyl groups at the para positions, linked by a carbon or sulphur bridge, and with substituents attached to this bridge that can vary and include, for example, alkyl groups and halogens (see Table 1). The similarities in structure of the alterative bisphenols to BPA make them ideal as replacements for use in polymers, however, these structural similarities also give cause for concern that they may also have estrogenic activity and induce similar associated health effects as BPA (Kitamura *et al.* 2005).

Table 1. Bisphenol A (BPA) and its structural analogues

Compound	Structure	Alternative names
BPA		2,2-Bis(4-hydroxyphenyl)propane 4,4′-Isopropylidenediphenol
BPF		Bis(4-hydroxyphenyl)methane 4,4′-Methylenediphenol,
BPAF		4,4′-(Hexafluoroisopropylidene)diphenol 2,2-Bis(4-hydroxyphenyl)hexafluoropropane Hexafluorobisphenol A
BPS		4,4′-Sulfonyldiphenol 4-Hydroxyphenyl sulfone Bis(4-hydroxyphenyl) sulfone

Production volumes of Bisphenol F (BPF), Bisphenol S (BPS) and Bisphenol AF (BPAF) have increased in recent years associated with their increasing widespread uses. Data on production volumes for these bisphenols is limited, but in the US in 2012, production volumes have been estimated at 161, between 453.6-4536, and 180 tonnes for BPF, BPS and BPAF, respectively (USEPA 2012). BPF and BPS are used as a monomer in polycarbonate and polyethersulfone, respectively, and in epoxy resins. BPS has also emerged as the leading replacement for BPA as a developer in thermal paper, while BPAF is commonly used in polycarbonate, electronic products and optical cables (Gardziella 2000, Biedermann *et al.* 2010, Delfosse *et al.* 2012).

BPF, BPS and BPAF are detected in food and beverage products (in the USA) at concentrations generally below 1ng/g (Liao and Kannan 2013). BPS has also been detected in thermal receipt papers, currency bills and other paper products, with the highest concentration of 22 mg/g in thermal paper (Liao *et al.* 2012). Indoor dust samples have been shown to carry bisphenols and BPA, BPF and BPS have been shown to account for >98% of the total bisphenol content in house dust. River and sediment samples also contain BPF and BPS, occasionally at concentrations similar to BPA (Fromme *et al.* 2002, Stachel *et al.* 2003, Liao *et al.* 2012). BPAF is generally found in rivers and sediments at lower concentrations than for BPA, BPF and BPS. (Yang *et al.* 2014). BPS has been detected in 81% of urine samples from American and Asian populations confirming human exposure (Liao *et al.* 2012).

Toxicity of bisphenol analogues has received little attention when compared to BPA. Studies assessing BPF, BPS and BPAF for estrogenic activity in *in vitro* reporter systems (Hashimoto and Nakamura 2000, Kitamura *et al.* 2005, Zhang *et al.* 2011) have reported varying potencies. Overall, however, findings appear to indicate BPF and BPA possess a similar estrogenic potency, and BPAF as more potent than BPA. Some *in vitro* studies have reported BPS as being weakly active as a ligand for the estrogen receptor, but others indicate an equal potency to BPA (Masuno *et al.* 2005, Grignard *et al.* 2012). BPA and BPS have been reported to induce DNA damage *in vitro* in HepG2 cells at 0.1 μM, whereas BPF and BPAF showed no such effects at 10μM (Fic *et al.* 2013). The mechanism(s) of action for this effect has not been established.

Few studies have assessed the endocrine disrupting potential of these alternatives to BPA *in vivo*. In mammals, BPS, BPF and BPAF have been shown to be estrogenic in the uterotrophic assay (Yamasaki *et al.* 2003, Yamasaki *et al.* 2004). Other effects of BPAF observed in mammals *in vivo* include reductions in cholesterol, testosterone and white blood cell counts, increase in serum thyroxin values and a disruption of the estrous cycle (Feng *et al.* 2012, Umano *et al.* 2012). In zebrafish, exposure to 0.5 μg BPS/l has been shown to impact negatively on reproductive endpoints and gonadosomatic index (Ji *et al.* 2013). An increase in concentrations of plasma E2 in males and females (at concentrations of 0.5 and 50 μg BPS/l, respectively) and reduced testosterone concentrations in males (at 50 μg BPS/l) have also been reported for aqueous exposures (Ji *et al.* 2013). These findings highlight that bisphenols marketed as safer alternatives to BPA, may similarly induce widespread and varied health effects.

In this study we aimed to determine if the *in vivo* toxicity and sub-lethal effects of BPA differed from that of its commonly used alternative analogues, BPS, BPF and BPAF to assess the possible health implications of the increasing use of these chemicals in BPA-free products. As the estrogenic nature of BPA has been attributed to receptor interactions by the hydroxyl groups at para positions on the phenol groups, we hypothesised that its related analogues that possess a similar structure would behave similarly. To test this we employed the use of an estrogen responsive transgenic fish, Tg(ERE:Gal4ff)(UAS:GFP) (Lee *et al.* 2012) to investigate for target tissues and potency for estrogenic effects.

Materials and methods

Fish source, culture and husbandry

Adult zebrafish for the provision of embryos were kept in aquaria (37.5L) at the University of Exeter in mixed-sex stock tanks, as described in (Paull *et al.* 2008). Effects of bisphenols on embryo development and toxicity were studied in wild-type WIK strain (originally from the Max Planck Institute, Tubingen, Germany). The estrogenic potency and tissue targets for estrogenic responses were analysed in Tg(ERE:Gal4ff)(UAS:GFP), a transgenic zebrafish developed at the University of Exeter (Lee *et al.* 2012). Fish were allowed to breed naturally and eggs were collected in egg collection chambers approximately 1 h post-fertilisation (hpf). Eggs were sorted to remove any unfertilised embryos prior to use.

Chemical exposures

Bisphenol A (purity >99%), Bisphenol F (purity 98%), Bisphenol S (purity 98%) and Bisphenol AF (purity 97%) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK). All stock chemicals were dissolved in ethanol in glass bottles and diluted in ethanol to the required stock concentrations prior to dilution in embryo test water. All tests were performed in reconstituted purified water in accordance with ISO (International Standard Organisation) guideline 7346-3. Chemicals in ethanol stock were dissolved in test water to give a final ethanol concentration of 0.01%. Solvent controls contained the equivalent of ethanol in ISO water without chemical. To confirm that the responses observed in the Tg(ERE:Gal4ff)(UAS:GFP) zebrafish exposed to the different bisphenol chemicals were mediated by an ER-dependent pathway, embryos were co-exposed with estrogen receptor antagonist ICI 182,780 (ICI) (Tocris Bioscience, Bristol, UK) ICI was also dissolved in ethanol and exposed to embryos at a concentration of 607 µg/l (1 µM).

Embryos were exposed to bisphenolic chemicals or to solvent control. Each experimental group consisted of 20 embryos exposed in 100 ml of water and each treatment group was run in triplicate. 20 embryos are in line with regulatory testing that follows OECD guidelines for Fish Embryo Acute Toxicity (FET) Test (Test No. 236). Although FET does not suggest a replicate number, tests were

run in triplicate to increase statistical power, whilst still maintaining a manageable workload, considering test implementation and data collection. All experiments were conducted in temperature controlled laboratories held at 28 ± 1 °C, under semi-static conditions. Exposures to determine toxic effects and morphological abnormalities were conducted from 0 hpf to 96 hpf in accordance with OECD FET testing. Throughout this period larvae were assessed on a regular basis (3, 24, 48, 72 and 96 hpf) and mortality, hatching rate and abnormalities recorded. Mortality was determined based on no visible heart beat in larvae. Morphological abnormalities were observed and photographed using an Olympus SZX16 microscope equipped with an Olympus XC10 camera. Exposures to determine estrogenic response by GFP induction assessments were conducted from 0 hpf (hours post fertilisation) to 120 hpf. At the end of this exposure period 120 hpf old larvae were processed for fluorescent imaging analysis.

Image analysis of Tg(ERE:Gal4ff)(UAS:GFP) zebrafish

Live Tg(ERE:Gal4ff)(UAS:GFP) larvae were anesthetised with 0.4% tricane, mounted in 3% methylcellulose in ISO water and placed onto a glass-bottom 35 mm dish (MatTek, Ashland, MA, USA). All larvae were observed in lateral view and images were obtained using a Zeiss Axio Observer.Z1 equipped with an AxioCam Mrm camera (Zeiss, Cambridge, UK). All photographs were taken using the same parameters using a X10 objective. Exposure times adopted were dependent on the region photographed due to differing levels of fluorescence intensity in different target organs, (50ms for head region, 20ms for mid body region and 400ms for tail section). Exposure times were selected based on generating a strong enough signal for clear visual interpretation without over-exposure (identified using the Axiovision Imaging software as over-exposed areas are shaded red) as this may affect intensity quantification. Exposure time was set by preliminary observations of 10 individual larvae of the highest concentration for each chemical, as this concentration would be most likely to cause over-exposure. Once set this was kept consistent for specific regions across the fish body. Photographs were processed using the Axiovision Imaging software and fluorescence quantification was calculated using the ImageJ software (http://rsb.info.nih.gov/ij/). For each picture intensity was measured as the mean grey value of all the pixels within a region of interest, and the region of interest 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).

Determination of chemical concentration in exposure water

For each compound (BPA, BPS, BPF and BPAF) various nominal concentrations were tested (Table 3), and water samples from control and bisphenol exposure tank were analysed in triplicate. Up to 100 mL of tank water was collected to which 2 % of methanol and 0.1 % of acid acetic were added. The water samples were then extracted through an Oasis HLB (6 mL, 200 mg) cartridge (Waters, Manchester, UK), which was previously conditioned with 5 mL of methanol and 5 mL ultrapure water at a flow rate of 5-10 mL/min. Prior to the SPE extractions, two internal standard (BPA-d8 and 2,2'-BPF) were added. The amount of internal standard (IS) added was calculated so that the ratio of compound/IS was 1/1. The cartridge was washed with 5 mL of distilled water, and was then dried under vacuum and elution was performed with 5 mL of methanol. Extracts were dried, reconstituted in water/acetonitrile (7/3, v/v) and passed through 0.22 µm centrifuge filters. Recovery test of the SPE protocol performed at a low and high concentration for each compound (n=4 for each concentration) gave values ranging from 83 ± 2 to 108 ± 9% (Table 2).

Ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analyses were carried out using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Samples were separated using a reverse phase Acquity UHPLC BEH C18 column (1.7 µm particle size, 2.1 mm × 100 mm, Waters, Manchester, UK) 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; then from 30:70 to 0:100 and held for 4 min, then return to initial condition at 12 min and equilibration for 6 min. Retention times, ionisation and fragmentation settings are reported in Table 2. 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 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 BP 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 ($R^2 > 0.99$) covered the range 125–2500 pg (injected on column) for all compounds, within the linear range of the instrument.

Table 2. LC-MS quantification of the target compounds performed by monitoring two characteristic multiple reaction monitoring (MRM) transitions

Compound	Parent ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
BPA	227.1	212.1	20
		133.1	28
BPF	199.1	149.1	22
		105.0	23
BPAF	335.0	265.1	24
		177.0	46
BPS	249.0	108.0	28
		92.0	40

Determination of internal chemical concentration

For a series of selected exposure concentrations, namely 1000 µg/l BPA, 100 µg/l BPAF, 1000 µg/l BPF and 50 000 µg/l BPS, analyses were run to measure internal whole body concentrations. The concentrations for these analyses were based on the production of a strong GFP signal in several tissues in the ERE-TG zebrafish, approximately comparable in intensity across treatments. Chemical analysis resulted in a known concentration present in an individual larvae per treatment, based on an estimated larval volume of 1µl BCF was then calculated based on the following calculation:

$$BCF = \frac{\text{Measured concentration per larvae (µg/L)}}{\text{Nominal external water concentration (µg/L)}}$$

Sample Preparation

Fish were exposed for 120 hpf for uptake assessment. Five zebrafish larvae, previously anaesthetised with 0.5 g/l solution of tricaine (non-recoverable), in 300 µL of test solution were transferred to a 96 well MultiScreen$_{HTS}$ BV Filter Plate (Merck Millipore, Ireland) The test solution was removed under vacuum and larvae were washed with culture water (containing tricaine), to eliminate residual test solution and transferred in 300 µL of pure water to a 96-well plate (Porvair Sciences, UK). Then 300 µL HPLC- grade acetonitrile was added and samples were homogenised for 3 minutes to achieve extraction of analytes. LCMS grade water (900ul) was added to each well and after mixing the plate was centrifuged at 4000 rpm for 30min. Supernatants from each well were transferred to a 96-well plate and removed for analysis.

LC-MS Method

Analyses were performed using Surveyor MS Pump Plus HPLC pump with HTC PAL autosampler coupled to TSQ Vantage triple quadrupole mass spectrometer equipped with heated electrospray (HESI II) source (all ThermoFisher Scientific, Hemel Hempstead, UK).

Chromatographic separation was achieved using 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) water and (B) methanol. The initial conditions for the gradient consisted of 10% solvent B, which was increased to 100% in 4.5 min and maintained for 1 min before returning to the initial 10% B. The flow rate was 500µL/min. Temperature of auto-sampler was set at 8˚C while column was kept at ambient room temperature.

HESI probe was operating in the negative mode; an ion-spray voltage of -4.0 kV was applied. The heated capillary temperature was set at 275 °C and the vaporizer temperature was 60 °C. Nitrogen was employed as sheath and auxiliary gas at a pressure of 30 and 5 arbitrary units, 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 (Table 2)

Data analysis

Concentration response curves were modelled using a generalised linear model (GLM) in R (http://www.r-project.org/) and allows for calculation of LC/EC50. For a given chemical, LC50 and EC50 were defined as the concentration inducing 50% mortality or of the maximal effect, respectively. Abnormality occurrence is expressed as mean percentage ± standard error of the mean (SEM). Fluorescence data is expressed as mean fold induction above the solvent control ± standard error of the mean (SEM). Statistical analyses of fluorescence data were performed in IBM SPSS Statistics 22.

Results

Bisphenol Exposure Concentrations and Uptake into Embryos

Chemical analysis of working solutions was determined to confirm bisphenol test concentrations at the start of the exposures. All bisphenols were determined to be within 20% of their nominal concentrations, indicating accurate dosing. Controls did not contain bisphenols at the limit of detection for the different bisphenols (40 ng/l for BPA, 10 ng/l for BPAF, 100 ng/l for BPF and 1.5 ng/l for BPS). Measured internal chemical concentrations in the larvae for the different bisphenols are summarised in Table 3. Uptake of BPA was determined to be 0.31 ± 0.011 and 4.50 ± 0.42 ng/larvae for fish exposures to 100 and 1000 μg/l respectively, for 1000 μ/l BPF, 17.8 ± 1.3 ng/larvae, for 100 μg/l BPAF, 0.53 ± 0.0035 ng/larvae and for 50 000 μg/l BPS, 3.34 ± 0.15 ng/larvae respectively. Based on these, albeit limited, analyses, the BCF for BPA, BPF, BPAF and BPS were 4.5 ± 0.42, 17.8 ± 1.3, 5.3 ± 0.035 and 0.067 ± 0.0030 respectively. These values do not necessarily indicate the amount of chemical that has entered cells, therefore binding with receptors, there is also no indication as to specific tissue distribution. However, it does give an indication as to the concentration that may be bioavailable internally in comparison to the exposure water, therefore aiding in the identification of possible effect mechanisms.

Table 3. Measured water and whole body concentrations in 120 hpf zebrafish larvae for each bisphenol. Data as shown are the mean of 3 replicates, each containing 5 larvae, repeated 3 times (SEM in brackets)

	Nominal external water concentration (μg/l)	Measured external water concentration (μg/l)	Measured concentration per larvae (ng/larvae)	Estimated bioconcentration factor
BPA	100	94.2 (10.1)	0.31 (0.011)	3.1 (0.11)
	1000	986.9 (15.4)	4.50 (0.42)	4.5 (0.42)
BPF	1000	1063.3 (63.5)	17.8 (1.27)	17.8 (1.27)
BPAF	100	93.0 (4.79)	0.53 (0.0035)	5.3 (0.035)
BPS	50 000	52 300 (318.0)	3.34 (0.15)	0.067 (0.0030)

Relative toxicity of Bisphenols on zebrafish embryo development

In controls mortality rates were between 0 and 10%. Acute toxicities of the bisphenol chemicals were significantly different from one another with 96hpf LC 50 values of BPA, BPF, BPAF and BPS of 11.84, 31.78, 1.59 and 198.76 mg/l, respectively (Fig. 2) and thus a rank order of BPAF>BPA>BPF>BPS from most toxic to least toxic.

Embryos in control groups developed normally with a hatch rate between 85-100% by 72hpf. All bisphenols were shown to delay the time of hatching with EC50 (72hpf) values of 5.71, 13.77, 0.92 and 155.47 mg/l for BPA, BPF, BPAF and BPS, respectively (Fig. 1). The same rank order for the bisphenols occurred for hatching delay as for mortality (BPAF>BPA>BPF>BPS).

The incidence and type of deformity differed for different exposure concentrations and bisphenol type. Common deformities observed included the presence of cardiac edema, craniofacial abnormality (including a broadened-head, asymmetrical eye formation and lack of anterior lower jaw protrusion), spinal/tail malformations, cranial haemorrhaging and yolk sac malformations (Fig. 3). Common malformations observed for exposure to BPA were cardiac edema and craniofacial abnormality, with significant effects on these endpoints at 5.0 and 10.0 mg/l and higher respectively. Ten percent of fish were also observed to have cranial haemorrhage at an exposure of 12.5 mg BPA/l. BPF induced a range of morphological defects including cardiac edema, craniofacial abnormality, spinal malformation, cranial haemorrhage and yolk sac deformity (Table 4 and Figure 4) with significant effects at exposures of 10.0, 20.0, 20.0, 20.0 and 20.0 mg/l and above respectively. There was also a marked decline in pigmentation in zebrafish exposed to 10 mg BPF/l (Figure 4). The most potent bisphenol for development effects was BPAF, causing cardiac edema at concentrations of 1.0 mg BPAF/l. BPS caused developmental effects only at very high exposure concentrations; at 200mg BPS/l cardiac edema, craniofacial abnormalities and pronounced spinal deformity were induced. No deformities were observed in any of the control groups.

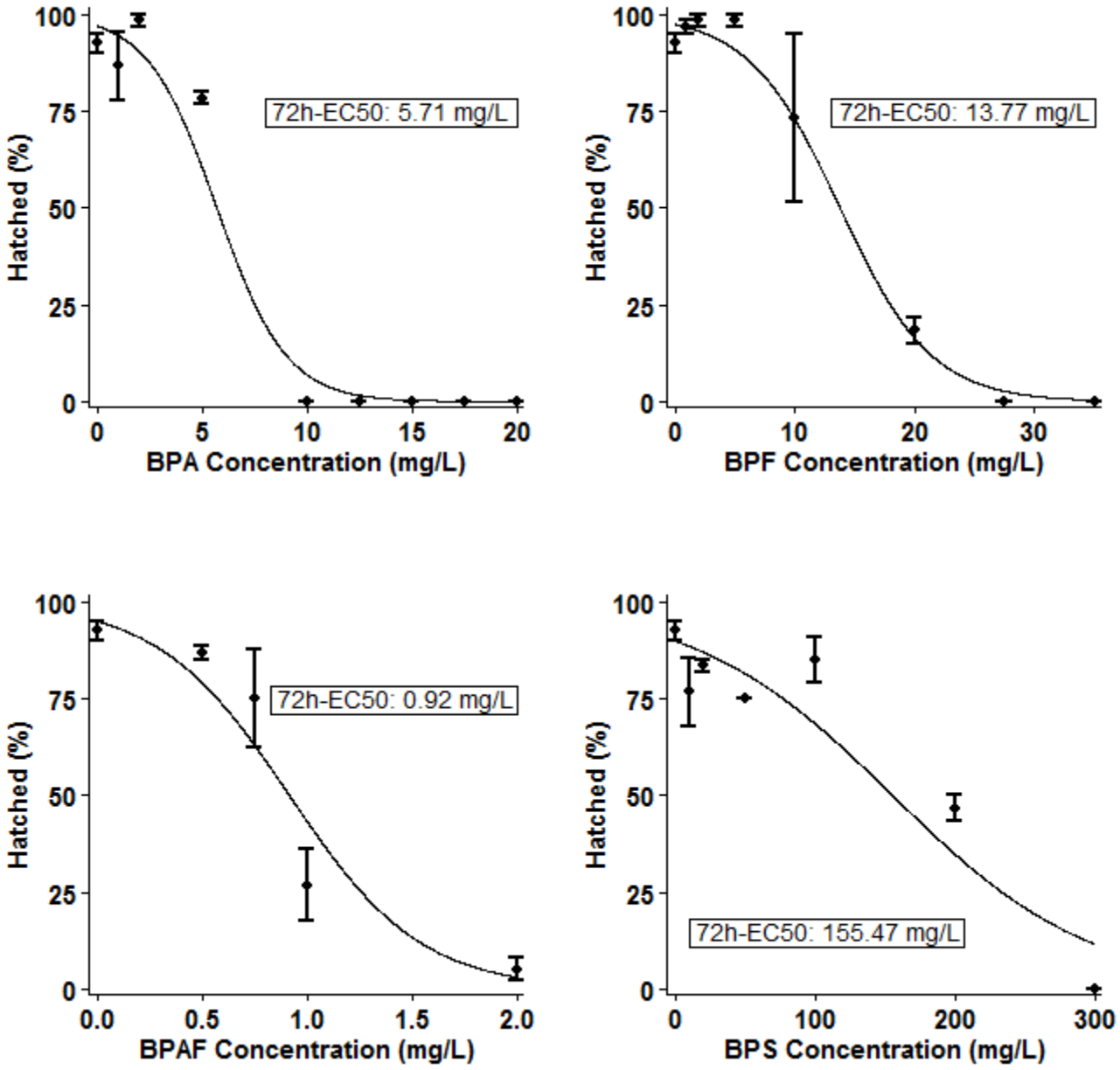


Figure 1. Hatching success rate of 72 hpf zebrafish larvae exposed to bisphenol chemicals

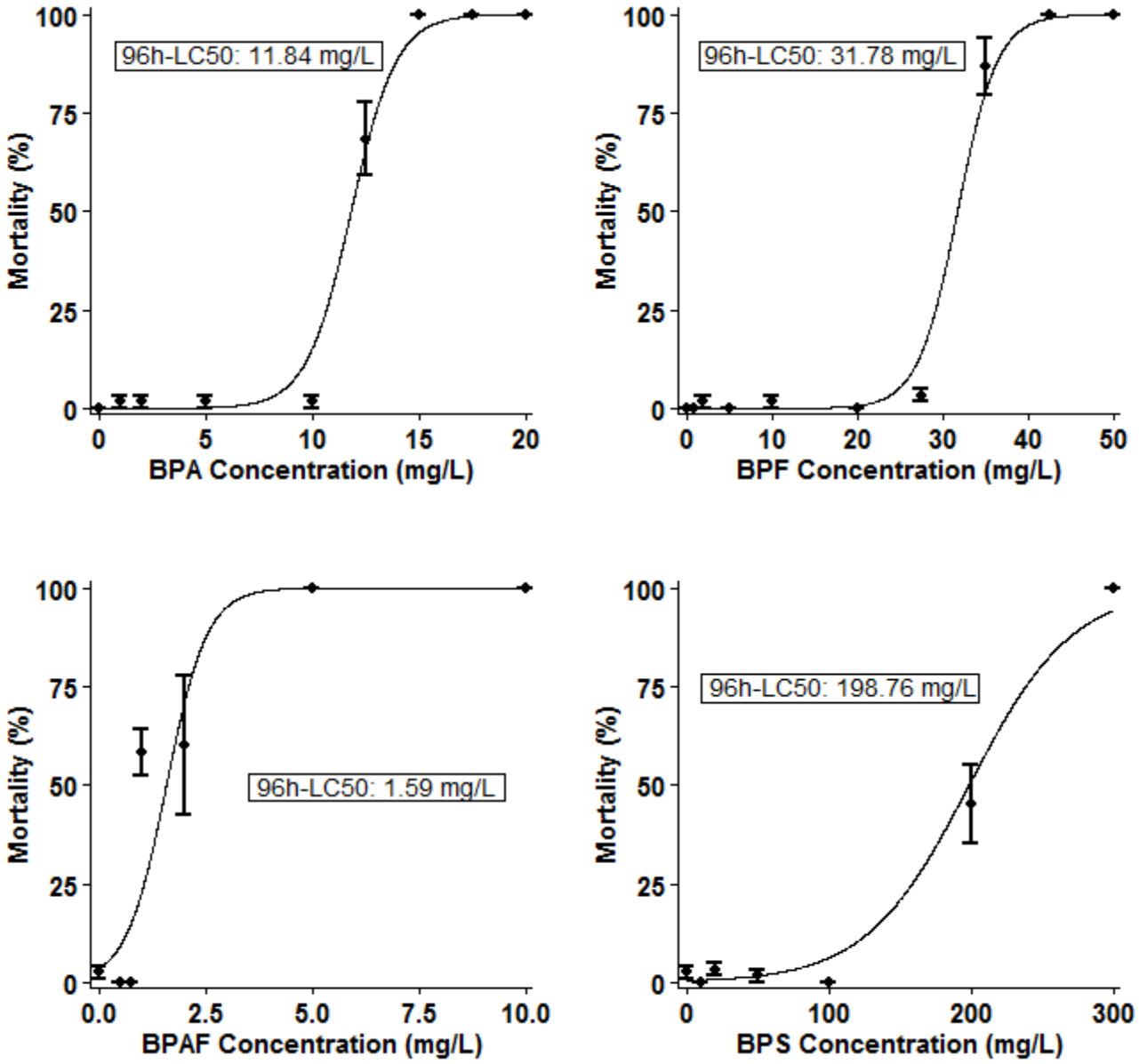


Figure 2. Mortality rates of 96 hpf zebrafish larvae exposed to bisphenol chemicals

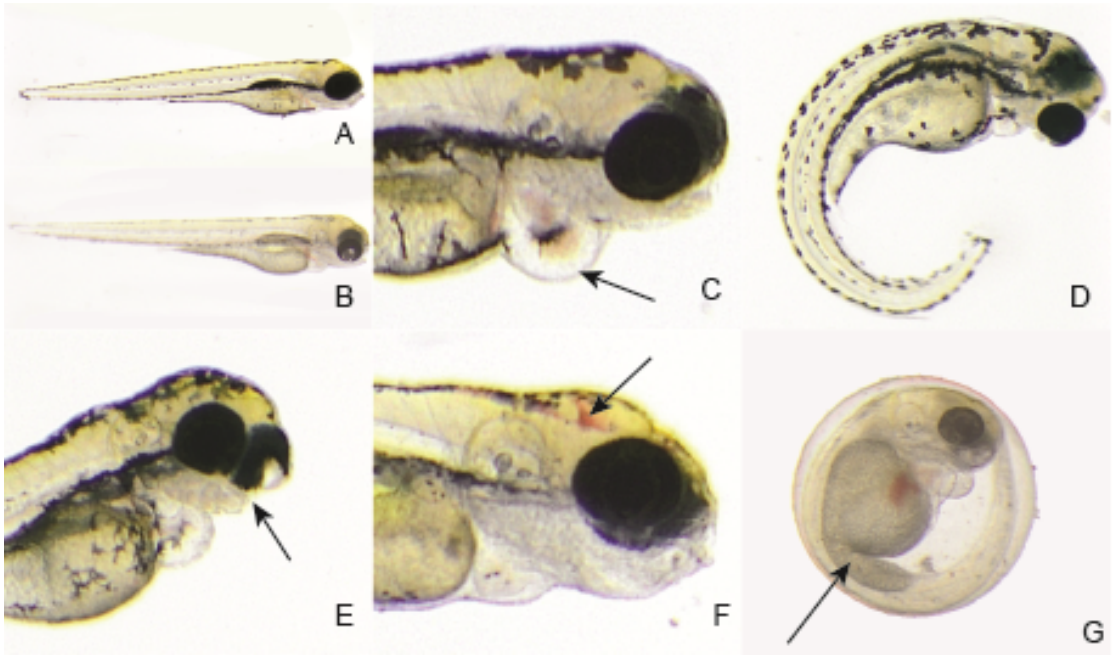
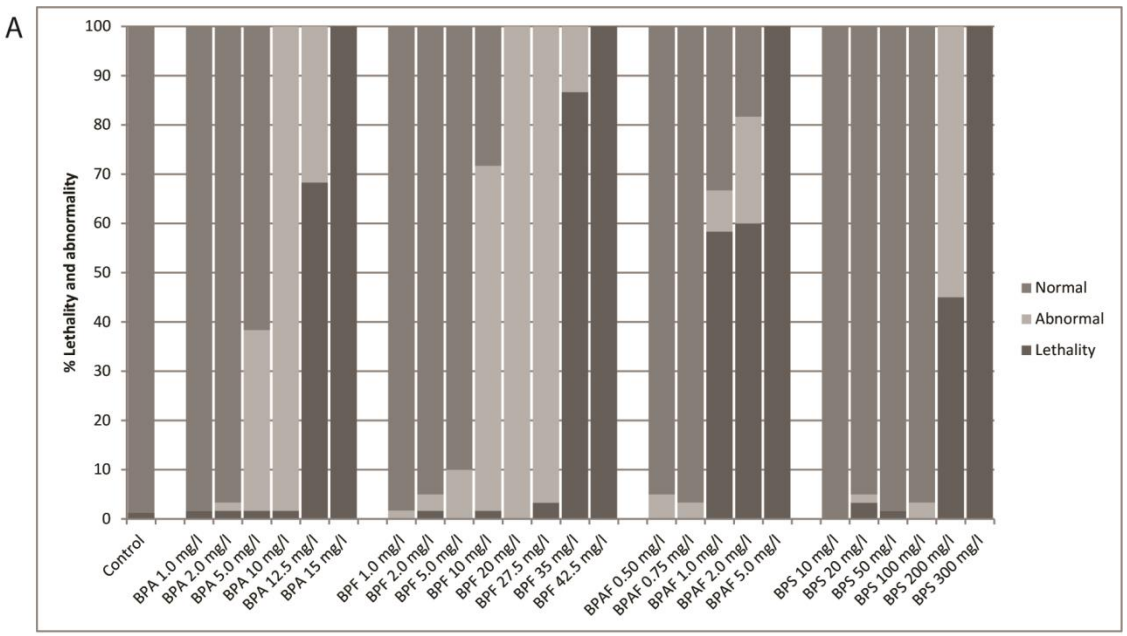


Figure 3. Examples of typical teratogenic responses of zebrafish larvae observed upon exposure to bisphenol chemicals: (A) normal, (B) pigment reduction, (C) cardiac edema, (D) spinal malformation, (E) craniofacial abnormality, (F) cranial haemorrhage, (G) yolk sac malformation.


A
% Lethality and abnormality
100
90
80
70
60
50
40
30
20
10
0
Normal
Abnormal
Lethality
Control
BPA 1.0 mg/l
BPA 2.0 mg/l
BPA 5.0 mg/l
BPA 10 mg/l
BPA 12.5 mg/l
BPA 15 mg/l
BPF 1.0 mg/l
BPF 2.0 mg/l
BPF 5.0 mg/l
BPF 10 mg/l
BPF 20 mg/l
BPF 27.5 mg/l
BPF 35 mg/l
BPF 42.5 mg/l
BPAF 0.50 mg/l
BPAF 0.75 mg/l
BPAF 1.0 mg/l
BPAF 2.0 mg/l
BPAF 5.0 mg/l
BPS 10 mg/l
BPS 20 mg/l
BPS 50 mg/l
BPS 100 mg/l
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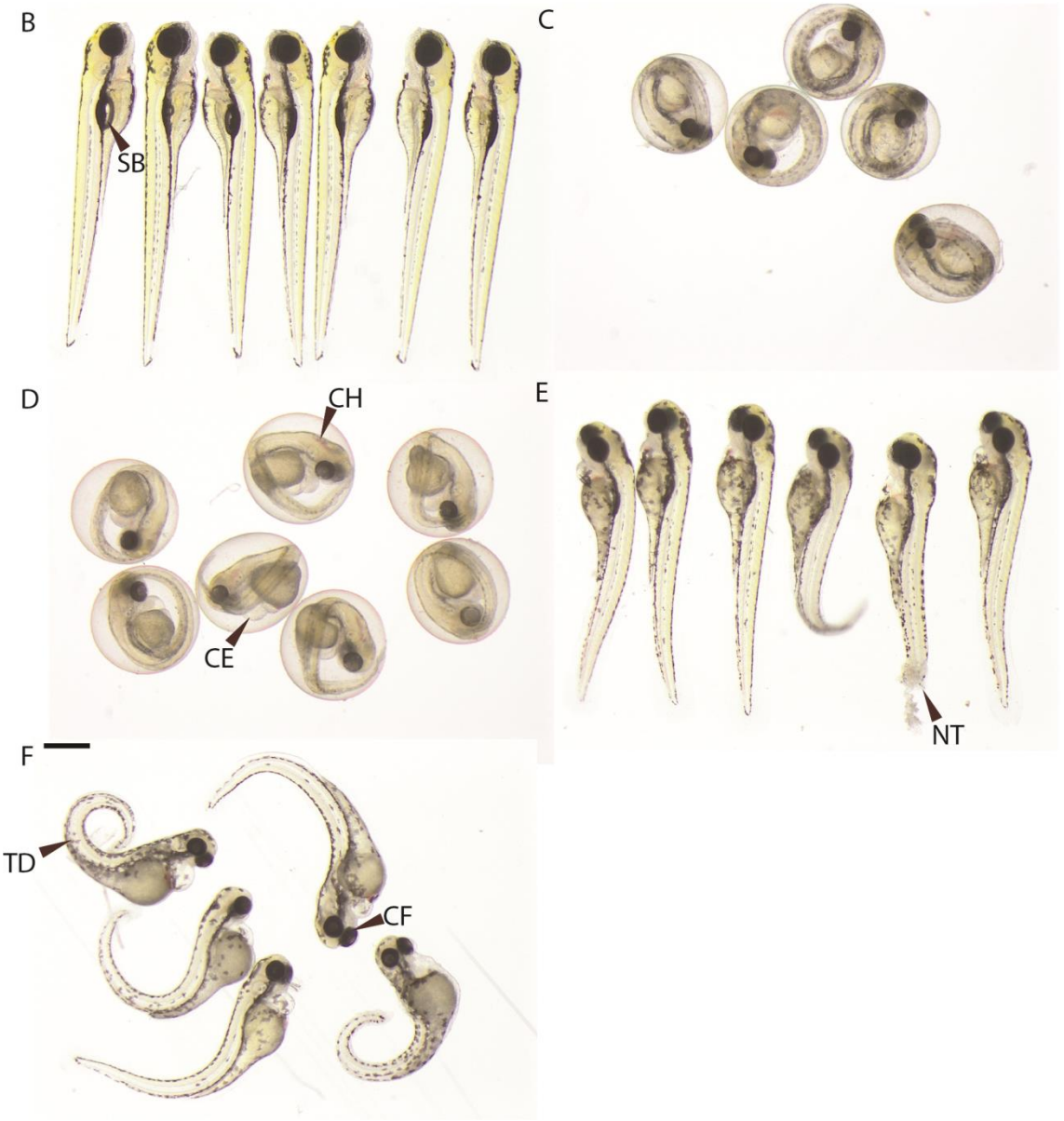
B
SB
C
D
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CF

Figure 4. (A) rates of developmental abnormality and lethality in 96 hpf zebrafish larvae exposed to bisphenol chemicals (B-F) and examples of observed abnormalities in exposed larvae: (B) Solvent Control, (C) BPA 12.5 mg/l, (D) BPF 27.5 mg/l, (E) BPAF 2.0 mg/l, (F) BPS 200 mg/l; Scale bar represents 500 μm; SB=swim bladder, CH=cranial haemorrhage, CE=cardiac edema, NT=necrotic tail tissue, TD=tail deformities, CF=craniofacial abnormalities

Table 4. Developmental abnormalities (%) observed in 96 hpf zebrafish larvae exposed to BPA, BPF, BPAF and BPS (mg/l). Data as shown are the mean of 3 replicates (SEM in brackets)

		Cardiac edema	Craniofacial abnormality	Tail development	Cranial haemorrhage	Yolk sac deformity
Control		0.0	0.0	0.0	0.0	0.0
BPA	1.0	0.0	0.0	0.0	0.0	0.0
	2.0	1.8 (1.8)	1.8 (1.8)	1.8 (1.8)	0.0	0.0
	5.0	37.4 (9.0)	0.0 (0.0)	0.0	0.0	0.0
	10.0	100.0 (0.0)	43.7 (11.7)	0.0	0.0	3.5 (3.5)
	12.5	100.0 (0.0)	100.0 (0.0)	0.0	10.0 (10.0)	0.0
BPF	1.0	1.7 (1.7)	0.0	0.0	0.0	0.0
	2.0	1.7 (1.7)	0.0	3.3 (1.7)	0.0	0.0
	5.0	10.0 (5.8)	3.3 (1.7)	1.7 (1.7)	1.7 (1.7)	0.0
	10.0	64.6 (7.3)	6.7 (4.4)	8.6 (3.6)	8.5 (4.4)	1.7 (1.7)
	20.0	100.0 (0.0)	11.7 (7.3)	11.7 (3.3)	36.7 (4.4)	21.7 (17.0)
	27.5	98.3 (1.7)	93.2 (3.4)	93.2 (14.0)	41.4 (0.70)	26.1 (11.0)
	35	100.0 (0.0)	90.0 (8.2)	90.0 (8.2)	40.0 (33.0)	40.0 (33.0)
BPAF	0.50	3.3 (1.7)	1.7 (1.7)	1.7 (1.7)	0.0	0.0
	0.75	3.3 (3.3)	1.7 (1.7)	0.0	0.0	0.0
	1.0	15.6 (8.7)	0.0	0.0	0.0	0.0
	2.0	34.6 (18.0)	2.8 (2.8)	2.8 (2.8)	0.0	0.0
BPS	10	0.0	0.0	0.0	0.0	0.0
	20	1.8 (1.8)	1.8 (1.8)	1.8 (1.8)	0.0	1.8 (1.8)
	50	0.0	0.0	0.0	0.0	0.0
	100	3.3 (1.7)	3.3 (1.7)	1.7 (1.7)	0.0	0.0
	200	94.1 (3.2)	96.3 (3.7)	82.2 (3.4)	3.7 (3.7)	0.0

Estrogenic effects of Bisphenol chemicals measured in ERE-TG zebrafish larvae

Transgenic zebrafish without chemical exposure had some detectable GFP expression in the otic vesicle (Fig. 5). This fluorescence was not found to be inducible by any of the bisphenol chemicals, with a similar level of occurrence and similar intensity across all treatments. No inducible GFP expression was detected in other tissues of unexposed larvae, however very low levels of autofluorescence were present in some tissues such as the yolk sac, but this did not affect quantitation of the estrogenic responses and were accounted for in background quantitations and subtractions for the individual tissues.

For BPA exposure, GFP expression was first detected for exposure to 100 µg BPA/l where a distinct statistically significant signal (3.2-fold GFP induction above controls) was observed in the pericardial region, confirmed to be the heart by periodical contractile movement of the expression domains. No significant GFP expression was observed in any other tissues at this exposure concentration. At the maximum concentration for BPA (1000 µg/l) a strong GFP signal was observed in the heart (23.6-fold GFP induction), liver (60.4-fold GFP induction), and tail (11.2-fold GFP induction) where somite muscle and corpuscles of stannius were observed to fluoresce. GFP expression in somite muscle was most apparent in posterior somites, with domains consisting of single muscle myotube expressing in a mosaic pattern.

BPAF, BPF and BPS demonstrated similar patterns of GFP expression to BPA, though concentrations necessary for comparative induction varied (Fig. 5). BPF had the most similar estrogenic potency with BPA, including a significant increase in fluorescence in the heart region at both 100 and 1000 µg BPF/l with GFP inductions of 3.4-fold and 29.9-fold, respectively. The heart was again the only tissue expressing GFP at 100 µg BPF/l. At 1000 µg BPF /l significant GFP induction was observed also in the liver and posterior tail region (124-fold and 13-fold, above controls, respectively). BPAF was observed to be the most potent estrogen of all bisphenol chemicals tested. Significant GFP was induced in the heart (2.4-fold increase) at 10 µg BPAF/l increasing to a 22-fold induction in GFP expression at 100 µg BPAF/l. BPAF at 100 µg/l also induced significant GFP

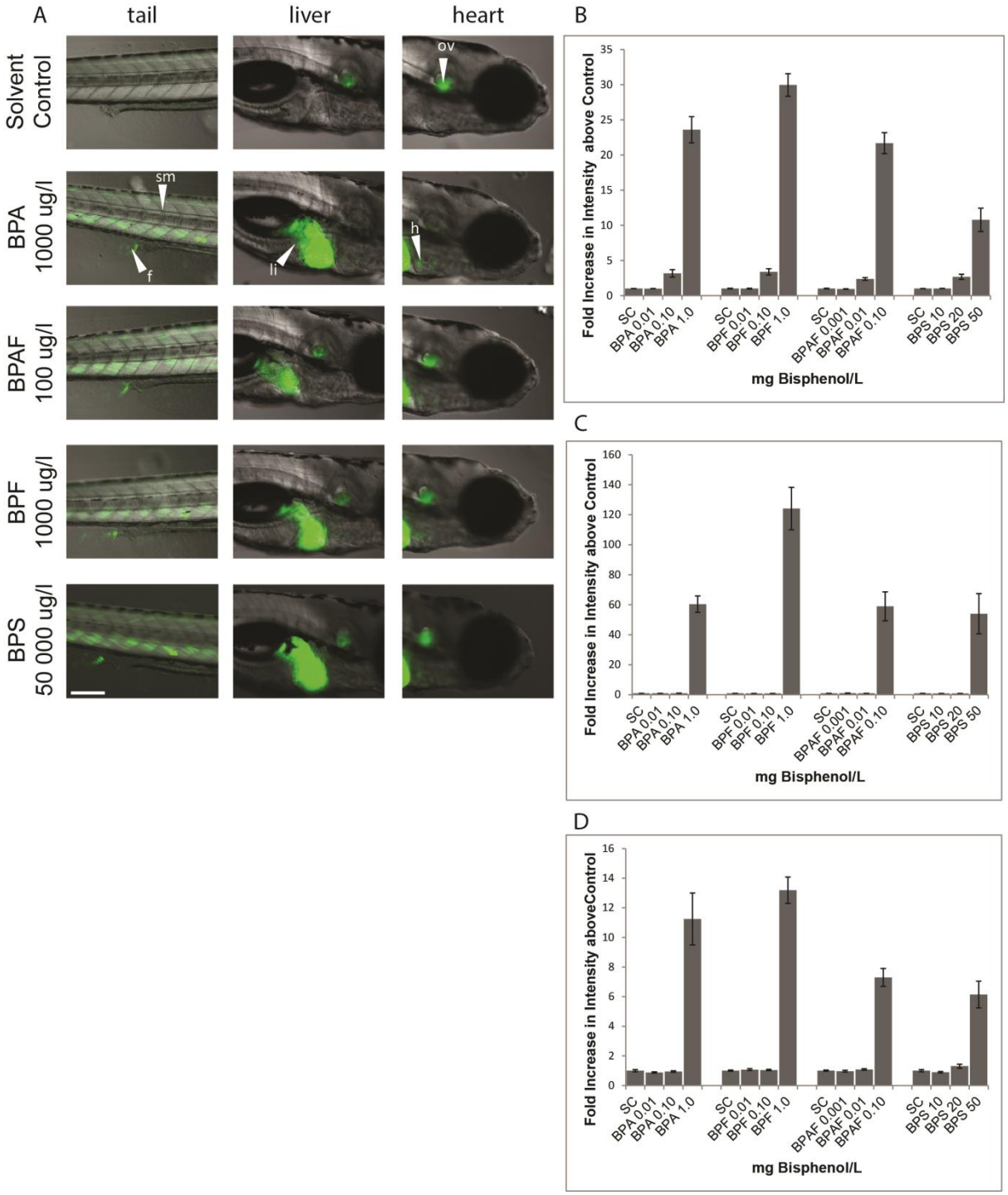

Figure 5. (A) Images of 120 hpf ERE-TG zebrafish larvae exposed to 1000µg/l BPA, 100 µg/l BPAF, 1000 µg/l BPF and 50000 µg/l BPS (images for exposures to lower concentrations not shown). Fluorescence observed in otic vesicle (ov), heart (h), liver (li), somite muscle (sm) and fin (f). Scale bar represents 200 µm; (B-D) GFP induction (fluorescence) in 120 hpf ERE-TG zebrafish larvae exposed to bisphenol chemicals measured by fold increase above control in the (B) heart, (C) liver, (D) tail somites.

induction in the liver and tail region (59-fold and 7.3- fold increases above controls, respectively). BPS was relatively weak as an estrogen in ERE-TG larvae; concentrations of 20mg/l and 50 mg/l induced 2.7-fold and 10.8-fold inductions in GFP in the heart, respectively. Somite muscle and corpuscles of stannius expression of GFP were also indicated for exposure to 20 mg BPS/l, occurring in 25.0% and 91.7% of the treated larvae, but these apparent inductions were not found to be statistically significant. Significant GFP induction occurred in the liver and tail regions only at the exposure to 50 mg BPS/l.

Estrogen Receptor Inhibitor suppression of GFP expression in ERE-TG zebrafish

Exposure of the estrogen inhibitor ICI 182,780 was carried out in tandem with various bisphenols to determine whether estrogen induced GFP expression in the ERE-TG larvae was dependent on the classic estrogen receptors (ERs). ICI 182,780 is a high affinity nonselective estrogen receptor antagonist, devoid of any partial agonism. Exposure to ICI completely removed the ability for BPA, BPF, BPAF and BPS to induce GFP expression in all tissues. Measurements of fluorescence intensity were determined to be significantly reduced for all chemicals in the heart, liver and tail regions (Fig 6). GFP intensity was determined to be no higher than control in the ICI and bisphenol exposed fish.

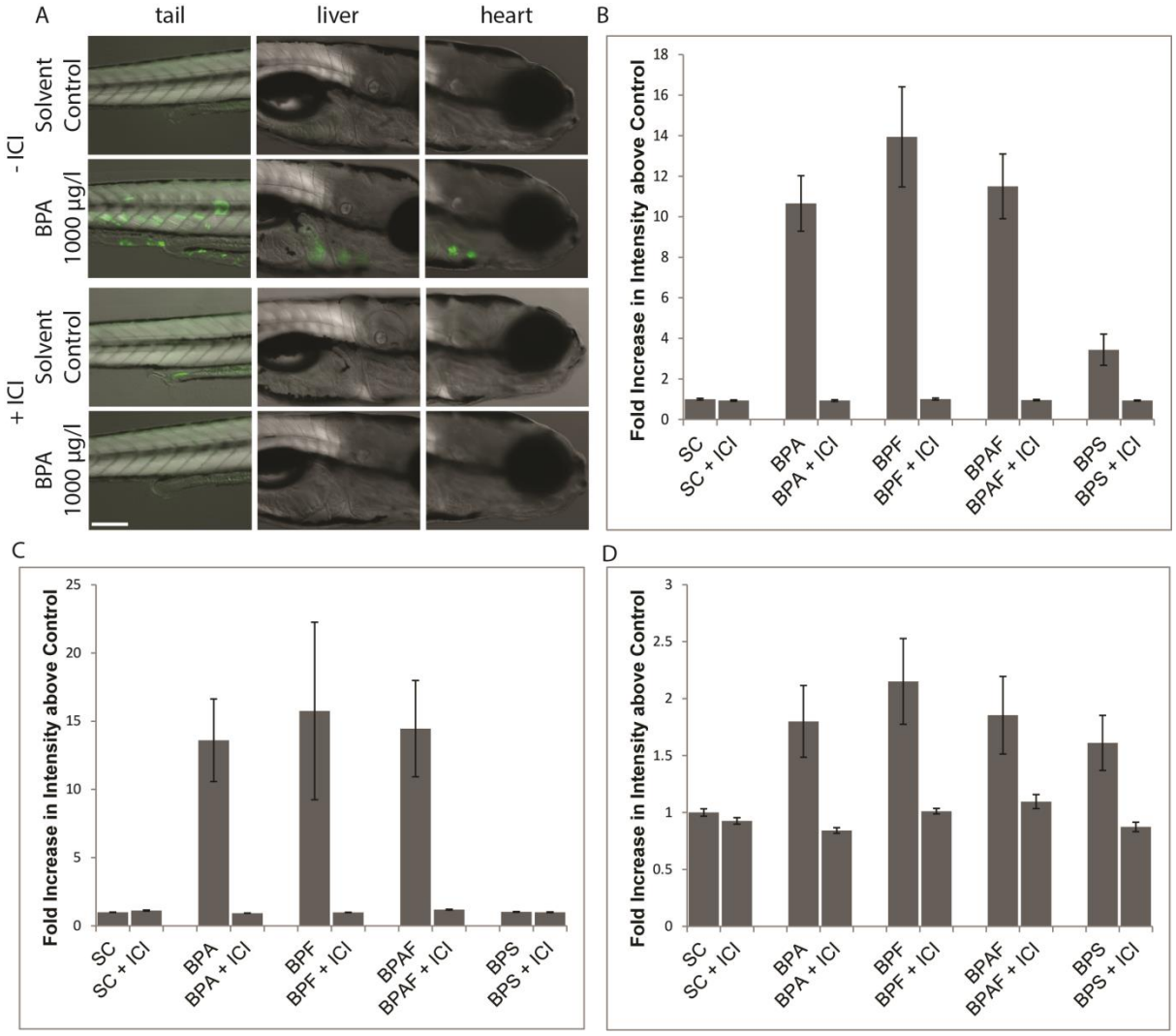

Figure 6. (A) Images of 120 hpf ERE-TG zebrafish larvae exposed to 1000µg/l BPA in the presences and absence of estrogen inhibitor ICI 182 780, scale bar represents 200 µM. (B-D) GFP induction (fluorescence) in 120 hpf ERE-TG zebrafish larvae exposed to bisphenol chemicals in combination with estrogen inhibitor ICI 182 780 measured as fold increase in GFP above control in the (B) heart, (C) liver, (D) tail somites. Bisphenol concentrations were 1000 µg/l for BPA and BPF, 100 µg/l for BPAF and 20 000 µg/l for BPS; when included ICI 182 780 concentration was 607 µg/l.

Discussion

BPA is a well-documented endocrine disruptor. Its ubiquitous use in many everyday products has led to exposure in humans and wildlife, and BPA is continuously discharged into the environment. To address environmental and health concerns for BPA, various products have recently become available marketing themselves as BPA free. These products however, often contain structurally similar monomers including the alternative bisphenols BPF, BPAF and BPS (Table 1) some of which have also been reported to be endocrine active (Liao *et al.* 2012, Yang *et al.* 2014). Comprehensive understanding on the comparative toxicity and estrogenic potency of these bisphenol A alternatives for exposures *in vivo* is still lacking. We addressed this knowledge gap and compared the *in vivo* toxic and estrogenic potencies and effects of BPA, BPF, BPAF and BPS, in zebrafish including through the use of a novel transgenic zebrafish developed for detecting environmental estrogens [Tg(ERE:Gal4ff)(UAS:GFP)].

We demonstrated that BPA and its commonly used alternatives, BPF, BPAF and BPS can be toxic in zebrafish larvae causing lethality but only for very high exposure concentrations (Fig. 2). Sub-lethal effects observed including, pericardial edema, craniofacial abnormality, pigment reduction, spinal malformation and yolk sac deformity (Table 4) occurred for lower exposure concentrations and there were distinct differences between bisphenol chemicals in their effects and potencies for these effects. The concentrations of the bisphenol chemicals required to induce toxic and developmental effects, however, was several orders of magnitude higher than those commonly measured in the environment (Fromme *et al.* 2002, Stachel *et al.* 2003, Kang *et al.* 2007, Liao *et al.* 2012). The highest levels of BPA reported in environmental waters are typically below 1 μg/l with higher concentrations more rarely measured up to 21 μg/l (Flint *et al.* 2012). For BPF, BPAF and BPS, concentrations in environmental samples, when detected, are generally below 1 ng/l, but have been recorded up to 18.99 ng BPS/l, 245.68 ng BPAF/l and 123 ng BPF/l (Fromme *et al.* 2002, Yang *et al.* 2014). The current trend for replacing BPA with its structurally similar alternatives however will inevitably lead to increased concentrations of these BPA alternatives in environmental and biological samples in the near future (Liao *et al.* 2012, Yang *et al.* 2014).

Despite their similar structure, the BPA analogues had different potencies for effects on hatching rate and mortality with BPAF approximately 6-7 fold more potent than BPA for these endpoints. In contrast, BPS showed effects at approximately 30 and 20- fold lower for delay on hatching and toxicity respectively, compared with BPA. The 72 h EC50 values for hatching rate (5.71 mg/l, Fig 1) and 96 h LC50 (11.84 mg/l, Fig 2) for BPA in our study in zebrafish are similar to those determined in previous studies (Lam *et al.* 2011, Chow *et al.* 2013).

Similar morphological abnormalities that included cardiac edema, spinal malformation and craniofacial abnormalities for the different bisphenols are consistent with those reported in the literature for BPA and may suggest similar modes of toxicity, albeit with variable potency (Duan *et al.* 2008, Gibert *et al.* 2011, Lam *et al.* 2011). However, lesions were also observed that were more associated with the different bisphenol analogues. For example, lack of pigmentation for exposure to BPF (at concentrations of 10 mg BPF/l and above; Fig. 4). This may arise as a consequence of an effect on thyroid signalling as there is evidence that BPA can bind directly to and block the TR (Moriyama *et al.* 2002, Seiwa *et al.* 2004, Zoeller *et al.* 2005) (Walpita *et al.* 2009). Pigmentation however also involves cross talk between estrogen and thyroid signalling, complicating possible effect mechanisms (Moriyama *et al.* 2002, Xu *et al.* 2007). Some pigment loss was observed in BPA exposed fish but the effect was much more pronounced for BPF exposures. Another phenotype that was more distinctive to an individual bisphenol analogue was intracranial haemorrhage for exposure to BPF (Fig. 4). This can arise through a weakening of local vasculature and previous studies have shown disruptions to thyroid and estrogen signalling can have these effects on vasculature (Lam *et al.* 2011). BPS was the least toxic of all the bisphenols tested, however, this analogue induced tail abnormalities not seen for other bisphenol and also induced the highest degree of pronounced curvatures of the spine (Fig. 4), again suggesting difference in some of the toxic mechanisms for the different bisphenols.

BPA has been shown to induce estrogen related effects in both fish and mammals for concentrations that include those that are environmentally relevant (Kang *et al.* 2007, Oehlmann *et al.* 2009, Flint *et al.* 2012). In the ERE-TG zebrafish we found the different bisphenol analogues induced similar target tissue response

patterns to that seen for BPA and included the heart, liver, tail muscle somites and corpus stannius (Fig. 5). For all bisphenols tested the most responsive tissue was the heart (Fig. 5), though at higher concentrations the greatest response level occurred in the liver. In the heart the tissues affected for the bisphenol A analogues were the atrioventricular valves and the bulbus arteriosus, as reported previously for BPA (Lee *et al.* 2012, Gorelick *et al.* 2014) Whether these heart valve responses relate to adverse health effects on cardiac function associated with BPA exposure (in humans) is a research question that needs addressing. Responses in the liver are consistent with reported effects for BPA, BPAF and BPS for inducing the hepatic synthesis of vitellogenin in fish (Flint *et al.* 2012, Naderi *et al.* 2014, Yang *et al.* 2016). Responses in the muscle somites are congruent also with the role of estrogen in muscle growth and for the corpuscles of stannius in calcium handling (Maltais *et al.* 2009).

BPAF was the most potent estrogen in the ERE-TG zebrafish inducing a response in the heart at 10 µg/l and other tissues at 100 µg/l compared with threshold concentrations at between 100 and 1000 µg/l for BPA and BPF and BPS was between 50 and 500-fold less potent than all other bisphenols. The potency order for the different bisphenols reflects that seen for their toxicity, but may operate mutually exclusive mechanisms.

Several *in vitro* studies have shown that BPAF is more potent (around 10 times more potent) than BPA as estrogen in *in vitro* cell systems (Kitamura *et al.* 2005, Bermudez *et al.* 2010, Zhang *et al.* 2011). The reported estrogenic activity of BPF and BPS in relation to BPA however are much more variable (Perez *et al.* 1998, Hashimoto and Nakamura 2000, Kanai *et al.* 2001, Chen *et al.* 2002, Stroheker *et al.* 2004, Kitamura *et al.* 2005, Kuruto-Niwa *et al.* 2005). Data from these *in vitro* studies are hard to align as little is known about the metabolic capabilities for most cell based assay systems, whether required co-factors for receptors are expressed in those cells, and they do not take into account the potential for bioconcentration, all of which can have a significant bearing on the biological effect of an EDC (Folmar *et al.* 2002, Murk *et al.* 2002). Few *in vivo* studies have investigated the BPA alternatives, but BPS has been reported to affect egg production, plasma steroid concentration and decreased hatching and survival in zebrafish from 10 µg/l (Ji *et al.* 2013, Naderi *et al.* 2014).

The data presented on the toxicity and estrogenic activity for bisphenols demonstrates that care should be taken when searching for BPA alternatives. BPF (Gardziella 2000) is a common replacement for BPA, but here we show that both chemicals share a similar level of toxicity and *in vivo* estrogenic potency, with other possible off target effects not seen for BPA. BPAF is not yet used as widely as BPF or BPS in BPA-free materials, and given its estrogenic and toxicity potencies it is not an appropriate alternative to BPA. BPS, the most commonly used monomer in thermal paper and BPA-free replacement products (Biedermann *et al.* 2010, Liao *et al.* 2012) has been reported to share a similar potency to BPA based on *in vitro* studies. Our data for zebrafish would suggest that this is not the case *in vivo*. There is, nevertheless, some remaining concern regarding BPS because of its reported resistance to degradation and persistence in the environment (Ike *et al.* 2006, Sakai *et al.* 2007, Danzl *et al.* 2009).

The mechanisms of action of BPA have been relatively well studied when compared with for its alternatives. BPA appears to be mechanistically pleiotophic. The best established mechanism is through its ability to bind to the ERs and modify gene expression, albeit at effective concentrations several orders of magnitude lower than that of 17β-estradiol (Gould *et al.* 1998, Matthews *et al.* 2001, Gertz *et al.* 2012). BPA may exert some effects through rapid nongeonomic pathways independently of classic ER signalling (Vasudevan *et al.* 2001, Alonso-Magdalena *et al.* 2005, Bouskine *et al.* 2009). BPA also has the ability to bind strongly to the estrogen related receptor ERRγ, while E2 by contrast is inactive on that pathway (Takayanagi *et al.* 2006, Okada *et al.* 2008). These ERRs can bind to EREs in ER target genes, inducing translational responses (Huppunen and Aarnisalo 2004), and it is therefore theoretically possible that fluorescence response observed in ERE-TG fish resulted partly from ERR induced promotion. Co-exposure of ERE-TG zebrafish larvae to ICI 182 780 however (an ER antagonist) abolished the GFP expression observed in all tissues for all chemicals tested (Fig. 6) strongly supporting the hypothesis that when activation of genes is induced via the ERE promoter for BPA, BPF, BPAF and BPS this activation is mediated through the classical ERs. It should be recognised however that the ERE-TG model only indicates presence of activity through ERE based gene activation, so cannot give evidence for nongeonomic effects. Also rapid signalling effects may have interactive effects with more traditional nuclear hormonal

receptor pathways (Vasudevan *et al.* 2001, Farach-Carson and Davis 2003). Whether these bisphenol analogues have effects on estrogen signalling either directly or indirectly through androgen and thyroid signalling pathways also warrants investigation. (Stroheker *et al.* 2004, Kitamura *et al.* 2005, Higashihara *et al.* 2007, Cabaton *et al.* 2009, Molina-Molina *et al.* 2013).

Potency differences between the bisphenols may also relate to differences in their bioavailability. Albeit a limited analysis, we show uptake differed between some of the bisphenol analogues (Table 3). As an example there was an approximate 4-fold higher uptake of BPF in exposed fish compared with uptake into BPA exposed fish (both with 1000ug/l exposure). In contrast uptake of BPAF was measured as possessing a similar BCF as BPA (5.3 and 4.5 respectively). BPS uptake concentration was similar to that for BPA; however, exposure concentrations were much higher for BPS, leading to a much lower BCF of 0.067. The similarities in the BCF of BPAF and BPA would suggest their marked differences in comparative estrogenic potency (10-fold higher for BPAF) predominantly relates to their comparative interactions with the ER(s). However the differences in the bioavailability of BPF and BPS compared to BPA, also appears to indicate that uptake may play a key role in the response of zebrafish to these chemicals. We do however emphasise that these analyses are based on whole body burdens of the bisphenols and do not consider comparative uptake into the different body tissues that could also affect interpretations on their comparative potency estimates.

Our findings show all the bisphenols tested are toxic to fish, albeit at concentrations that exceed those in most, if not all, environmental compartments. Potency in toxicity and estrogenic activity varied across the bisphenols tested and these probably relate principally to ability of individual chemicals to bind to and activate the ERs, albeit difference in uptake and bioconcentration observed may play a role for some analogues. Co-treatment with an ER inhibitor indicated that estrogenic activity of BPA and all the analogues tested in our ERE-TG zebrafish was mediated by the classical ER(s) signalling pathway.

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CHAPTER 3

Estrogenic mechanisms and effects of Bisphenol A and its metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) on the heart in zebrafish

Manuscript in preparation

CHAPTER 3: Estrogenic mechanisms and effects of Bisphenol A and its metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) on the heart in zebrafish.

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Abstract

Bisphenol A was originally developed as a synthetic estrogen and inadvertent environmental exposure to BPA through its subsequent use in plastics and resins in various consumer products has been associated with adverse health effects in humans, which include heart defects. There is lack of agreement on its mechanism(s) of action, some of which stem from comparisons between *in vivo* and *in vitro* test systems. *In vivo* metabolic activation of BPA produces a metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) that is reported to be up to 1000-fold more potent as an estrogen than BPA.

We investigated the comparative estrogenic effects of BPA and MBP in a transgenic, estrogen responsive (ERE-TG) zebrafish and applied a targeted morpholino approach to knockdown the three fish estrogen receptor (ERs) subtypes to determine the ER signalling pathways. We then assessed if BPA's estrogenic stimulation of heart valves in ERE-TG fish had cardiovascular functional consequences in zebrafish larvae at 5 and 14 dpf.

We demonstrate that the atrioventricular valves and the bulbus arteriosus were the primary target tissues for both BPA and MBP in the ERE-TG zebrafish. MBP was approximately 400-fold more potent than BPA. ER knock down with morpholino indicated that the signal transduction in the heart for both BPA and MBP were both mediated via an estrogen receptor 1 (esr1) dependent pathway. Measures of cardiovascular function, including blood flow/velocity, beat rate, stroke volume and blood vessel diameter at 5dpf and 14dpf showed no signs of adverse effects compared with controls except at the highest exposure concentration tested (2500 μg/l) where variation in atrial:ventricular beat rate ratio

was indicative of arrhythmia in 5dpf larvae and there was a significantly reduced heart beat rate in larvae at 14dpf.

Introduction

Bisphenol A (BPA) originally developed as a synthetic estrogen (Dodds and Lawson 1936) has subsequently been used as a monomer for the production of plastics and resins. These materials are used in a wide range of consumer products, including plastic drink bottles, food and beverage can linings, thermal paper and dental sealants (Geens *et al.* 2011). BPA can leach from these materials and is taken up into the human body either via dermal contact or via the gut through ingestion. BPA enters aquatic environments predominantly through effluent discharge from wastewater treatment plants (WWTP), but also directly from manufacturing plants, landfill leachate, and degradation of plastic litter (Corrales *et al.* 2015). Concentrations of BPA in the aquatic environment are generally below 1µg/l (Flint *et al.* 2012) but reported up to 21, 19 and 12µg/l in the Netherlands, Japan and USA, respectively (Flint *et al.* 2012). Much higher concentrations of BPA have been reported in paper-mill effluent (370 µg/l) and in landfill leachates (17, 200 µg/l) (Yamamoto *et al.* 2001, Fukazawa *et al.* 2002).

BPA possesses the ability to bind to and activate the estrogen receptor(s), mimicking the actions of the endogenous estrogen 17β-estradiol. Various adverse health effects have been attributed to BPA in mammals, including decreases in sperm production and fertility (Li *et al.* 2010, Liu *et al.* 2013, Zhang *et al.* 2013), polycystic ovarian syndrome (Signorile *et al.* 2010, Vo *et al.* 2010), obesity and diabetes (Rubin *et al.* 2001, Alonso-Magdalena *et al.* 2006) and cancer (Weber Lozada and Keri 2011, Song *et al.* 2015, Seachrist *et al.* 2016). Epidemiological data have indicated positive correlations between BPA exposure in human populations and various risk factors pertaining to cardiovascular disease (Melzer *et al.* 2010, Bae *et al.* 2012, Melzer *et al.* 2012, Shankar and Teppala 2012). BPA however has not been proven to be the causative factor in these heart disease conditions. Nevertheless, these reports have led to approaches by government bodies to regulate the use and discharge of BPA.

Several experimental studies have reported effects of BPA on the cardiovascular system in rodents, *in vitro* cell lines and *ex vivo* hearts. Lifelong exposure to BPA (0.5 – 5 mg/kg/day) in mice was shown to modify cardiac structure and function with sex specific effects (Patel *et al.* 2013). In males there was concentric remodelling, whereas in females there were increases in systolic and diastolic

blood pressure. There was also modified calcium handling protein expression, suggesting increased and reduced calcium mobility in males and females respectively, supporting changes in cardiac function. Further female specific effects reported include arrhythmia, which is exacerbated in the presence of 17β-estradiol (Yan *et al.* 2011, Belcher *et al.* 2012, Poidatz *et al.* 2012, Yan *et al.* 2013). This arrhythmic effect was abolished when animals were treated with an ER antagonist, suggesting mediation via ER signalling (Yan *et al.* 2011). BPA has been shown to decrease atrial contraction rate and force in *ex vivo* rat hearts at exposure concentrations of 10^{-6} and 10^{-4} M (Pant *et al.* 2011).

Several studies have shown that BPA can affect the function of ion channels in the cardiovascular system including via activation of Ca^{2+}/voltage-sensitive K channels (Maxi-K) (Asano *et al.* 2010), blocking of human heart sodium channels (O'Reilly *et al.* 2012), inhibition of L-type Ca^{2+} channels in mouse ventricular myocytes (Deutschmann *et al.* 2013) and inhibition of T-type Ca^{2+} channels in HEK 293 cells (Michaela *et al.* 2014). Acute exposure to BPA (dose and exposure period) has been shown to decrease ventricular conduction velocity and increase action potential duration in female hearts, however the underlying mechanisms for these effects was not explored (Posnack *et al.* 2014). Most of the studies on the effects of BPA on the heart have been conducted for exposures that far exceed those with environmental relevance. There is also a lack of knowledge for effects of BPA on heart development *in vivo* and whether this has any functional consequence.

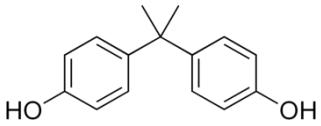
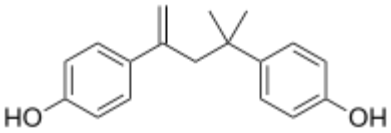
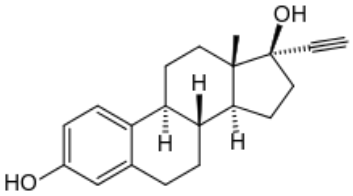
The molecular mechanisms for BPA effects in the heart are not well established. BPA has been demonstrated to act through a variety of different mechanisms including via the nuclear estrogen receptors ERα and ERβ (three isoforms exist in fish ERα, ERβ1 and ERβ2) (Gaido *et al.* 1997, Gould *et al.* 1998, Cosnefroy *et al.* 2009, Delfosse *et al.* 2012) and the orphan receptor Estrogen Related Receptor γ (ERRγ). ERRγ is thought to be capable of binding to the Estrogen Response Element (ERE) of estrogen responsive genes, however, activation of ERRγ by BPA displays a greater potency than for E2. BPA may also bind the androgen (Sohoni and Sumpter 1998, Fic *et al.* 2014) and thyroid (Moriyama *et al.* 2002) receptors and has also been reported to be capable of interacting with membrane bound receptors to induce rapid cell responses, for conditions

associated with breast cancer, and the pancreas, pituitary, cerebellar cortex and heart (Quesada *et al.* 2002, Zsarnovszky *et al.* 2005, Gao *et al.* 2013).

ERs occur in a very wide range of body tissues but the abundance of different isoforms varies and this can affect the cell sensitivity to a particular ligand (Böttner *et al.* 2014). In mammalian models moderate to high level expression of ERα occurs in uterus, testis, pituitary, ovary, kidney and epididymis, while higher expression for ERβ occurs in prostate, ovary, lung, bladder, brain, uterus and testis (Kuiper *et al.* 1998). In the heart, both ER subtypes occur in rodent and human cardiomyocytes (Grohé *et al.* 1997, Böttner *et al.* 2014). In fish too, ER subtype localisation in body tissues varies. In the fathead minnow (*Pimephales promelas*) the different ERs were all expressed in brain, pituitary, liver, gonad, intestine, and gill; esr1 and esr2b were expressed predominantly in the liver, while esr2a was expressed predominantly in the intestine (Filby and Tyler 2005). Expression of the ER subtypes can also vary during ontogeny in fish (Halm *et al.* 2004).

Responses to BPA *in vivo* are not always supported by *in vitro* studies. A possible explanation for this in mammals involves the metabolic activation of BPA that occurs *in vivo* producing a metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) that is more potent as an estrogen compared with BPA (Yoshihara *et al.* 2004). This metabolic activation may also help to explain the non-monotonic response sometimes observed for BPA exposure (Lagarde *et al.* 2015). Incubation of BPA with S9 liver fractions from mouse, monkey and humans all produce metabolites more potent as estrogens than the parent BPA compound and are thought to be MBP (Yoshihara *et al.* 2004). In rats and medaka (*Oryzias latipes*) estrogenic effects of MBP *in vivo* are between 400-1000 times greater than for BPA (Ishibashi *et al.* 2005, Nagae *et al.* 2005, Yamaguchi *et al.* 2005, Okuda *et al.* 2010). 3D modelling of human ERs and binding strength assessments for BPA, MBP and 17β-estradiol suggest that the increased spacing between the phenolic rings in MBP compared with BPA (see Table 1) allows key amino acid contacts with the ERs that are important in binding of E2 by these receptors (Baker andChandsawangbhuwana 2012). Despite these findings MBP has not yet been measured as a natural *in vivo* metabolite in any previous study and its presence is likely to be at very low concentrations. There is little information on the metabolism of BPA to MBP *in vivo* in fish, but one report

Table 1. Bisphenol A, MBP and EE2 chemical structures

Compound	Structure
BPA 2,2-Bis(4-hydroxyphenyl)propane	
MBP 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene	
EE2 17α-ethinylestradiol	

indicates an inability to produce the conjugated metabolite bisphenol a glucuronic acid in juvenile trout (Bjerregaard *et al.* 2008).

In this study we aimed to more clearly understand the specific signalling mechanisms by which BPA acts in the heart, specifically if a single ER subtype was responsible. To do this we exposed fish to estrogenic chemicals in combination with morpholino injection specific to individual ER subtypes to knockout function. We also investigated if the potent BPA metabolite MBP, described in mammalian cells was estrogenic and if it demonstrated a similar tissue specific response to the parent compound in our ERE-TG fish. If metabolic activation is a possible mode of action for BPA we hypothesised that we would see a similar GFP induction pattern but at a much lower concentration. We also aimed to determine if we could detect MBP in larvae after exposure to BPA to provide evidence this metabolite is produced *in vivo*. Finally we set out to establish whether there were subsequent detectable effects on heart function in juvenile fish as a result of early life developmental exposure to BPA.

Materials and methods

Fish source, culture and husbandry

Adult zebrafish for the provision of embryos were kept in aquaria (37.5L) at the University of Exeter in mixed-sex stock tanks, as described in (Paull *et al.* 2008). All zebrafish embryos used in this study were obtained from tg(ERE:Gal4ff)(UAS:GFP) adults, a transgenic zebrafish developed at the University of Exeter (Lee *et al.* 2012). Fish were kept on a 12/12 light/dark cycle. Fish were allowed to breed naturally and eggs were collected in egg collection chambers approximately 1h post-fertilisation (hpf). Eggs were sorted to remove unfertilised and non-viable embryos prior to use.

Chemical preparations.

Bisphenol A (purity >99%) and 17α-Ethinylestradiol (≥98%) (EE2) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK). 4-methyl-2,4-bis(4-hydroxyphenol)pent-1-ene (MBP) was synthesised by Mark Wood at the University of Exeter and purity of the compound was certified as described in (Cipelli *et al.* 2014). All stock chemicals were dissolved in ethanol in glass bottles and diluted in ethanol to the required stock concentrations prior to dilution in embryo test water. All tests were performed using reconstituted purified water in accordance with ISO (International Organisation for Standardisation) guideline 7346-3. Chemicals in ethanol stock were dissolved in test water to give a final ethanol concentration of 0.01%. Solvent controls contained the equivalent of ethanol in ISO water without chemical.

Tissue responses to chemical treatments

All exposures were conducted in a temperature controlled laboratory held at 28 ± 1 ^{o}C, under semi-static conditions. To determine tissue differences in response to BPA, MBP and EE2 embryos were exposed to these chemicals at a series of different concentrations or to solvent control. Concentration ranges for EE2 were 2.0 and 20 ng/l, BPA 10, 100 and 1000 μg/l, and MBP 0.025, 0.25 and 2.5 μg/l. Each experimental group consisted of 20 embryos exposed in 100 ml of ISO water and each treatment group was run in triplicate. The exposures were conducted from 0 hpf to 120 hpf. At the end of the exposure period 120 hpf old larvae were processed for fluorescent imaging analysis.

To investigate specific responses in the hearts of developing zebrafish to BPA and its putative metabolite MBP a similar exposure regime was undertaken as described above across a narrower chemical dosing range. In order to better visualise the location of GFP expression in the heart, larvae were stained with MF-20 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA), which stains cardiac myosin.

Embryos were anaesthetized in 0.016% tricaine and fixed for 4 hours at room temperature using 4% PFA in Phosphate Buffered Saline (PBS). Following fixation, embryos were rinsed in PBS with 0.5% TX-100 (PBSTx). Embryos were then dehydrated in a series of graded methanol concentrations, before being rehydrated and further permabilised using a Proteinase K digestion. Embryos were rinsed in PBSTx and placed in a blocking solution (1% skim milk with 1% DMSO in PBSTx) for 3 hours at room temperature, then incubated in 1:20 monoclonal mouse antibody MF-20 and 1:1000 rabbit anti-GFP antibody (ams Biotechnology) in blocking solution overnight at 4^{o}C. After rinsing in PBSTx embryos were incubated for 4 h at room temperature in the dark with 1:300 AlexaFluor 546 goat anti-mouse IgG secondary antibody and AlexaFluor 488 goat anti-rabbit IgG in antibody buffer as before. This protocol allowed visualization of cardiac muscle in red and GFP in green. Prior to heart dissection, embryos were rinsed in PBSTx. In order to visualise hearts most effectively they were dissected from fixed and stained zebrafish using tungsten wire.

Morpholino analysis and injection

Esr1 morpholino (MO) was designed against the zebrafish esr1 translational start site, with the sequence 5'-AGGAAGGTTCCTCCAGGGCTTCTCT-3'. MOs for esr2a and esr2b have the sequences 5'-AGCTCATGCTGGAGAACAC AAGAGA-3' and 5'-GAGTTCAGAGCTGTCGCATCAGTAA-3', respectively. These MOs were obtained from GeneTools, LLC (Philomath, Oregon, USA). Each MO was used at a dilution of 1:4 of a 1mM stock, with 2nl of solution injected into the yolk proximal to the blastomeres of the embryo at the one- to four-cell stage using a microinjector (INTRACEL, PICOSPRITZER®III). Fluorescence imaging of MO injected and chemically exposed larvae was conducted at 72 hpf. Timing of the chemical exposures was slightly shorter that to those conducted previously as pilot studies for the morpholino demonstrated that for a combination

of all three esr morpholinos the maximum inhibitory effect (fluorescence intensity) occurred up to 72hpf.

Image analysis of Tg(ERE:Gal4ff)(UAS:GFP) zebrafish

For fluorescence intensity measurements live Tg(ERE:Gal4ff)(UAS:GFP) larvae were anesthetised with 0.016% tricane, mounted in 3% methylcellulose in ISO water and placed onto a glass-bottom 35 mm dish (MatTek, Ashland, MA, USA). All larvae were observed in lateral view and images were obtained using a Zeiss Axio Observer.Z1 equipped with an AxioCam Mrm camera (Zeiss, Cambridge, UK). For comparison of chemicals and MO injected larvae all photographs were taken using the same parameters under a X10 objective; images of zebrafish used for cardiovascular analysis were taken using the same parameters under a X20 objective. Exposure times adopted for image capture were dependent on the stage of the larvae and the body tissue studied as the response varied in different target organs. Exposure time for image capture was kept consistent for specific regions across the fish body. Photographs were processed using the Axiovision Imaging software and fluorescence quantification was calculated using the ImageJ software (http://rsb.info.nih.gov/ij/). For each picture intensity was measured as the mean grey value of all the pixels within a region of interest, and the region of interest 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).

Confocal microscopy was used on stained dissected hearts. The hearts were mounted in 0.7 % agarose (low melting point) and ISO water was added once the agarose was set to cover them. Hearts were photographed under X20 magnification on a confocal-laser scanning microscope (ZEISS LSM510). Images were reconstituted using LSM510 Meta.

Cardiovascular functional analysis

5dpf zebrafish were maintained in ISO water in the presence or absence of BPA at 28^{o}C ± 1 ^{o}C as described for the previous expression studies. For zebrafish exposed for 14 dpf larvae were raised in 250ml test medium with a light aeration to reduce risk of oxygen depletion and build-up of ammonia from the breakdown of food waste. Fry were fed on a microencapsulated diet (ZM Advanced Fry feed,

ZM Ltd, Hampshire, UK) from 5 dpf. All uneaten food was cleared on a daily basis and exposure water was changed on a semi-static basis.

Following chemical exposure, larvae were anaesthetised (0.016% tricaine) individually, then transferred into low melting point agarose (10 mg/mL in the same tricane) before being placed in a total volume of 80 µL in a single well created by a press-to-seal silicon isolator (Sigma-Aldrich, Poole, UK) on a clear microscope slide. The orientation of the larva was then adjusted so that it lay on its side with its head to the left. Larvae were left for one minute before observation to acclimatise to handling; previous studies have demonstrated a highly reproducible fluctuation in CV activity across various treatments shortly after mounting (Parker *et al.* 2014). The slide was then transferred to an inverted light microscope (Leica DM IRB, Leica Microsystems UK Ltd., Milton Keynes, UK, 5 × objective) fitted with two high speed video cameras. One camera was positioned to capture the whole heart at 30 frames per second (fps. Grasshopper® GRAS-50S5C-C, Point Grey, Richmond, Canada), and the second to capture the dorsal aorta, caudal to the swim bladder, at 120 fps (Grasshopper® GRAS-03K2M-C, Point Grey, Richmond, Canada). Both cameras were independently focused on their respective regions of interest to ensure optimal image quality, and set to record simultaneously for 5 min.

To determine atrial and ventricular beat rates (ABR and VBR respectively), heart videos were analysed using MicroZebraLab™ (v3.5, ViewPoint, Lyon, France). This software detects changes in pixel density associated with cardiac muscle contraction and chamber filling, and registers this as contractions of the cardiac muscle in beats per minute (bpm). Analysis involved selecting two clear (with little overlying pigmentation) tracking zones over the atrium and the ventricle. From this, beat frequencies were provided for each chamber independently to allow a global heart rate output and also the detection of certain arrhythmias, e.g. A–V decoupling. Normally stroke volume is precisely calculated using the difference between end systolic and end diastolic volumes. However, using this system a surrogate measure of cardiac stroke volume (SSV) can be calculated by dividing the dorsal aorta flow rate (in nL/s), by the VBR per second (bpm/60).

Blood flow videos were analysed using ZebraBlood™ (v1.3.2, ViewPoint, Lyon, France), which also operates by detecting changes in pixel density and combining

them with vessel diameter to generate a flow rate in nl/s for every frame. An area of the blood vessel was selected with care to exclude other neighbouring capillaries in which blood flow would interfere with the assessment of the main vessel. The software detects the motion of erythrocytes within the tracking area to provide a measure of blood flow. In addition vessel diameter was provided simultaneously at 10 cross-sections that were subsequently averaged to provide one value per 20 s period.

Measured chemical exposure concentrations

Sample preparation:

For water samples, 1 mL of test media for concentrations 1000 μg BPA/l and 0.25, 2.5 and 25 μg MBP/l was removed from the tank to a glass vial and 1 mL of HPLC-grade acetonitrile was added. Aliquots were vortex-mixed and diluted with mixture of HPLC-grade acetonitrile and water (1:3 v/v) before LC-MS/MS analysis.

For measurement of uptake into the exposed fish, a parallel set of replicates was run alongside those for fluorescence analysis. In addition replicates for fish exposed to 25 μg MBP/l were also run for uptake analysis. Twenty zebrafish larvae, previously anaesthetised with 0.5 g/l solution of tricaine (non-recoverable), in 300 µL of test solution were transferred to a 96 well MultiScreen$_{HTS}$ BV Filter Plate (Merck Millipore, Ireland). The test solution was removed under vacuum and larvae were washed with culture water (containing tricaine), to eliminate residual test solution and transferred in 300 µL of pure water to a 96-well plate (Porvair Sciences, UK). Then 300 µL HPLC- grade acetonitrile was added and samples were homogenised in their wells for 3 minutes to achieve extraction of analytes LCMS grade water (900ul) was added to each well and after mixing the plate was centrifuged at 4000 rpm for 30min. Supernatants from each well were transferred to a 96-well plate and removed for analysis.

LC-MS Method

Measurement of BPA and MBP were performed using Surveyor MS Pump Plus HPLC pump with HTC PAL autosampler coupled to TSQ Vantage triple quadrupole mass spectrometer equipped with heated electrospray (HESI II) source (all ThermoFisher Scientific, Hemel Hempstead, UK).

Chromatographic separation was achieved using 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) water and (B) methanol. The initial conditions for the gradient consisted of 10% solvent B, which was increased to 100% in 4.5 min and maintained for 1 min before returning to the initial 10% B. The flow rate was 500µL/min. Temperature of auto-sampler was set at 8˚C while column was kept at ambient room temperature.

HESI probe was operating in the negative mode; an ion-spray voltage of -4.0 kV was applied. The heated capillary temperature was set at 275 °C and the vaporizer temperature was 60 °C. Nitrogen was employed as sheath and auxiliary gas at a pressure of 30 and 5 arbitrary units, 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 (Table 2).

Table 2. LC-MS quantification of the target compounds performed by monitoring two characteristic multiple reaction monitoring (MRM) transitions

Compound	**Parent ion (*m/z*)**	**Product ion (*m/z*)**	**Collision energy (eV)**
BPA	227.1	212.1	20
		133.1	28
MBP	267.1	133.1	23
		117.0	49

Data analysis

Fluorescence data are expressed as mean fold induction above the solvent control ± standard error of the mean (SEM). Data quoted in the text, or shown graphically, for cardiac function parameters are presented as the mean ± SEM over the 5 minute observation period. Data for A:V beat ratio at 5 dpf was separated into 0.5 minute time segments to clearly visualise observed fluctuations in A:V ration in exposure groups. Significant differences between groups were analysed using a one-way ANOVA, and a Tukey posthoc test was carried out where significant differences were detected *(*$P\text{-}value < 0.05$*)*. A Kolmogorov-Smirnov test was used to compare the distribution curves between treatments for A:V beat rate data and differences were reported as significant if $p < 0.05$.-Statistical analyses of data were performed in IBM SPSS Statistics 22.

Results

Measured exposure concentrations

Chemical analysis of working solutions was determined to confirm test concentrations of BPA and MBP at the start of the exposures. Bisphenol A was determined to be within 20% of nominal concentrations, indicating accurate dosing. The analytical method could not detect MBP at a concentration of 0.25 µg/l as this was below the limit of detection. However accurate dosing (±20%) was confirmed at 2.5 and 25 µg MBP/l. No chemical was detected in control exposure medium.

Uptake and metabolism of BPA in exposed embryos

Larvae exposed to BPA at a concentration of 1000 µg/l were determined to have an internal load of 2.3 ± 0.17 ng BPA/larvae, equating to an estimated bioconcentration factor (BCF) of 2.3 (Table 3). MBP was not detected in 5 dpf zebrafish embryos exposed to BPA. Similarly in larvae exposed to MBP (0.25 µg/l and 2.5 µg/l) MBP was not detected with the analytical method applied. MBP was detected in larvae exposed to the highest MBP concentration (25 µg/l) with an internal concentration of 0.66 ± 0.16 ng MBP/larvae, equating to a BCF of 26.4.

Table 3. Measured exposure water and internal (whole body) concentration of BPA and MBP in 120 hpf zebrafish larvae. Data for zebrafish larvae are the mean of 3 replicates, each replicate consisting of 20 larvae, (SEM in brackets). Larvae exposed to BPA were assessed for internal concentration of BPA and MBP, larvae exposed to MBP were assessed for internal concentration of MBP only.

	Nominal external concentration (µg/l)	Measured external concentration (µg/l)	Concentration BPA per larvae (ng/larvae)	Concentration MBP per larvae (ng/larvae)
SC	0.0	<LOQ	<LOQ	<LOQ
BPA	1000	980.3 (0.12)	2.3 (0.17)	<LOQ
MBP	0.25	<LOQ		<LOQ
	2.5	2.1 (0.15)		<LOQ
	25	28.2 (0.53)		0.66 (0.16)

Tissue expression of GFP in response to BPA, MBP and EE2 in ERE-TG zebrafish larvae

Some ERE-TG zebrafish were observed to show detectable GFP expression in the otic vesicle in the absence of chemical treatment (Fig. 1). This fluorescence was not inducible by the chemicals tested, and had a similar level of occurrence across treatments. No GFP fluorescence was detected in other tissues of unexposed control larvae.

There was no significant induction of GFP as detected by fluorescence in ERE-TG zebrafish exposed to 10 µg/l BPA (Fig 1). At 100ug BPA/l there was a significant increase in GFP fluorescence in the heart (3.2 ± 0.7 fold above control). Exposure to 1000 µg BPA/l resulted in a GFP signal in the heart, liver and tail region, with fold increases above control of 25.7 ± 2.0, 55.6 ± 5.5 and 10.4 ± 1.8, respectively. Fluorescence in the tail was associated with individual muscle myotubes expressing in a mosaic pattern and in the corpuscles of stannius.

Exposure to MBP resulted in a very similar pattern of fluorescence expression, to that for BPA, but MBP was considerably more potent. A response in the heart was detected at an MBP concentration of 0.25 µg/l (3.5 ± 0.6 -fold increase above control) increasing to 32.6 ± 1.6 fold above controls at 2.5 µg MBP/l. At 2.5 µg MBP/l fluorescence was also detected in the liver and tail regions at levels of 78.4 ± 13.1 and 12.1 ± 0.9 fold above the control, respectively.

Responses to EE2 differed to the phenolic compounds. Here the most responsive tissue was the liver, at an exposure of 2 ngEE2 /l (8.6 ± 2.1 fold increase above controls). At an exposure of 20 ng EE2/l fluorescence was detected in the heart (9.6 ± 0.9 fold above controls), liver (128.2 ± 16.3 fold above controls) and tail regions (13.2 ± 0.6 fold above controls). Responses to EE2 were much more pronounced in liver and somites compared with the heart.

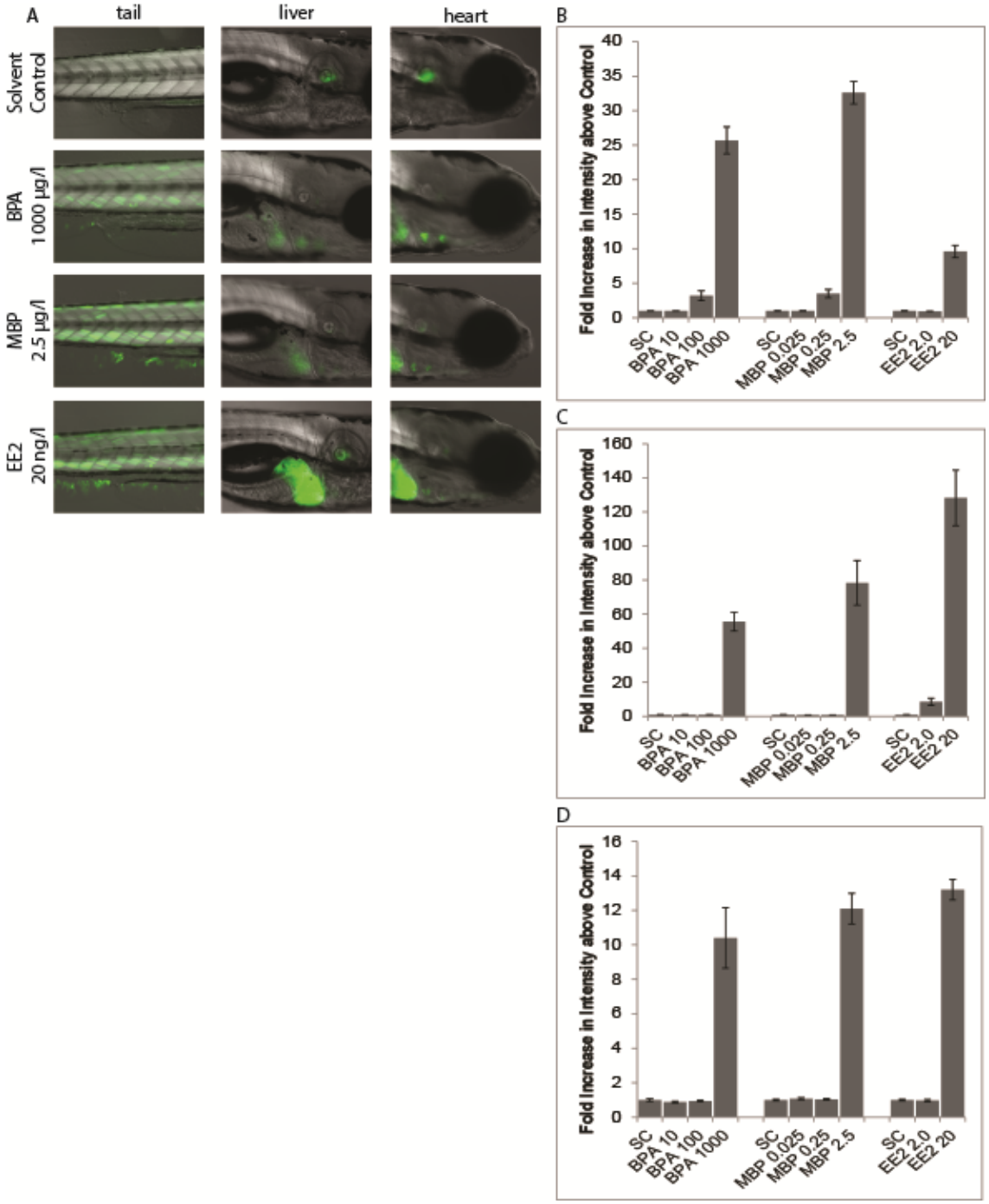


Figure 1. (A) Representative images of 120 hpf ERE-TG zebrafish larvae for controls and exposures to 1000µg BPA/l, 2.5 µg MBP/l and 20 ng EE2/l (images for lower exposure concentrations are not shown); (B-D) GFP induction in 120 hpf ERE-TG zebrafish larvae exposed to test chemicals (BPA and MBP concentrations indicated in µg/l, EE2 concentrations in ng/l) is presented as fold-increase above controls for the (B) heart, (C) liver, (D) tail somites.

Concentration dependent chemical responses in dissected hearts

To better identify regions in the heart responding to BPA and MBP, zebrafish hearts were stained to identify myocardium tissue and dissected out for microscopy analysis (Fig. 2). This approach identified the main GFP expression domains within the heart as the atrioventricular valve and the bulbus arteriosus structural features that are essential for regulating blood flow in fish. At the higher exposure concentrations (1000 µg/l for BPA and 2.5 µg/l for MBP) GFP fluorescence was seen to extend from the atrioventricular value throughout the ventricular tissue. No evidence was observed to indicate that the overall morphology of valves or bulbus arteriosus was affected by the chemical treatments.

Concentration dependent responses in the heart were observed for both BPA and MBP as detected by fluorescence intensity. Equivalent fold inductions above control for BPA and MBP occurred at concentrations of 1000 µg/l and 1 µg/l respectively (fold increase above control 23.6 ± 2.6 and 23.4 ± 3.5 respectively) giving MBP a relative potency of 1000 times greater than BPA for effects on the heart valves.

Estrogen Receptor signalling pathways for BPA and MBP in ERE-TG zebrafish

To determine which ER(s) was responsible for the tissue specific responses to the test chemicals, we treated the ERE-TG zebrafish larvae with morpholinos for the different ER subtypes in the presence of BPA, MBP and EE2.

Non-injected fish exposed to BPA, MBP and EE2 at 1000 µg/l, 2.5 µg/l and 20 ng/l respectively, showed significant increases in GFP signal in the heart, liver, anterior trunk and tail regions (Fig 3). Injection with the morpholino for esr1, induced a suppression of GFP signal in the heart, liver and trunk, but there was no observed suppression of GFP in the tail region for these morphants. GFP expression was reduced for all chemical exposures in the heart and liver to levels that did not differ from controls (i.e. complete suppression of the GFP). GFP was reduced in the trunk region in fish exposed to BPA. However, although reduced MBP and EE2 exposed fish still demonstrated significantly higher fluorescence than unexposed fish.

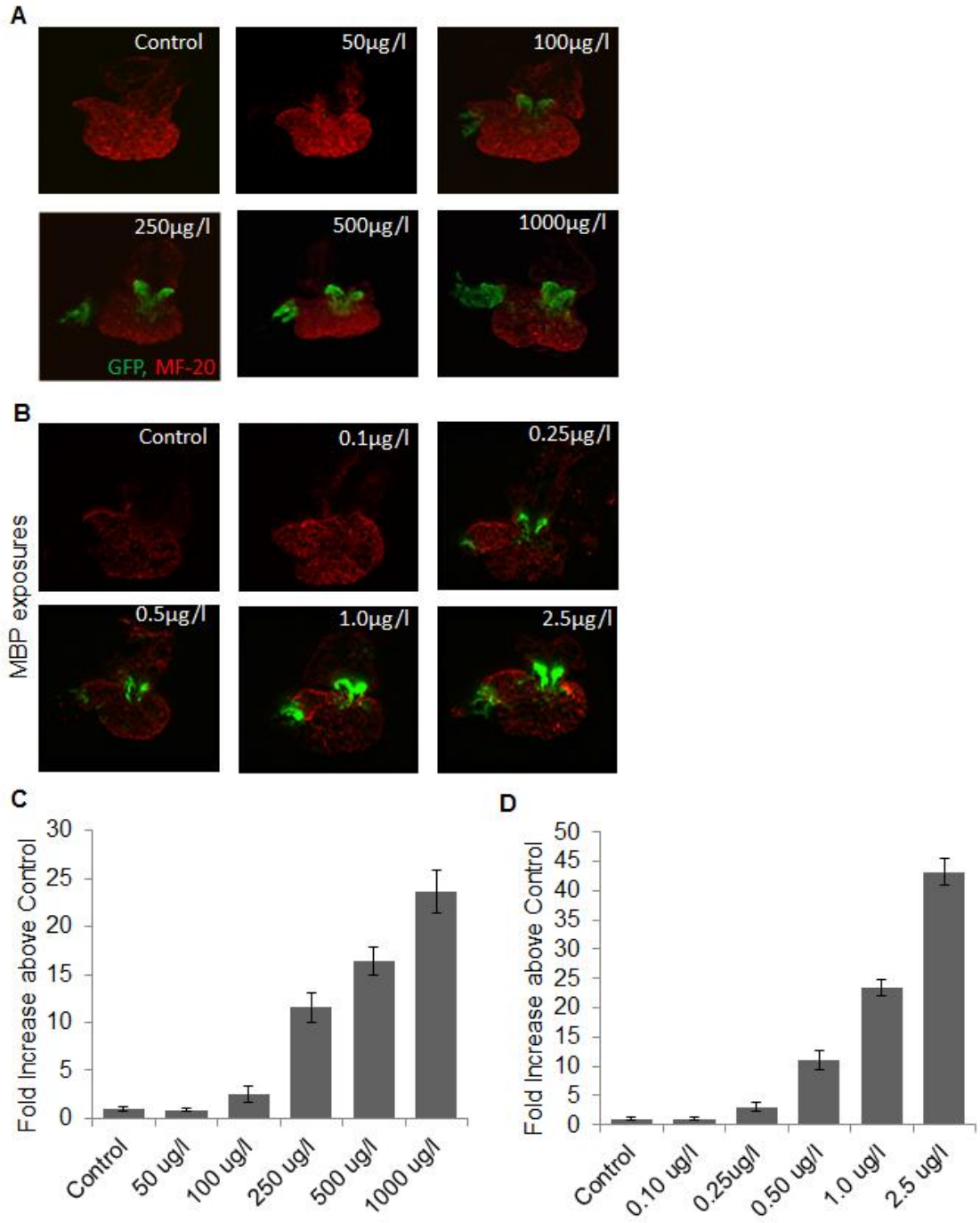


Figure 2. Composite z stacks of hearts dissected from 120 hpf ERE-TG zebrafish larvae exposed to a gradient of concentrations of BPA (A) and MBP (B). Hearts were immunostained with MF-20 to identify cardiac tissue. GFP induction (fluorescence) was measured and quantified using fluorescence microscopy and image analysis in zebrafish hearts for BPA (C) and MBP (D) exposures.

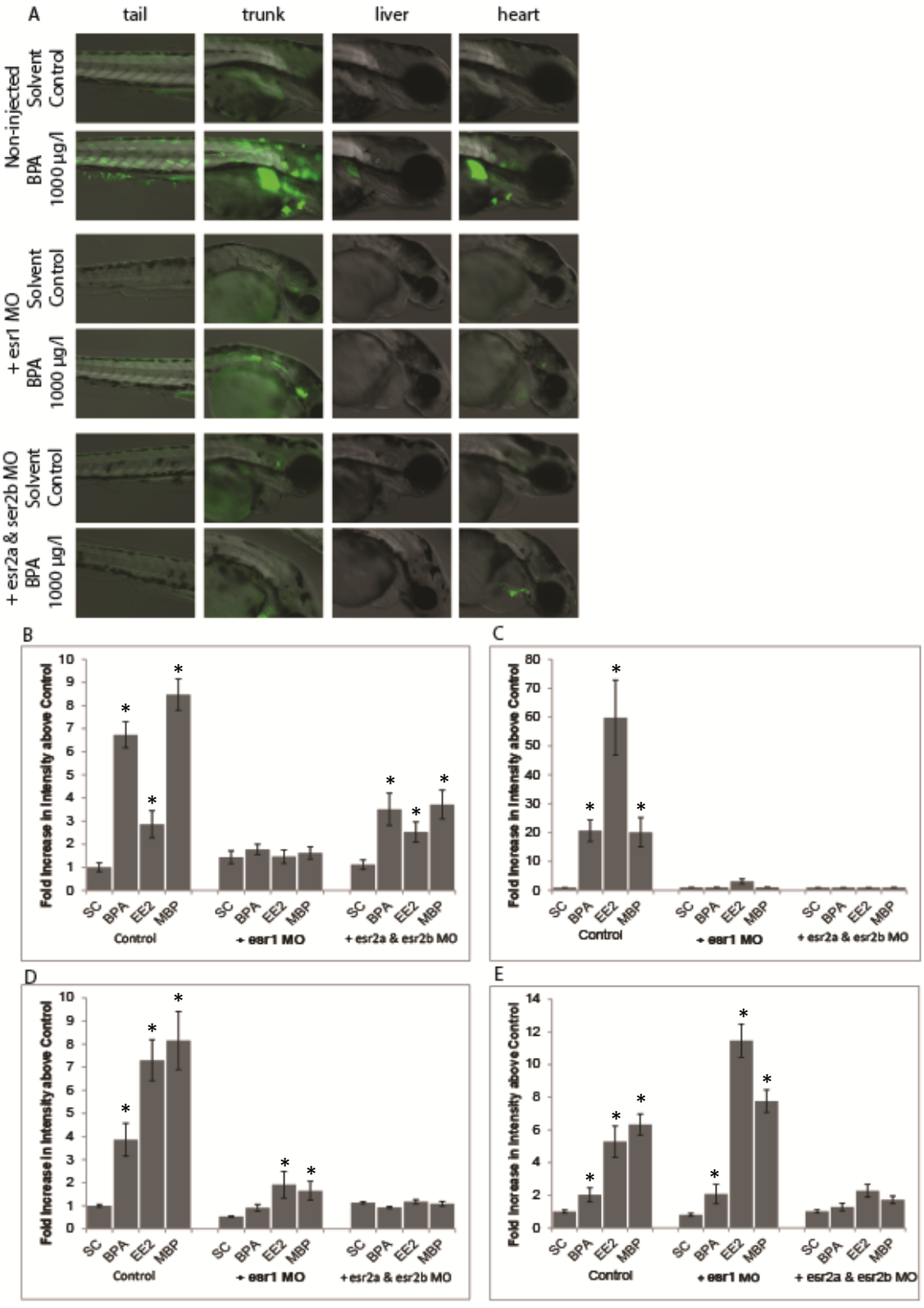


Figure 3. (A) Images of ERE-TG zebrafish larvae at 72hpf for controls and larvae exposed to 1000 µg BPA /l, 2.5 µg MBP/l and 20 ng EE2/l injected with morpholinos against esr1, or esr2a & esr2b at 1-4 cell stage; (B-E) Fluorescence response in 72 hpf ERE-TG zebrafish larvae exposed to BPA, MBP and EE2 in combination with the injected morpholino(s) against esr1 or esr2a & esr2b was measured by fluorescence microscopy and image analysis and expressed as fold

increase above controls for target tissues; (B) heart, (C) liver, (D) trunk muscle, and (E) tail somites (* indicates a significant difference from relevant control treatment p=<0.05).

In contrast with the esr1 morphants, zebrafish injected with morpholinos for esr2a and esr2b still exhibited high induction of GFP in the heart when exposed to the estrogenic chemicals (BPA, MBP and EE2). There was no significant increase observed in GFP fluorescence for BPA, MBP and EE2 exposed morphants in the trunk, tail and liver regions when compared to the controls, thus, indicating that a combined knockdown of esr2a and esr2b may prevent estrogen signalling in these tissues.

Effects of BPA on heart function in 5 and 14 dpf zebrafish

To assess if the ER signalling responses in atrioventricular valves and bulbus arteriosus for BPA translated to any significant effects on heart function and blood flow, we used non-invasive video analysis of the heart and dorsal aorta to measure multiple cardiovascular endpoints; including beat rate, atrial:ventricular beat ratio, blood flow/velocity, stroke volume and blood vessel diameter. These studies were conducted on fish at 5dpf and 14dpf for exposures to both 1000 µg BPA/l, and 2500 µg BPA/l. For the 5dpf fish GFP induction in the heart was 8.9 ± 1.2 fold above controls for 1000 µg BPA /l and 10.8 ± 1.4 fold above controls for 2500 µg BPA /l and for the 14dpf fish, 50.7 ± 11.5-fold above controls for 1000 µg BPA/l and 44.7 ± 7.7- fold above controls for 2500 µg BPA/l (Fig. 4).

At 5 dpf, there were no significant effects of BPA exposure at either exposure concentration on heart beat rate, dorsal aorta diameter, speed or volume of blood flow (Fig. 5). There appeared to be a slight reduction in the surrogate stroke volume in fish treated with 2500 µg/l, however this was not statistically significant. No significant differences were found between the means of the atrium to ventricle (A:V) beat ratio values. However, a Kolmogorov-Smirnov analysis which compares the distribution pattern around the means showed that the 2500 µg/l treatment group had a significantly different distribution from the control and 1000 µg/l treatment group. This may be seen more clearly when measurements are split into time bins (Fig 5H), where A:V ratio fluctuates erratically throughout the 5 minute observation period for those fish.

Pigment formation at 14 days precluded accurate detection of atrial and ventricular beat rates due to an inability to clearly visualise the heart. Heart beat rate however was calculated from the blood pulses of the dorsal aorta. There was no effect of BPA exposure on volume or speed of blood flow, dorsal aorta

diameter, or surrogate stroke volume (Fig 6). However, there was a significant reduction observed in the arterial beat rate of fish exposed to 2500 µg/l BPA.

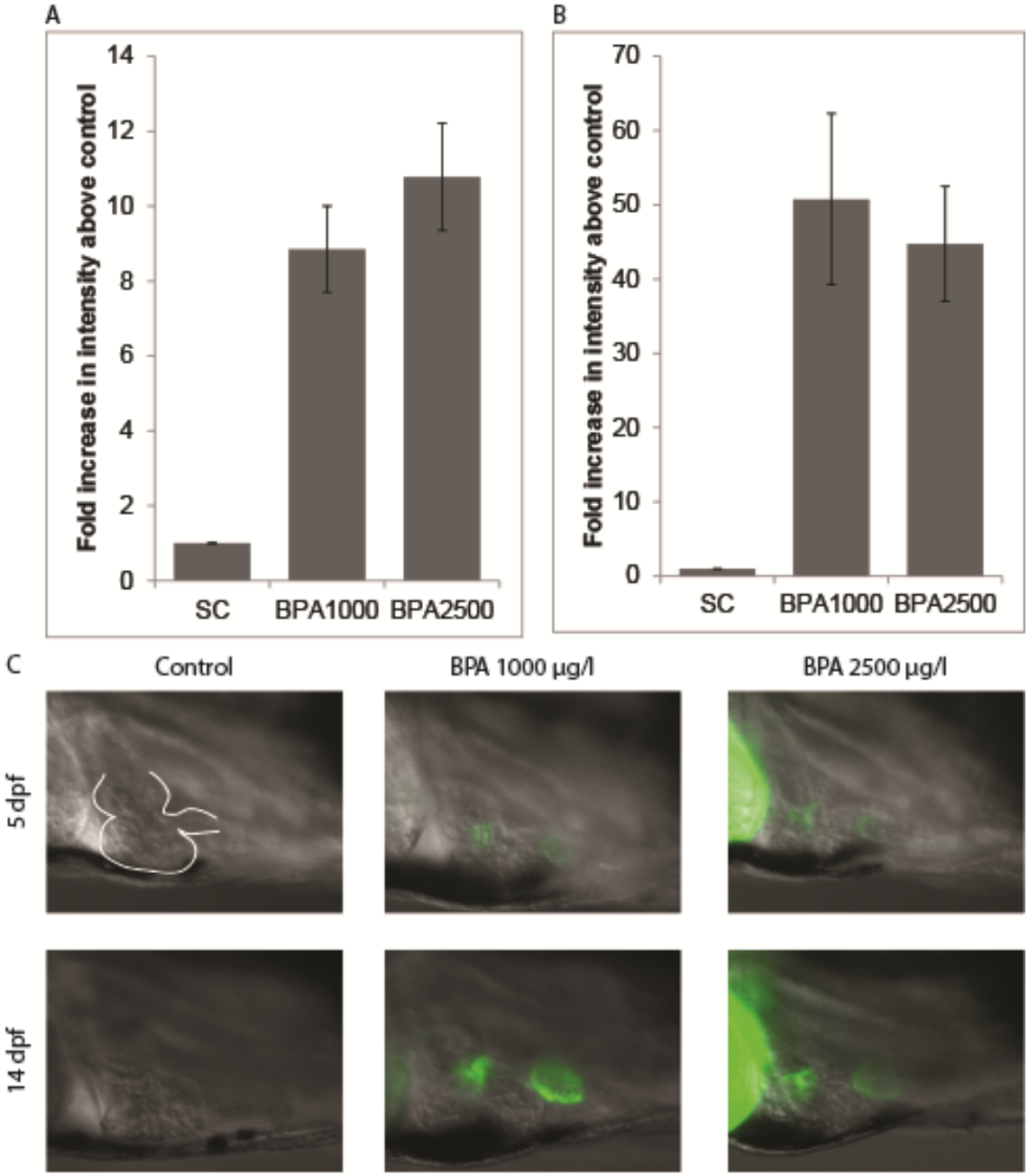


Figure 4. GFP induction (fluorescence) in hearts of ERE-TG zebrafish used for analysis of cardiac function at 5 dpf (A) and 14 dpf (B) measured as fold- increase above controls; (C) images of hearts in ERE-TG zebrafish exposed to 1000 and 2500 µg/l BPA at 5 dpf and 14 dpf; 5 dpf control includes heart outline.

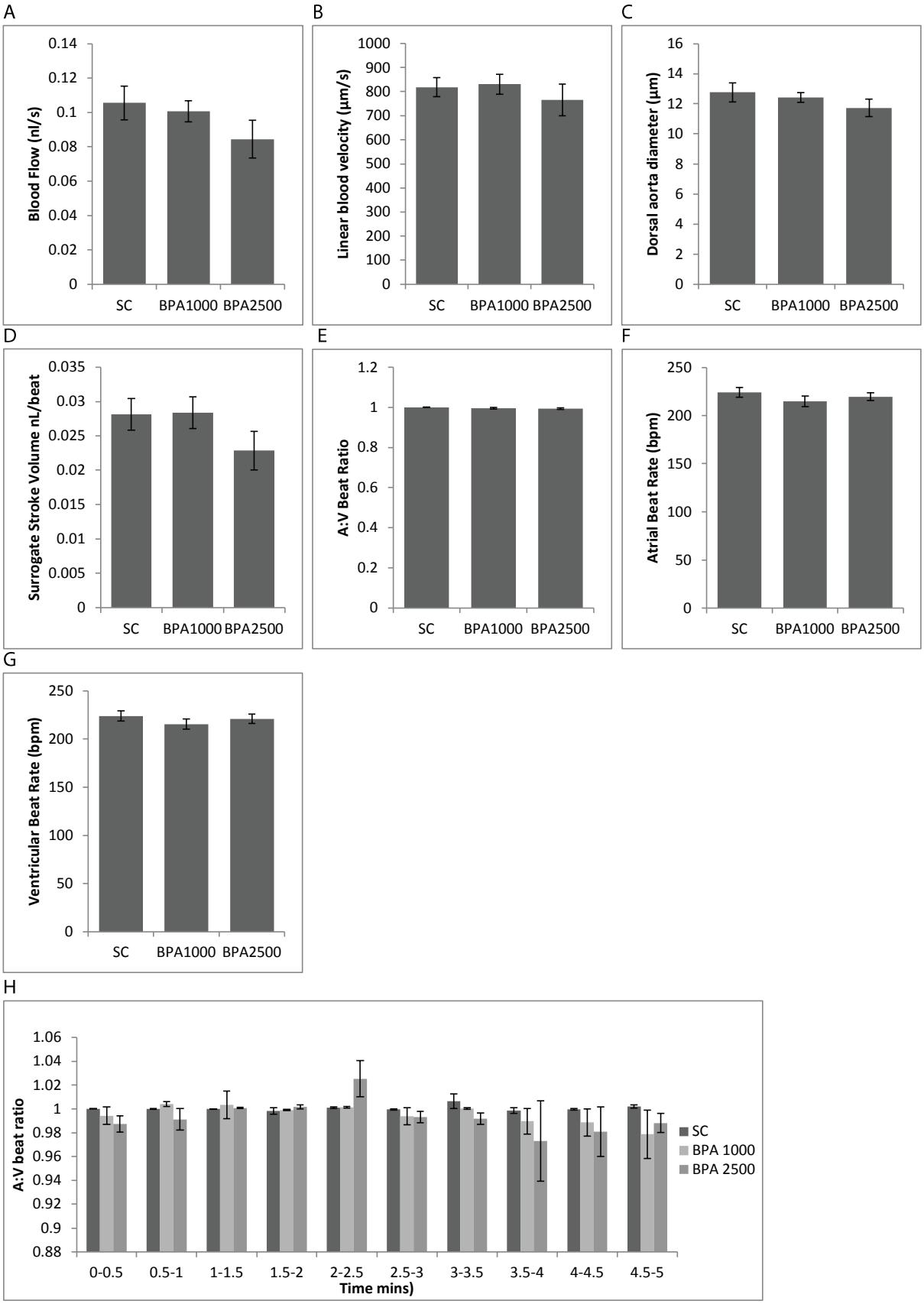


Figure 5. Measurements of cardiovascular function in 5 dfp zebrafish for control and BPA exposed fish; flow rate (A) and linear velocity (B) of blood through dorsal aorta, dorsal aorta diameter (C), surrogate stroke volume (D), atrial and

ventricular beat rates (E-F) and atrial to ventricular beat ratio (G); (H) splits data for atrial to ventricular beat rate into time segments for 30s

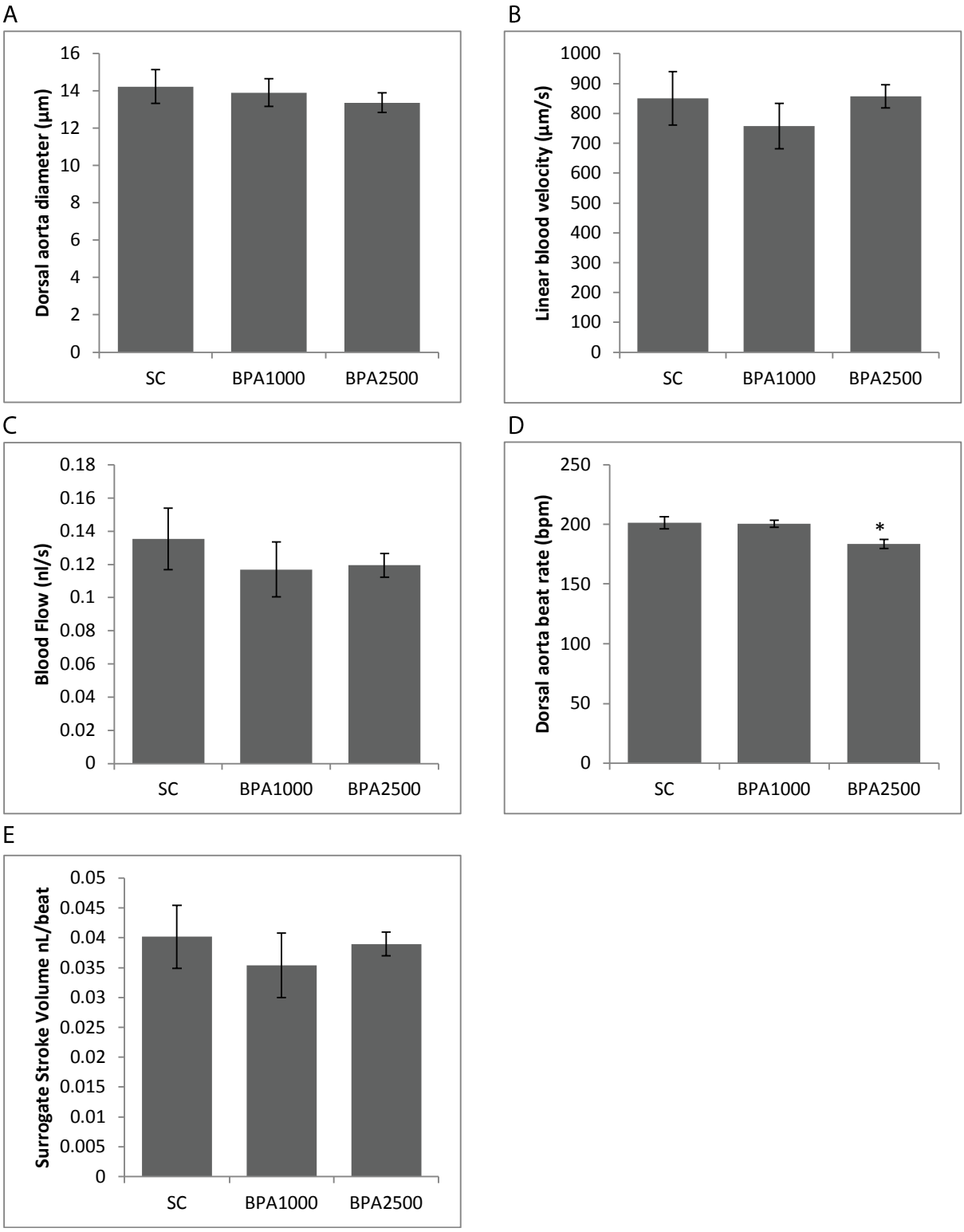


Figure 6. Measurements of cardiovascular function in 14 dfp zebrafish for control and BPA exposed fish; dorsal aorta diameter (A), linear velocity (B) and blood flow (C), estimated beat rate based on arterial blood flow (D) and surrogate stroke volume (E) (* indicates a significant difference from relevant control treatment p=<0.05).

Discussion

Several epidemiological studies have inferred that BPA exposure in humans is linked to an increased occurrence of cardiovascular disease (Melzer *et al.* 2010, Bae *et al.* 2012, Melzer *et al.* 2012, Shankar and Teppala 2012), however, there is little direct experimental evidence to support this and the mechanisms by which this may occur are not known. Here we used an ERE-TG zebrafish in combination with morpholino knockdown of ER subtypes to determine the signalling mechanisms for BPA and its metabolite MBP in the heart and across other body tissues and assessed for effects of early life exposure to BPA on subsequent heart function.

Estrogenic responses to BPA occurred in the heart, liver, muscle somites and corpus stannius as identified previously in different lines of estrogen responsive transgenic zebrafish (Lee *et al.* 2012, Gorelick *et al.* 2014). In the heart, the atrioventricular valves and bulbus arteriosus, were the primary targets for BPA (Fig 2). BPA's metabolite in mammals, MBP, showed similar patterns of estrogenic responses to BPA in the ERE-TG zebrafish, including for the heart valves and bulbus arteriosus, but with higher estrogenic potency. Across the different responding body tissues, MBP was between 400-to 1000 times more potent than BPA, which is highly consistent with findings reported for relative estrogenic potencies in mammals *in vivo* (Ishibashi *et al.* 2005, Nagae *et al.* 2005, Yamaguchi *et al.* 2005, Okuda *et al.* 2010). For the heart the threshold concentration for induction of GFP was 100 µg/l for BPA, compared with 0.25 µg/l for MBP. It is possible that some effects for BPA in the ERE-TG zebrafish derived from its metabolism, however, we did not detect MBP in 5 dpf zebrafish exposed to BPA even at 1000 µg BPA/l. This may indicate that zebrafish larvae do not possess the effective liver function to metabolise BPA to MBP, or that the level of MBP in these larvae was below the detection limit for our analysis method. We were though able to detect MBP in larvae on exposure to the highest concentration of MBP tested (25 µg MBP/l). Thus, from our study we are not able to conclude whether or not zebrafish are able to metabolise BPA to MBP.

Induction of GFP by both BPA and MBP was inhibited in the hearts of ERE-TG larvae injected with morpholinos against esr1 (Fig 3). In the body trunk muscle and liver, morpholinos against esr1 and against esr2a and esr2b together

inhibited GFP expression, whereas in the tail myotubes esr2a and esr2b morpholinos were effective, but the esr1 morpholino was not. These findings indicate different ER response pathways for the bisphenols in the different body tissues. Studies on a zebrafish liver cell line, transfected with different ERs found that BPA most strongly activated ERα (esr1), with some activation of ERβ2 (esr2a) and no detectable effect on ERβ1 (esr2b) (Cosnefroy *et al.* 2012). It is possible that the preferences indicated for different ERs could lead to different chemicals activating ERs preferentially in specific tissues, leading to the differing response patterns observed. In most studies in mammals, it has been indicated that BPA preferentially binds to and activates ERβ (Kuiper *et al.* 1997, Matthews *et al.* 2001, Yoshihara *et al.* 2004, Takemura *et al.* 2005). However, binding activity to specific ERs in these studies has not been confirmed as occurring in particular tissues *in vivo* but rather in cell lines.

Whole mount *in situ* hybridisation of esr transcripts conducted in 5 dpf zebrafish by Gorelick and co-workers has shown esr1 transcripts present in the atrioventricular valves and bulbus arteriosus and esr2a (reported as esr2b) transcripts in the liver; esr2b (reported as esr2a) transcripts were not detected; (Gorelick *et al.* 2014). Esr1 therefore appears to play a crucial role in transducing the estrogenic signal in the cardiac tissue during early life and would explain why estrogenic chemicals with a higher affinity for esr1 (over esr2a or esr2b) show activation responses in the heart before that seen in other tissues. In the liver the fact that the GFP signal was knocked down using the esr1 morpholino, as well as for esr2a and esr2b, indicates hepatic expression of esr1 transcripts, which contrasts with the studies reported for another transgenic zebrafish line for the same life stage (Gorelick *et al.* 2014). It has been shown that esr1 is expressed in livers in many fish species (including the zebrafish) at later life stages, where is plays a role in regulating the expression of estrogen responsive genes, including signalling pathways for induction of vitellogenin (Nelson and Habibi 2010, Chen and Chan 2016). Cross-talk between the esr sub-types is likely to occur in the liver (and other body tissues) and there may be involvement of other factors in estrogen signalling, that lead to a reduced GFP signal when either receptor subtype is knocked down/out.

The fact that BPA and MBP target the atrioventricular valves and bulbus arteriosus, tissues responsible for regulating blood flow from an early life stage,

and here during a critical stage of their development, could have functional consequences. The valves of the atrioventricular canal are crucial for maintaining unidirectional blood flow in the heart, completely blocking retrograde blood flow by 72 hpf (Scherz *et al.* 2008) with the valve leaflets developed by 102 hpf (Staudt and Stainier 2012). The bulbus arteriosus functions as a type of capacitor to maintain a continuous blood flow (Hu *et al.* 2000). The first five days of life are crucial for correct valve development in zebrafish with the valves forming from migration of endocardial cells in this time (Stainier 2001). Further development serves to lengthen and thicken the valves (Martin and Bartman 2009). In humans it has been estimated that 5% of children may carry some form of congenital valve deformation, and 10-20% of all congenital heart diseases are caused by defects in the atrioventricular canal (Loffredo 2000, Pierpont *et al.* 2007). This places a strong emphasis on the need to better establish whether chemicals such as BPA, that are active in these tissues during their development, have any effect on valve function or can contribute to congenital heart disease, and to establish the mechanisms for any such effects.

To determine if the fluorescent signal observed in zebrafish heart valves in response to BPA had any effect on cardiac function or output we used non-invasive video capture to measure various endpoints (Fig 5&6). This method has previously been used effectively in the assessment of integrated cardiovascular function following drug treatment (Parker *et al.* 2014). Endocardial cushion (precursors to functioning valves) development is thought to be complete around 5dpf while valve elongation is thought to occur from around 6 dfp completing around 14 dpf (Martin and Bartman 2009, Staudt and Stainier 2012). It was therefore postulated that if BPA was significantly impacting heart valve function in zebrafish larvae, alterations would be detected at this time. At 5dpf the only significant endpoint measured was the difference in A:V beat ratio distribution. A:V beat ratio was relatively conserved in control larvae with little variation observed throughout the 5 minute observation window. However, in fish exposed to 2500 µg/l BPA A:V beat ratio differed over the assessment period (Fig 5H), indicating it is possible that, although not decoupling atrial and ventricular beat rates in a uniform manner, BPA exposure could lead to an erratic beat in either or both chambers. It is unlikely that A:V beat ratio would be linked to a disruption of the heart valves, however in dissected hearts fluorescent cells extending into

the ventricle were observed, possibly indicating BPA action here. Previous studies have indicated that BPA may promote arrhythmia in mammalian hearts by alteration of myocyte Ca^{2+} handling, mediated by ERβ-signalling (Yan *et al.* 2011, Belcher *et al.* 2012, Gao *et al.* 2013). Estrogen responsive fluorescent signalling in our ERE-TG fish appears to be mediated by an esr1 dependent pathway, however, it may be possible that at the higher concentration of 2500 µg/l further pathways are being induced. The only significantly affected endpoint at 14 dpf was a reduction in arterial beat rate in zebrafish exposed to 2500 µg/l. The mechanism for his is not known. BPA however has been shown to affect thyroid signalling (Moriyama *et al.* 2002, Zoeller *et al.* 2005) and reduced circulating thyroid hormones leads to a reduction in heart rate in humans (Cingoz 2015).

In conclusion, we demonstrate that the estrogenic BPA metabolite MBP targets the same tissues and has a mode of action similar to its parent compound and distinct from that of the classic steroidal estrogen EE2, but the potency of MBP is several orders of magnitude higher than for BPA. We demonstrate that key targets in the heart for both BPA and MBP appear to be the developing atrioventricular valves and bulbus arteriosus, but that early life stage exposure to these bisphenols, even at high concentrations and exceeding those in most environments by several orders of magnitude, did not subsequently affect heart function, up to 14dpf. We cannot say whether chronic, lifelong exposure to these bisphenols has functional consequences for the heart in fish, as has been reported in humans.

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CHAPTER 4

Transcriptomic responses in the hearts of larval zebrafish in response to Bisphenol A exposure

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Introduction

Bisphenol A is an organic synthetic chemical used mainly in the production of plastics and epoxy resins. The uses for these materials are diverse and widespread making BPA one of the most commonly produced industrial chemicals. As a result of the application of BPA in materials that have human contact, exposures by consumers, including infants has resulted in the detection of BPA, or BPA metabolite in human blood, urine and breast milk. BPA has been determined to be present in urine of more than 90% of individuals as reported by biomonitoring studies conducted in the USA, China and Korea (Ye *et al.* 2015, Zhang *et al.* 2015, Park *et al.* 2016). As the ultimate sink for various environmental contaminants the aquatic environment receives BPA from Waste Water Treatment Plants, landfill leachates, ground runoff and natural degradation of BPA containing products. In general BPA has been measured at below 1 µg/l in environmental waters. However, higher concentrations have been recorded, up to 21 µg/l in a river water sample from the Netherlands, 12 µg/l in stream water from the USA, and 3.9 µg/l in estuary water in China (Belfroid *et al.* 2002, Kolpin *et al.* 2002, Dong *et al.* 2009). The widespread use of this chemical and the frequent measurements in humans and environmental samples has raised concerns about its toxicity and the risks that it may pose for human health and wildlife.

Originally developed as a synthetic estrogen before being utilised as an industrial monomer, most studies have focussed on the disruptive effects of BPA as an estrogenic chemical. By mimicking the actions of the endogenous estrogen 17β-estradiol (E2) BPA may bind to and activate the estrogen receptor (ER). The ER is part of the family of nuclear receptors, ligand-activated transcription factors that bind directly to DNA (in the case of ERs at estrogen response elements (EREs)) and regulate the expression of estrogen dependent genes. In mammals there are two ER subtypes esr1 and esr2, whereas the majority of teleost fish possess a third distinct esr2, therefore comprising esr1, esr2a and esr2b (Filby and Tyler 2005). These ERs display distinct expression patterns. In zebrafish larvae esr1 transcripts have been found to be highly expressed in the heart valves while

esr2a transcripts are largely found to be expressed in the liver (Gorelick *et al.* 2014). Both esr2a and esr2b transcripts have also been detected in the developing brain and lateral line system (Tingaud-Sequeira *et al.* 2004). In addition to the classic nuclear-receptor genomic signalling pathway, BPA can also exert actions through rapid response non-genomic signal transduction pathways (Gao and Wang 2014). This rapid signalling of BPA has been reported in various cell types including the pituitary, pancreas, cerebellar cortex and the heart (Alonso-Magdalena *et al.* 2005, Wozniak *et al.* 2005, Zsarnovszky *et al.* 2005, Gao *et al.* 2013). A further possible mode of action for BPA that may explain some of the discrepancies reported between *in vivo* and *in vitro* studies could be metabolic activation, which may not occur in many *in vitro* systems (Welshons *et al.* 2006, Vandenberg *et al.* 2009). Previous studies have reported that in mammals BPA may be metabolised to the more potent estrogen 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) in the liver (Yoshihara *et al.* 2001, Yoshihara *et al.* 2004, Okuda *et al.* 2011). We also demonstrated in Chapter 3 that MBP is several orders of magnitude stronger in inducing a fluorescent response in our ERE-TG zebrafish. Interestingly, we found MBP displays a highly conserved expression pattern with its parent compound, with the heart appearing to be a key tissue target in larval zebrafish.

Several epidemiological and experimental studies have suggested potential links between BPA exposure and disease including diabetes, obesity, reproductive disorders, kidney disease and cancer (Fenichel *et al.* 2013, Rochester 2013, Rezg *et al.* 2014). An increasing amount of evidence has also identified the cardiovascular (CV) system as a potential target for BPA. These findings suggest that BPA exposure may be a risk factor for a range of CV abnormalities such as vascular diseases and cardiac arrhythmias, and highlight the need for further assessment of the impact of BPA and other environmental EDCs on the CV system, with particular respect to the underlying molecular mechanisms. We have also demonstrated in previous chapters in this thesis that BPA targets the heart valves in estrogen responsive transgenic zebrafish, demonstrating that BPA is binding to and activating ERs in these tissues and promoting transcriptional responses.

A limited number of studies have shown that BPA is able to alter transcriptional responses in the heart. Foetal rhesus monkeys exposed during gestation

(mothers fed 400 µg/kg/day) were found to have significant down regulation of myosin heavy chain, cardiac isoform alpha (*Myh6*) in the left ventricle and upregulation of *Adam 12-l* in atrium and ventricles (Chapalamadugu *et al.* 2014). Male rats exposed to 50 µg/kg/day from their first feed were determined to have a decrease in expression of PGC-1α in hearts at 24 and 48 weeks (Jiang *et al.* 2015). Functional impacts were also measured at both time points and they included cardiomyopathy and significantly reduced ATP production. Proteomic analysis of left ventricles from females fed 250 µg/l BPA in their drinking water found an increase in proteins involved in fatty acid transport and oxidation (Ljunggren *et al.* 2016).

As ERs are present in cardiac tissue, it is perhaps not surprising to find transcriptional responses on exposure to BPA. Several studies have also noted responses in cardiac tissue/cells to the natural estrogen 17β-estradiol (E2) and the manufactured steroidal estrogen 17α-ethinylestradiol (EE2) (Nakajima *et al.* 1999, Cong and Ni 2014, Pugach *et al.* 2016, Salla *et al.* 2016). It appears that ERα may be a key transcription factor in the heart and BPA has been described to interact with ERα in a manner that differs compared with E2 (Gould *et al.* 1998). It is therefore possible that global transcriptional responses observed in the heart may differ between BPA and the more potent steroidal estrogens.

Transcriptomic approaches can comprehensively investigate global molecular mechanisms of toxicity and therefore, potentially provide valuable information for understanding or predicting adverse health outcomes to exposure. Historically qPCR and microarray technologies have been used successfully to conduct transcriptional profiling in model organisms exposed to a wide range of chemical stressors. High-throughput RNA sequencing (RNA-Seq) has more recently emerged as a sensitive and reproducible tool that can be used to conduct non-biased transcriptomic analysis (Rapaport *et al.* 2013, Uren Webster *et al.* 2015). RNA-Seq has been used to determine transcription responses in a number of studies using larval zebrafish, however, whole larvae tend to be analysed which does not allow assessments on tissue specific responses.

In this project, we employed RNA-Seq on an Illumina HiSeq 2500 platform to characterize the global cardiac transcriptomic responses of larval zebrafish following exposure to BPA, MBP and EE2 to help characterise possible

toxicological effects of these compounds. Specifically, we aimed to determine if response pathways were conserved between BPA and the classical steroidal estrogen EE2 or if there were distinct differences in their gene targets possibly brought about by their reported differing interactions with the ERs. We also aimed to determine if BPA induced similar transcriptional responses to its potent metabolite MBP. Previous studies have focussed on whole body transcriptional responses when using larvae, we aimed to demonstrate it is possible to isolate DNA from individual tissues, giving a clearer indication as to specific tissue dependant mechanisms of toxicity.

Materials and Methods

Fish source, culture and husbandry

Adult zebrafish for the provision of embryos were kept in aquaria (37.5L) at the University of Exeter in mixed-sex stock tanks, as described in (Paull *et al.* 2008). All zebrafish embryos used in this study were obtained from tg(ERE:Gal4ff)(UAS:GFP) adults, a transgenic zebrafish developed at the University of Exeter (Lee *et al.* 2012). Fish were kept on a 12/12 light/dark cycle. Fish were allowed to breed naturally and eggs were collected in egg collection chambers approximately 1h post-fertilisation (hpf). Eggs were sorted to remove unfertilised and non-viable embryos prior to use.

Chemical exposures and sampling

Bisphenol A (purity >99%) and 17α-Ethinylestradiol (≥98%) (EE2) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK). 4-methyl-2,4-bis(4-hydroxyphenol)pent-1-ene (MBP) was synthesised by Mark Wood at the University of Exeter and purity of the compound was certified as described in (Cipelli *et al.* 2014). All stock chemicals were dissolved in ethanol in glass bottles and diluted in ethanol to the required stock concentrations prior to dilution in embryo test water. All tests were performed using reconstituted purified water in accordance with ISO (International Organisation for Standardisation) guideline 7346-3. Chemicals in ethanol stock were dissolved in test water to give a final ethanol concentration of 0.01%. Solvent controls contained the equivalent of ethanol in ISO water without chemical.

Chemical exposure concentrations were based on pilot studies which aimed to determine a threshold concentration for exposure that produced a fluorescent GFP signal of equal intensity across all treatments for a given exposure time period. These concentrations were determined to be 150 μg BPA/l, 0.50 μg/l MBP and 10 ng EE2/l over a 96 hr exposure. Financial and practical constraints limited this study to one concentration of each chemical treatment only. 96hpf was chosen as a time point as there are various genetic and morphological changes in the endocardial cushions as they form into valves between 72hpf and 120hpf. By selecting a threshold concentration at this time point we aimed to capture gene expression changes in the mid period of the response cascade of signalling.

All exposures were conducted in a temperature controlled laboratory held at 28 ± 1 ^{o}C, under semi-static conditions. Each experimental group consisted of 50 embryos exposed in 100 ml of test media and each treatment group was run in triplicate. Hearts removed from larvae were pooled within treatment groups to ensure sufficient RNA was obtained; the experiment was repeated twice to obtain three experimental replicates per treatment. The exposures were conducted from 0 hpf to 96 hpf. At the end of the exposure period five 96 hpf old larvae from each vessel were processed for fluorescent imaging analysis, remaining larvae were processed for heart extraction.

Hearts were extracted and isolated from other tissue from 96 hpf zebrafish larvae as described in (Burns and MacRae 2006), with the alteration that we used Gibco Leibovitz's L-15 Medium, no phenol red due to possible estrogenic activity of phenol red. Following purification hearts were snap frozen in liquid nitrogen and stored at -80^{o}C prior to transcript profiling.

Image analysis

For fluorescence intensity measurements live Tg(ERE:Gal4ff)(UAS:GFP) larvae were anesthetised with 0.016% tricane, mounted in 3% methylcellulose in ISO water and placed onto a glass-bottom 35 mm dish (MatTek, Ashland, MA, USA). All larvae were observed in lateral view and images were obtained using a Zeiss Axio Observer.Z1 equipped with an AxioCam Mrm camera (Zeiss, Cambridge, UK). All photographs were taken using the same parameters and exposure time under a X10 objective. Photographs were processed using the Axiovision Imaging software and fluorescence quantification was calculated using the ImageJ software (http://rsb.info.nih.gov/ij/). For each picture intensity was measured as the mean grey value of all the pixels within a region of interest, and the region of interest was kept consistent between individuals. Low level background fluorescence from excitation source, camera noise, ambient light etc. was subtracted using the ImageJ rolling ball algorithm which removes any spatial variations of the background intensities as described in (Sternberg 1983).

RNA extraction, library preparation and sequencing

Transcript profiling was conducted on the hearts of 3 replicates per treatment group. RNA was extracted from hearts using an RNeasy Micro extraction kit

(Qiagen), incorporating on-column DNase treatment, according to the manufacturer's instructions. The concentration, purity and integrity of RNA were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). All RNA used for library construction was of high quality with RIN scores > 7. cDNA libraries from all samples were then prepared using the epicentre TotalScript^{TM} RNA-Seq Kit (Epicentre, Madison, WI, USA), multiplexed with 12 samples per lane and sequenced using an Illumina HiSeq 2500, to generate 150 bp paired reads.

Transcriptome analysis

Raw reads were subject to quality-related processing where low quality (phred score <30) and adapter sequences were removed using Fastq-mcf (https://expressionanalysis.github.io/ea-utils/). Filtered reads were then aligned to the zebrafish reference genome (Ensembl Danio rerio Zv9 release 79) using Tophat2 (v2.0.13) (Trapnell *et al.* 2009) with standard settings. Cufflinks (v2.2.1) (Trapnell *et al.* 2010) was used to quantify the transcripts, followed by differential expression analysis using CuffDiff2 (Trapnell *et al.* 2013). Transcripts with a false discovery rate (FDR) < 0.1 were considered differentially expressed (Benjamini−Hochberg correction). Functional analysis was performed for differentially expressed genes from each treatment group using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) (Huang da *et al.* 2009) with all genes detected as being expressed in the heart as a background. Gene ontology (GO) terms for Biological Process, Cellular Component and Molecular Function and Kyoto Encyclopaedia of Gene and Genomes (Kegg) pathways were considered significantly over-represented when $P < 0.05$.

As the sequence for the GFP gene contained in our ERE-TG zebrafish was not contained in the Ensembl zebrafish genome the aligner bowtie2 (v2.2.5) (Langmead and Salzberg 2012) was used to align all the reads to the eGFP sequence (Table 1). This determined the abundance of fragments that aligned to the sequence allowing calculation of fragments per kilobase of exon per million fragments mapped (FPKM) for each replicate, providing a quantification of transcript abundance.

Table 1. eGFP sequence used to determine transcript expression based on FPKM values

GAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAG
CTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCTGGCGAGGGCG
AGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC
GGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACG
GCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTC
TTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTT
CAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGC
GACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG
ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAAC
GTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGCGAACTTCAA
GATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTAC
CAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACC
ACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCG
CGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCG
GCATGGACGAGCTGT

Data analysis

Data for FPKM values, and fluorescence intensities were expressed as mean ± standard error of the mean (SEM) and significant differences were analysed with either a Student's *t*-test or one-way ANOVA, depending if testing between two or more groups respectively. Data were tested for normality of distribution (Shapiro–Wilk test) and homogeneity of variance (Levene's test) prior to analysis. Where a significant difference was detected a Tukey posthoc test was carried out *(*P-value < 0.05*).* Analyses were performed with SPSS Statistics 23.0.

Repeat study

Following the above experiment we lacked some confidence in the initial results as read number was not as high as expected, there were also a high number of genes where no expression was detected in some treatments. It was unclear if

this was due to practices with the sequencing, including multiplexing samples with a separate study, or if it was due to the low levels of RNA used in this study compared to traditional library preparation methods; TotalScript library preparation uses 5ng total RNA. We therefore conducted a further study repeating the exposure to BPA with also a relevant control in an attempt to determine library preparation efficiency.

Preparation of samples adopted an identical approach to that described above for the original experiment. Analysis was also as described above with the only difference being that quality processing and adapter trimming was performed using fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) as in this case a higher number of filtered reads was obtained.

Results

Fluorescence Intensities in hearts of exposed ERE-TG zebrafish

In order to determine if a measured fluorescence response across the different treatments was related to the expression of any specific identifiable gene/s in hearts a sub set of fish were sampled and fluorescence images captured to determine intensity (Fig. 1). ERE-TG fish without chemical exposure were not observed to possess any induced GFP fluorescence, any weak GFP detected as autofluorescence was accounted for as background level expression. In the first study ERE-TG fish exposed to 150 µg/l BPA, 0.50 µg/l MBP and 10 ng/l EE2 were all determined to have GFP induction in atrioventricular valves and bulbus arteriosus, indicating estrogen dependent transcription present in these tissues. GFP induction was measured to be 4.2 ± 0.7, 4.0 ± 1.8 and 2.6 ± 0.2 fold increase above the controls for ERE-TG fish exposed to 150 µg/l BPA, 0.50 µg/l MBP and 10 ng/l EE2 respectively. These values were not found to be significantly different from each other, indicating a similar level of intensity induced across all treatments.

In the study where we repeated the exposure to BPA only, there was again no induced signal observed in the hearts of unexposed fish, whereas hearts from fish exposed to 150 µg/l were found to express GFP in atrioventricular valves and bulbus arteriosus, GFP intensity was measured as being 4.2 ± 0.7-fold increase above controls.

RNA-Seq Analysis

Following waterborne exposure of larvae, zebrafish hearts were removed and we extracted total RNA to analyse for transcriptional responses using RNA-Seq. For the first exposure study we obtained between 9,418,706 and 11,954,691 paired end raw reads, reduced to between 8,911,315 and 11,148,867 paired end reads after filtering through quality control analysis using Fastq-mcf. The mapping level to the reference genome (Zv9 Ensembl release 79) for the samples ranged from 88.9% to 92.0%. For the second exposure to BPA quality control analysis was carried out using fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Paired end raw reads obtained were between 29,950,946 and 44,558,632 while filtered

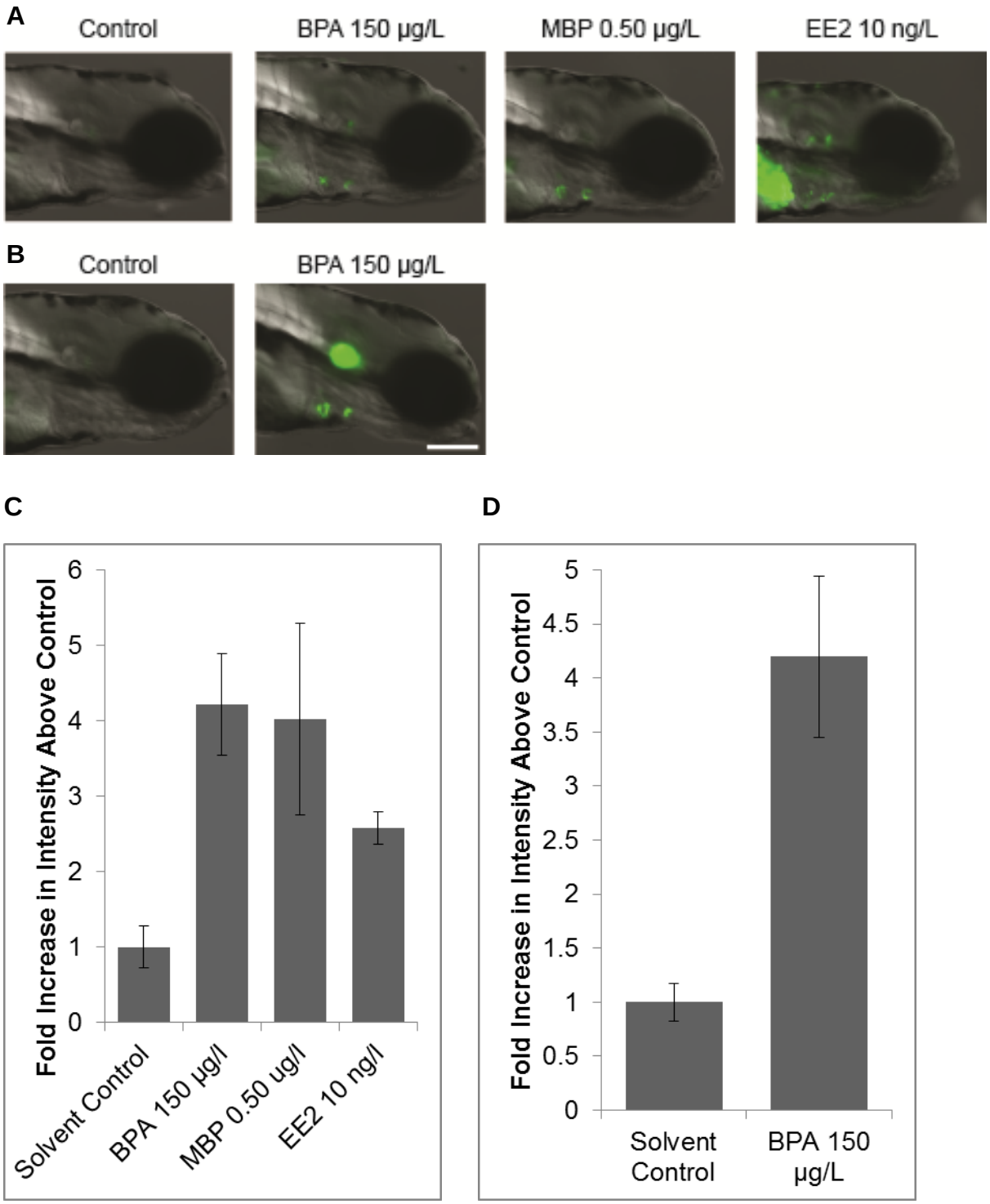

Figure 1. Images of 96 hpf ERE-TG zebrafish larvae used for transcriptomic analysis, exposed to either 150 µg BPA/l, 0.50 µg MBP/l and 20 ng EE2/l, in the first study (A) or 150 µg BPA/l in the second study (B); control fish were exposed to solvent vehicle only; Scale bar represents 200 µm. (C-D) GFP induction (fluorescence) in the hearts of 96 hpf ERE-TG zebrafish larvae exposed to 150 µg BPA/l, 0.50 µg MBP/l and 20 ng EE2/l in the first study (C) or 150 µg BPA/l in the second study (D).

reads were between 27,585,887 and 40,937,865. Percentages of genes mapped to the reference genome were between 89.3% and 94.0%.

Differentially expressed transcripts were determined using the CuffDiff2 programme. The total number of transcripts differentially expressed in hearts exposed to BPA, MBP and EE2 treatment groups compared to the controls in the first study was 26 (16 of which were up-regulated), 54 (33 of which were up-regulated) and 64 (33 of which were up-regulated) respectively (Fig. 2 and Table 2). Only one transcript was found to be differentially expressed across all treatment groups. Transcripts for the gene *slc4a1a* were up-regulated in all treatments. Of the 26 differentially expressed transcripts observed in BPA exposed zebrafish there were 4 transcripts in common with MBP (3 upregulated and 1 downregulated) and 4 transcripts in common with EE2 (2 upregulated and 2 downregulated). MBP and EE2 exposed fish we found to also have 5 (3 upregulated and 2 downregulated) differentially expressed transcripts in common. In the repeat study the total number of transcripts differentially expressed in the 150 µg BPA/l treatment group compared to the controls was 130 (23 of which were up-regulated) (Table 3). No common transcripts were found differentially expressed after exposure to 150 µg BPA/l in both the first and repeat study. The only common differentially expressed transcript that was observed between a treatment group and the control in the original and second BPA exposure study was for the gene *krt4*. *krt4* was found to be up-regulated 1.9-fold after exposure to 0.5µg MBP/l, however in the second study it was down-regulated 2.7-fold from the control after exposure to 150 µg BPA/l.

As the GFP gene did not appear in the reference genome we separately calculated the transcript abundance of GFP in the hearts of fish in this study. Differences in GFP expression were compared using fragments per kilobase per million (FPKM) (Fig. 3). FPKM is calculated with the following formula $X_i/(L_i*N) * 10^9$, where X_i and L_i are the total number of reads mapping to a particular gene and the length of the gene in bases, N is the total number of reads sequenced. FPKM therefore, gives a value to the number of transcripts out of all those sequenced that map to a gene of a given sequence, indicating expression level. In the first study FPKM values for GFP were determined for the Control, 150 µg BPA/l, 0.5 µg MBP/l and 10 ng EE2/l treatment groups to be 2.6 ± 1.3, 110.9 ± 22.8, 22.7 ± 1.8 and 21.4 ± 5.2. In the second BPA exposure study

FPKM values for GFP were determined to be 0.59 ± 0.4 and 42.1 ± 4.7 for the control and BPA treatment groups respectively. Although there were no significant differences in transcript expression between controls and treatment groups we also calculated FPKM values for the 3 estrogen receptor (ERs) genes *esr1*, *esr2a* and *esr2b*, to expand on our understanding of estrogen signalling in the heart (Fig. 3). In the first exposure study *esr1* transcript FPKM values were determined to be 1.7 ± 0.3, 2.3 ± 0.9, 4.9 ± 1.1 and 1.7 ± 1.0 for control, BPA, MBP and EE2 groups respectively. No transcripts were detected in any treatment group for esr2a. Only one replicate in each of the Control, BPA and EE2 treatment groups were found to have transcripts present for *esr2b* with FPKM values of 0.040, 0.53 and 0.28, respectively. In the second BPA exposure study transcripts for all 3 ER genes were observed in all replicates for both treatments FPKMs were 4.2 ± 0.5 and 3.8 ± 0.5 for esr1, 0.46 ± 0.2 and 0.30 ± 0.07 for esr2a and 0.77 ± 0.1 and 0.63 ± 0.1 for esr2b for Control and BPA treated groups, respectively.

GO Functional Analysis and KEGG pathways

We performed functional analysis of Gene Ontology (GO), whereby differentially expressed genes may be assigned functional terms which may be enriched. The list of over-represented GO terms and Kegg pathways are shown in Table 4. In the original study no significantly enriched terms were determined for differentially expressed genes in the hearts of zebrafish exposed to either BPA or MBP. The only enriched term found for those fish exposed to EE2 was *protein complex* within the cellular component classification.

In the second BPA exposure study a number of significantly ($p<0.05$) enriched terms were determined in the hearts of zebrafish exposed to BPA. In the biological processes classification, the majority of terms were associated with membrane transport and localisation. Terms associated with membrane transport activity were also significantly enriched in the molecular function classification. The most significantly enriched terms in the cellular component classification were involved in cell to cell gap junctions. Three KEGG pathways were also identified as being over-represented, these are associated with phenylalanine biosynthesis and metabolism and drug metabolism (Table 4).

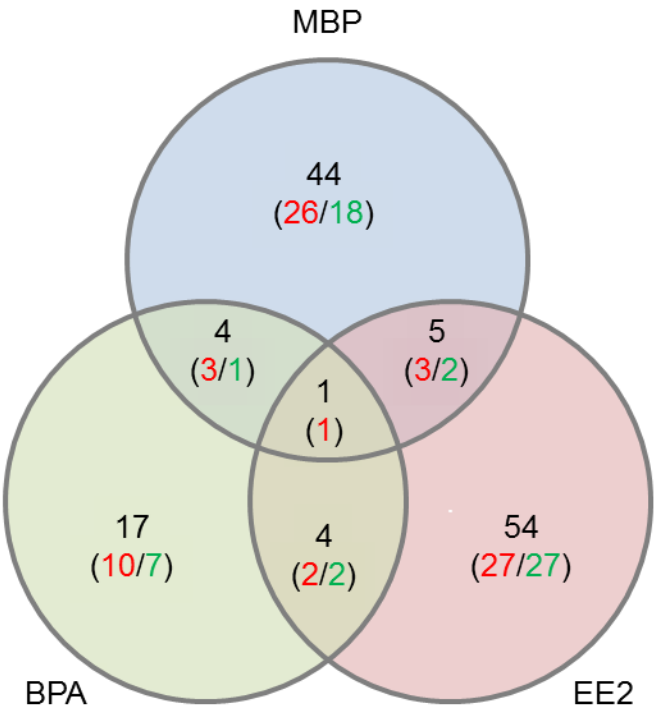

Figure 2. Venn diagram displaying the numbers of differentially expressed transcripts in each treatment group. Red and green numbers represent up- and down-regulated genes respectively

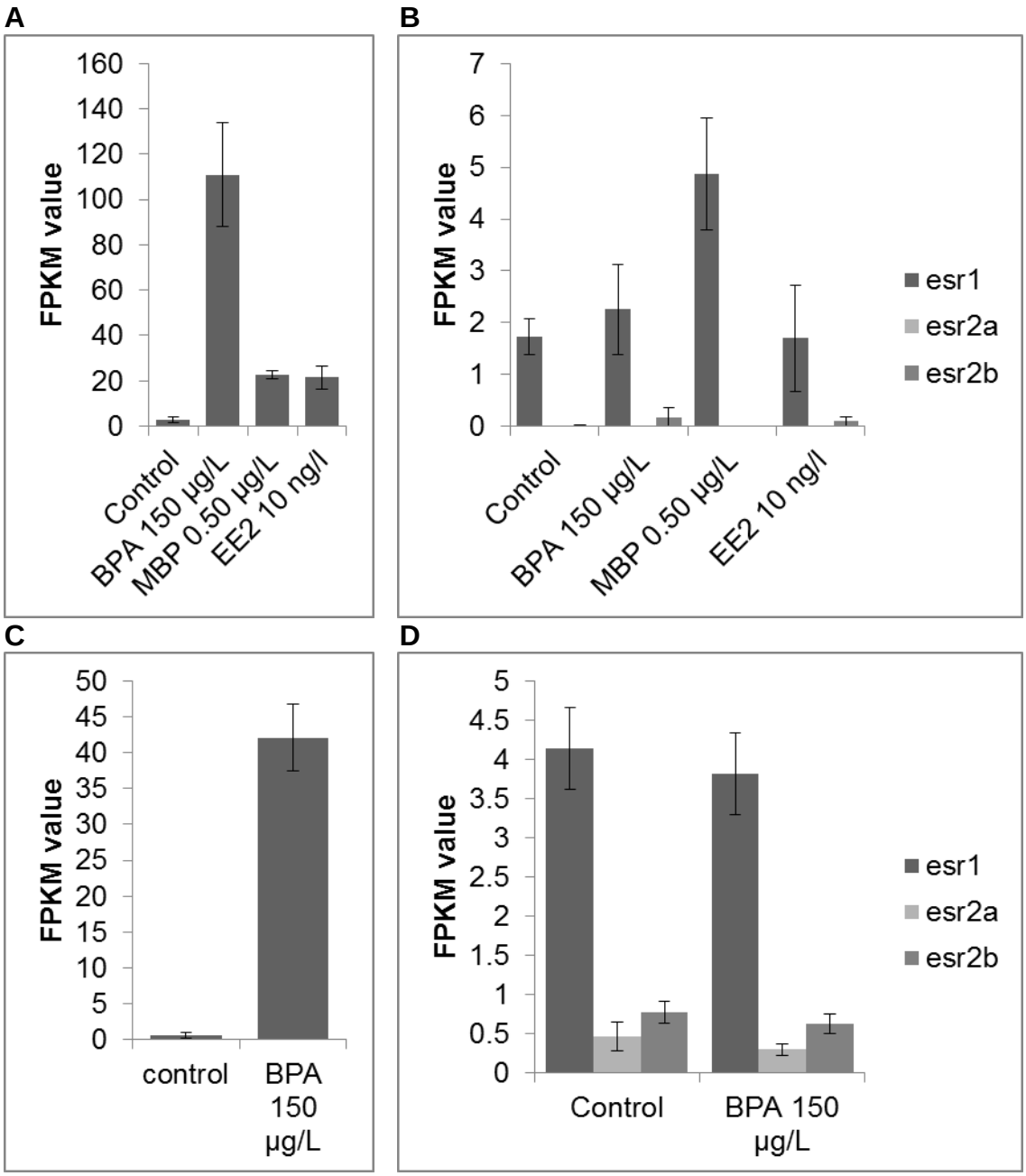

Figure 3. Fragments Per Kilobase of transcript per Million fragments (FPKM) values demonstrates expression level of various transcripts; (A and C) GFP; (B and D) Estrogen Receptor genes *esr1, esr2a* and *esr2b*; for original (A and B) and repeat (C and D) study.

Table 2. Fold changes of all differentially-regulated transcripts in the hearts of 96 hpf zebrafish larvae exposed to either 150 µg BPA/l, 0.50 µg MBP/l or 10 ng EE2/l. Values presented are log2 transformed fold changes calculated by CuffDiff2. Significant differences in expression (FDR <0.1) are indicated by red (up-regulated) and green (down-regulated) shading.

Name	Ensembl ID	BPA Log2 FC	MBP Log2 FC	EE2 Log2 FC
acaa2	ENSDARG00000038881	1.08	0.50	1.00
acacb	ENSDARG00000061994	-1.99	-1.30	-1.32
actn2	ENSDARG00000038785	-0.42	-0.73	-0.20
anapc1	ENSDARG00000075687	-1.98	-1.76	-1.29
angptl4	ENSDARG00000035859	0.64	0.84	1.37
ank3b	ENSDARG00000077582	-0.13	-2.44	-2.82
ap3s2	ENSDARG00000039882	0.14	-1.18	-6.87
apc	ENSDARG00000058868	1.77	1.29	1.98
arf1	ENSDARG00000009484	-0.59	4.09	-1.63
asb2a.1	ENSDARG00000096748	2.17	4.72	2.56
b2ml	ENSDARG00000015887	-1.48	-2.14	-3.22
bcl11aa	ENSDARG00000061352	0.08	-2.98	-0.20
BOLA2B	ENSDARG00000051780	-1.34	-1.82	-2.09
btbd10b	ENSDARG00000020252	0.77	0.44	1.29
BX323876.2	ENSDARG00000052948	-0.60	-1.37	-0.47
c1qbp	ENSDARG00000039887	0.92	0.92	0.50
C25H12orf73	ENSDARG00000045797	0.03	-1.42	2.49
calm1a	ENSDARG00000021811	-0.39	-0.59	-1.13
caprin1b	ENSDARG00000054272	-0.34	-1.45	0.14
capza1a	ENSDARG00000034240	-0.07	0.03	-1.59
cd151	ENSDARG00000038288	-0.54	-0.94	-0.49
CDCA2	ENSDARG00000068759	-0.15	2.47	0.33
cep97	ENSDARG00000102407	4.05	5.08	3.28
cfd	ENSDARG00000039579	0.20	0.67	0.87
cltca	ENSDARG00000043493	0.10	-0.88	-0.01
cnbpb	ENSDARG00000070922	-0.56	-1.35	-0.85
COL5A2 (2 of 2)	ENSDARG00000031678	-0.19	-0.71	-0.04
cpt1b	ENSDARG00000058285	-0.33	-0.09	1.63
dis3	ENSDARG00000060559	3.03	-0.61	3.19
dkc1	ENSDARG00000016484	-0.33	-0.37	-1.06
dolpp1	ENSDARG00000036915	2.47	1.52	1.42
eif2s1a	ENSDARG00000005827	-0.64	-0.15	-1.86
ell2	ENSDARG00000006251	-0.23	1.26	1.81
emc1	ENSDARG00000057255	0.80	1.61	0.71
ENSDARG00000051775	ENSDARG00000051775	1.27	2.58	0.02
ENSDARG00000059604	ENSDARG00000059604	1.69	4.42	1.55
fabp11a	ENSDARG00000017299	0.31	0.78	0.44
FAM188B,nexn	ENSDARG00000077696	-0.67	0.26	-1.10

FAM96A	ENSDARG00000104940	2.07	0.56	2.88
farsb	ENSDARG00000016864	0.93	0.48	1.52
fbxw11b	ENSDARG00000017230	1.83	0.02	2.51
fnbp4	ENSDARG00000051800	-0.68	-0.82	-1.68
fndc3bb	ENSDARG00000062023	-1.41	-1.03	-0.74
fosl2	ENSDARG00000040623	0.09	-0.35	2.60
foxk1	ENSDARG00000037872	-1.75	-0.30	-1.13
fzd7a	ENSDARG00000060004	-0.46	-0.92	-1.61
gfer	ENSDARG00000070061	-1.78	-0.58	-5.27
gps1	ENSDARG00000040650	0.73	1.89	0.22
h1fx	ENSDARG00000054058	-0.02	0.83	-0.55
hbae1	ENSDARG00000089475	0.39	0.76	0.89
hbae3	ENSDARG00000079305	0.35	0.71	0.85
hbbe1.1	ENSDARG00000089963	0.40	0.31	0.84
hbbe2	ENSDARG00000045143	0.41	0.53	0.63
hdac9b	ENSDARG00000056642	-1.73	-0.64	-1.84
hivep1	ENSDARG00000103658	0.72	0.60	2.15
hmgb3b	ENSDARG00000006408	1.15	1.70	0.65
ivd	ENSDARG00000042853	-0.84	-1.26	-0.40
jam3b	ENSDARG00000061794	1.68	0.26	-0.07
kansl2,mlh3	ENSDARG00000056318	-0.19	0.01	3.12
kmt2d	ENSDARG00000037060	-1.60	0.02	-1.84
krt4	ENSDARG00000017624	-1.06	1.93	0.27
lmna	ENSDARG00000013415	1.09	1.66	0.89
lmo2	ENSDARG00000095019	2.57	2.71	2.16
lmod2b	ENSDARG00000045864	-0.16	-0.59	-0.99
lrrc15	ENSDARG00000070792	0.27	1.33	0.35
mcamb	ENSDARG00000005368	-0.17	-0.19	0.75
minos1	ENSDARG00000101619	-1.11	-0.89	-0.41
mtch2	ENSDARG00000019732	-0.37	-0.66	-1.06
mtmr3	ENSDARG00000093364	0.82	2.44	2.17
myom1b	ENSDARG00000104836	0.32	0.31	0.89
napab	ENSDARG00000020405	0.67	1.61	0.12
nars	ENSDARG00000061100	-0.54	-0.42	-0.77
ncl	ENSDARG00000002710	-0.43	-0.65	-0.78
ndufv1	ENSDARG00000036438	0.78	0.65	0.61
PARD3B (1 of 2)	ENSDARG00000092671	-0.09	-2.87	-1.16
pbx4	ENSDARG00000052150	0.99	0.32	0.16
pcid2	ENSDARG00000013802	0.82	-4.39	0.26
PET100,brd4	ENSDARG00000098017	1.58	1.95	1.38
pfkpb	ENSDARG00000012801	0.67	1.92	-0.56
pfn2l	ENSDARG00000012682	-0.29	0.07	-1.03
pkma	ENSDARG00000099730	-0.62	-0.89	-0.50
plekhg5a	ENSDARG00000025902	-2.32	-2.70	-1.79
psmd3	ENSDARG00000018124	1.06	0.17	0.57
ptenb	ENSDARG00000056623	0.84	-0.21	1.67
qpctla	ENSDARG00000061691	-1.75	-0.68	-0.23
RAPGEF2 (2 of 2)	ENSDARG00000005482	0.02	3.32	0.37

rb1	ENSDARG00000006782	0.93	1.64	1.86
rfc1	ENSDARG00000054799	2.51	1.45	0.36
sap130a	ENSDARG00000056924	1.52	3.02	0.43
sap130b	ENSDARG00000079352	-3.25	-2.18	-3.17
sbf1	ENSDARG00000062968	1.95	1.02	0.91
sdad1	ENSDARG00000105117	0.59	0.64	1.80
sec11a	ENSDARG00000008936	-0.98	-1.10	-1.33
sema6dl	ENSDARG00000011533	0.77	2.07	-0.72
sept7b	ENSDARG00000052673	-0.65	-0.85	-1.44
sept8a	ENSDARG00000032606	1.08	0.94	1.87
serf2	ENSDARG00000074340	-0.05	-0.21	-0.89
si:ch211-281l24.3	ENSDARG00000068637	0.43	1.87	0.25
si:ch211-286b5.5	ENSDARG00000096661	0.70	1.96	0.05
si:ch211-5k11.2	ENSDARG00000088330	0.45	1.53	1.37
si:dkey-199f5.8	ENSDARG00000018853	0.28	1.19	-0.49
si:dkey-228a15.1	ENSDARG00000095698	0.21	-1.29	-5.17
si:dkey-261m9.6	ENSDARG00000093195	0.91	1.46	1.52
si:dkey-4c15.13	ENSDARG00000095704	-3.93	-0.19	-0.96
si:dkeyp-117h8.4	ENSDARG00000043004	-1.50	-5.94	-3.21
slc12a7b	ENSDARG00000062058	0.78	2.42	1.94
slc38a2	ENSDARG00000045886	-0.31	-1.03	-0.60
slc4a1a	ENSDARG00000012881	0.69	0.73	1.14
soga1	ENSDARG00000063075	-0.82	-1.68	-0.64
sptb	ENSDARG00000030490	0.02	-1.17	-0.70
srgap2a	ENSDARG00000032161	0.40	2.48	0.69
ssr1	ENSDARG00000101627	-0.57	-1.91	-1.07
ssr2	ENSDARG00000005230	0.26	0.89	0.44
st3gal2l	ENSDARG00000007494	0.08	-0.25	1.16
stard13b	ENSDARG00000098954	0.17	2.99	0.26
thop1	ENSDARG00000013776	1.19	1.16	1.64
tie1	ENSDARG00000004105	-0.67	-1.67	-1.79
timm23a	ENSDARG00000071396	2.16	1.80	0.85
tspo	ENSDARG00000026655	0.71	0.68	1.09
ttna	ENSDARG00000028213	-0.14	-0.28	-0.77
ubr5	ENSDARG00000018192	0.86	0.56	1.54
uchl3	ENSDARG00000030177	1.00	1.19	1.74
uck2b	ENSDARG00000022213	0.29	0.86	1.58
wasf2	ENSDARG00000024209	-0.65	-1.89	-0.31
wwp2	ENSDARG00000061345	-1.03	-1.25	-2.24
zfhx3	ENSDARG00000103057	-0.27	-0.34	-1.05
zfp36l2	ENSDARG00000021806	-0.24	-0.41	-0.91
zgc:101560	ENSDARG00000099870	-0.45	0.14	-1.43
zgc:112966	ENSDARG00000095369	5.38	3.94	-

Table 3. Fold changes of all differentially-regulated transcripts in hearts of 96 hpf zebrafish exposed to 150 μg BPA/l in second study. Values presented are log2 transformed fold changes calculated by CuffDiff2. Significant differences in expression (FDR <0.1) are indicated by red (up-regulated) and green (down-regulated) shading.

Name	Ensembl ID	BPA Log2 FC
adamtsl7	ENSDARG00000055439	-2.59
ahcyl1	ENSDARG00000056331	-3.48
akr1b1	ENSDARG00000006215	-2.32
and1	ENSDARG00000088514	-3.56
and2	ENSDARG00000079302	-4.19
and3	ENSDARG00000056873	-4.77
apoda.1	ENSDARG00000060345	-1.96
apoea	ENSDARG00000102004	2.33
apoeb	ENSDARG00000040295	-3.3
aqp3a	ENSDARG00000003808	-3.34
asah2	ENSDARG00000012829	2.2
aspa	ENSDARG00000005154	-2.63
ass1	ENSDARG00000103044	-3.09
aste1	ENSDARG00000070132	-3.37
atp8b5b	ENSDARG00000079235	2.16
ca2	ENSDARG00000014488	-1.83
CABZ01007794.1	ENSDARG00000088655	-3.04
CABZ01045617.2	ENSDARG00000091047	3.14
CABZ01048956.1	ENSDARG00000089465	-2.18
CABZ01074130.1	ENSDARG00000105274	-4.55
MGAM	ENSDARG00000101629	4.19
camk2d1	ENSDARG00000043010	-1.33
capn12	ENSDARG00000010758	-2.87
cd36	ENSDARG00000032639	2.31
CES1 (1 of 3)	ENSG00000198848	2.01
cfl1l	ENSDARG00000012972	-2.71
ciarta	ENSDARG00000058094	-1.67
ckmt1	ENSDARG00000016598	2.95
cldn7b	ENSDARG00000014047	-3.04
cldnb	ENSDARG00000009544	-2.61
cldnh	ENSDARG00000069503	-2.46
cldni	ENSDARG00000054616	-2.86
CR385063.1	ENSDARG00000044212	-2.12
cspg5a	ENSDARG00000069981	-2.03
CT583700.1	ENSDARG00000090579	-2.56
CU019646.2	ENSDARG00000091234	-1.9
CU856173.1	ENSDARG00000091560	1.58
cubn	ENSDARG00000087013	-3.63
cx32.3	ENSDARG00000041787	-2.82

cxcl12a	ENSDARG00000037116	-2.37
cyp2k16	ENSDARG00000102981	1.98
cyt1l	ENSDARG00000036832	-2.26
dgat2	ENSDARG00000018846	2.55
dpys	ENSDARG00000079543	-3.13
egfl6	ENSDARG00000045958	-2.47
si:dkey-110c1.10	ENSDARG00000061810	-0.93
ENSDARG00000067760	ENSDARG00000067760	-2.7
ENSDARG00000075668	ENSDARG00000075668	-2.77
ENSDARG00000078528	ENSDARG00000078528	1.95
epcam	ENSDARG00000040534	-1.65
f2	ENSDARG00000036041	-0.87
fa2h	ENSDARG00000090063	2.54
fbln1	ENSDARG00000103311	-2.28
fmodb	ENSDARG00000010294	-2.53
FOS	ENSDARG00000031683	-2.84
fras1	ENSDARG00000054619	-1.76
frem2a	ENSDARG00000076856	-2.58
ftcd	ENSDARG00000007421	-2.27
glis3	ENSDARG00000069726	-2.38
gpx1b	ENSDARG00000006207	-2.76
grid2ipa	ENSDARG00000076103	-2.12
GSTAL	ENSDARG00000039832	2.01
hnf1ba	ENSDARG00000006615	-3.4
hoga1	ENSDARG00000018944	-1.89
hpdb	ENSDARG00000044935	-3.38
IM:7150988	ENSDARG00000098058	-3.14
isg15	ENSDARG00000086374	-3.7
jdp2b	ENSDARG00000020133	-2.93
kcnj13	ENSDARG00000043443	-1.81
krt4	ENSDARG00000017624	-2.68
krt5	ENSDARG00000058371	-2.7
lgmn	ENSDARG00000039150	-2.15
mid1ip1a	ENSDARG00000041051	-2.41
mslna	ENSDARG00000098949	-0.9
mxra5b	ENSDARG00000076309	-2.88
myh6	ENSDARG00000090637	-2.35
myh9a	ENSDARG00000063295	-1.65
MYO7B (1 of many)	ENSDARG00000044441	2.47
nr1d1	ENSDARG00000033160	-2.61
pah	ENSDARG00000020143	-3.65
pax2a	ENSDARG00000028148	-1.31
pdzk1	ENSDARG00000022261	-2.22
PDZK1IP1L	ENSDARG00000017127	-5.01
pnp4b	ENSDARG00000029230	1.62
prlra	ENSDARG00000016570	-2.03
ptgdsb	ENSDARG00000027088	-3.41
pyroxd2	ENSDARG00000014101	-1.57

rasd1	ENSDARG00000019274	-2.13
rbm47	ENSDARG00000061985	-1.8
s100a10a	ENSDARG00000037425	1.98
si:ch211-153b23.5	ENSDARG00000058206	-3.86
sgk1	ENSDARG00000025522	-1.66
si:ch211-117m20.5	ENSDARG00000091996	-2.33
si:ch211-125o16.4	ENSDARG00000056836	-3.44
si:ch211-157c3.4	ENSDARG00000087093	-1.02
si:ch211-220g18.3	ENSDARG00000092905	-1.94
si:ch211-251b21.1	ENSDARG00000007275	-2.36
si:ch73-65n21.1	ENSDARG00000098037	1.86
si:dkey-188i13.7	ENSDARG00000078389	-3.23
si:dkey-188i13.7	ENSDARG00000078389	-3.11
si:dkey-33c14.3	ENSDARG00000097118	-2.1
slc13a2	ENSDARG00000053853	2.92
slc13a3	ENSDARG00000069478	-3.37
slc22a7b.1	ENSDARG00000056643	-3.44
slc26a1	ENSDARG00000029832	-4.53
slc2a12	ENSDARG00000036865	-3.21
slc4a2a	ENSDARG00000028173	-3.77
slc4a4a	ENSDARG00000013730	-2.52
slc4a4b	ENSDARG00000044808	-1.75
slc5a8l	ENSDARG00000003697	-2.65
slc6a19b	ENSDARG00000056719	-2.22
socs3a	ENSDARG00000025428	-2.86
sp8b	ENSDARG00000056666	-1.33
stat1b	ENSDARG00000076182	-1.83
tat	ENSDARG00000069630	-1.74
tfap2a	ENSDARG00000059279	-2.27
tmc4	ENSDARG00000031757	-3.32
tmem176l.4	ENSDARG00000074390	-2.56
TMEM27	ENSDARG00000041644	-3.89
tmprss2	ENSDARG00000098686	-3.42
tnnc1b	ENSDARG00000037539	-3.26
ugt1a1	ENSDARG00000006220	4.12
upp1	ENSDARG00000040869	-1.82
vil1	ENSDARG00000040466	2.23
zgc:100868	ENSDARG00000004748	-1.93
zgc:123068	ENSDARG00000017489	-4.86
ZGC:158614	ENSDARG00000061634	1.58
zgc:172079	ENSDARG00000056762	2.27
ZGC:64085	ENSDARG00000025757	-3.66
zgc:65811	ENSDARG00000100904	-2.25

Table 4. Gene Ontology Terms and Kegg Pathways over-represented in the list of differentially expressed transcripts for each treatment group in original and repeat exposures. Values presented are the P-values and adjusted P-values associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6 .7 (Huang da *et al.* 2009) using the total number of transcripts observed to be expressed in the heart across all treatments as a background.

BIOLOGICAL PROCESS (BP ALL)	**First Study**						**Second Study**	
	150 µg BPA/l		**0.5 µg MBP/l**		**10 ng EE2/l**		**150 µg BPA/l**	
	P Value	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**
Localisation							3.4E-3	7.5E-1
Aromatic amino acid family metabolic process							5.4E-3	6.6E-1
Ion transport							6.0E-3	5.5E-1
Transport							6.4E-3	4.8E-1
Establishment of localisation							7.0E-3	4.3E-1
Sodium ion transport							9.5E-3	4.7E-1
Transmembrane transport							3.7E-2	8.8E-1
Monovalent inorganic cation transport							4.1E-2	8.8E-1
MOLECULAR FUNCTION (MF ALL)	**150 µg BPA/ L**		**0.5 µg MBP/ L**		**10 ng EE2/ L**		**150 µg BPA/ L Repeat**	
	P Value	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**
Secondary active transmembrane transporter activity							1.2E-3	2.1E-1
Transporter activity							1.9E-3	1.7E-1
Active transmembrane transporter activity							1.2E-2	5.6E-1
Substrate-specific transmembrane transporter activity							1.9E-2	6.1E-1
Structural molecule activity							2.2E-2	5.9E-1
Transmembrane transporter activity							3.0E-2	6.3E-1
Substrate-specific transporter activity							4.0E-2	6.8E-1
Anion:anion antiporter activity							4.1E-2	6.4E-1
Actin binding							4.8E-2	6.6E-1
CELLULAR COMPONENT (CC ALL)	**150 µg BPA/ L**		**0.5 µg MBP/ L**		**10 ng EE2/ L**		**150 µg BPA/ L Repeat**	
	P Value	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**
protein complex					4.9E-2	9.4E-1		
Cell-cell junction							3.3E-3	1.8E-1
cell junction							3.9E-3	1.1E-1

occluding junction							4.4E-3	8.5E-2
tight junction							4.4E-3	8.5E-2
apical junction complex							5.5E-3	7.9E-2
apicolateral plasma membrane							5.5E-3	7.9E-2
plasma membrane part							6.7E-3	7.7E-2
plasma membrane							2.1E-3	1.9E-1
keratin filament							2.7E-3	2.1E-1
intermediate filament cytoskeleton							3.7E-2	2.5E-1
intermediate filament							3.7E-2	2.5E-1
KEGG PATHWAY	**150 µg BPA/ L**		**0.5 µg MBP/ L**		**10 ng EE2/ L**		**150 µg BPA/ L Repeat**	
	P Value	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**
Phenylalanine metabolism							8.5E-3	3.4E-1
Drug metabolism							2.2E-2	4.2E-1
Phenylalanine, tyrosine and tryptophan biosynthesis							4.2E-2	5.0E-1

Discussion

BPA has been identified through epidemiological studies as a possible causative agent in increasing cardiovascular disorders (Melzer *et al.* 2010, Bae *et al.* 2012, Melzer *et al.* 2012, Shankar and Teppala 2012). A number of *in vitro* and *in vivo* studies in mammalian systems also appear to support this hypothesis (Yan *et al.* 2011, Belcher *et al.* 2012, O'Reilly *et al.* 2012, Poidatz *et al.* 2012, Yan *et al.* 2013, Posnack *et al.* 2014). However, experimental evidence is still lacking concerning the mechanisms by which this may occur. The zebrafish has emerged as a model organism useful for studying the vertebrate heart, with larval stages particularly useful for investigating developmental abnormalities that may arise. Several studies have also revealed that the core molecular mechanisms driving zebrafish cardiac valve formation are highly conserved with those found in mammalian hearts. (Beis *et al.* 2005). Using a transgenic zebrafish that responds to estrogenic stimulus with a fluorescent protein, we have previously demonstrated that exposure to BPA causes a fluorescent response in the heart valves of larval zebrafish, indicating an estrogenic related transcriptomic response. This resulted in an unstable atrial:ventricular beat ratio at 5dpf and a reduced beat rate in larvae at 14dpf. However, concentrations tested far exceeded those typically found in the environment (2500 µg/l). Here a transcriptomic approach utilising RNA-Seq was taken to determine if exposure to BPA, the potent BPA metabolite MBP and the classic steroidal estrogen EE2 cause alterations in gene expression in hearts dissected from exposed larval zebrafish. Previous RNA-Seq studies on larval zebrafish have used whole larvae, making our approach particularly novel.

Using the ERE-TG zebrafish,it was determined that at the exposure concentrations tested a similar fluorescent response was observed (Fig. 1), indicating a similar level of transcriptional activity based on GFP response. Using the bowtie2 aligner it was possible to determine the concentrations of GFP mRNA using FPKM values. FPKM value in control hearts was very low, representing low levels of GFP present as background, possibly due to the actions of endogenous estrogens (Fig. 3). FPKM values for GFP mRNA were significantly higher in fish exposed to BPA than those exposed to MBP and EE2, the reasons for this are unclear. This may result from the fact that the GFP protein is relatively stable compared to mRNA values, therefore fluorescence images indicate estrogen

activity over a longer period of time, which may differ from the smaller window indicated by mRNA. There was no significant difference between the GFP mRNA measured in MBP and EE2 exposed fish indicating a similar level of transcriptional response. Though it should be noted GFP production is not dependent on a particular estrogen receptor (ER) signalling pathway and these chemicals could be acting through different mechanisms. Though previous work in this thesis has indicated in the heart all three chemicals tested appear to mediate their effects via an esr1-dependent pathway (Chapter 3).

Analysis of mRNA of the different ERs in these zebrafish hearts may help to further explain our previously determined results whereby morpholino (MO) knockdown of esr1 was sufficient to inhibit the fluorescence response in ERE-TG fish exposed to several different estrogens. In the first exposure study in this chapter there were no transcripts detected for esr2a across any treatment and only one replicate in each of the Control, BPA and EE2 treatments were found to have very low levels of esr2b transcripts (Fig. 3). In contrast transcripts for esr1 were seen across all treatments, with FPKM values of 1.7 ± 0.3, 2.3 ± 0.9, 4.9 ± 1.1 and 1.7 ± 1.0 for control, BPA, MBP and EE2 groups, respectively. Transcripts for both esr2a and esr2b however, were detected in the second BPA exposure study, possibly due to a larger depth of coverage generated by the sequencing run. FPKM values were several times lower than those seen for esr1. These results further indicate that during this early life stage estrogenic responses in the heart of zebrafish appear to be mediated via an esr1 dependent pathway as this is the dominant ER expressed. It could be argued therefore that chemicals more likely to preferentially target esr1 may be more likely to exert effects on heart development.

Atrioventricular cardiac valve development in zebrafish (and other phyla) requires a multitude of specific processes, including initiation of endothelial-to-mesenchymal transition (EMT), EMT migration and valve maturation (Beis *et al.* 2005). The molecular cues that modulate these early processes in valvulogenesis have been studied extensively. BMP modulates several important genes and pathways in atrioventricular valvulogenesis, such as Notch1 and TGF-β, which are known to play significant roles in endothelial-to-mesenchymal transition. Notch1 signalling in atrioventricular endothelial cells is also known to induce the expression of Snail, a transcription factor that promotes EMT. Specific cells and

tissue interactions are also expected to enlist a wide array of protease actions. In particular, the novel serine protease PRSS23 has been identified as essential for the Snail-dependent EMT during valvulogenesis (Chen *et al.* 2013). Interestingly PRSS23 has also been demonstrated to be regulated by ERα in human MCF-7 cells, possibly suggesting a key role for ERα in valve development (Chan *et al.* 2012). After EMT FGF signalling is important for the regulation of growth and development of cardiac cushions (Person *et al.* 2005). Cellular mechanisms involved in the formation of bulbus arteriosus are less well studied. However, BMP signalling has been identified as being evident in the proximal cells of the bulbus arteriosus during formation (Person *et al.* 2005, Hami *et al.* 2011). As we observed a GFP fluorescent signal in the atrioventricular valves and developing bulbus arteriosus in larval zebrafish we considered that BPA, MBP and EE2 may have some effect on development. However, in both the first and second (BPA only) studies we found no significant effect on the expression of any genes known to possess a role in formation of these tissues. This would suggest that at the concentrations tested at 96 hpf there is no measurable effect on valve formation, this may help to explain the results observed in Chapter 3 where there was no observable effect on cardiac parameters that may be affected by abnormalities in valve formation, namely, blood flow, blood speed and stroke volume. However, it should also be noted that many genes, particularly those involved in valve formation are temporal and window of response may have been missed.

The number of differentially expressed genes observed was generally low. In the original study there were 26, 54 and 64 differentially expressed genes in groups exposed to 150 µg BPA/l, 0.50 µg MBP/l and 10 ng EE2/l respectively (Fig. 2). There was also little overlap in differentially expressed genes across treatments with only one gene *slc4a1a* significantly up-regulated by all treatments (Table 2). As many transcripts were found to not be present across all treatments it was considered possible that this library preparation may be less effective at detecting changes in transcript level in these tissues. However, RNA quality was sufficient as recommended by the preparation guidelines and the library preparation method has been optimised for experimental conditions such as these. We therefore ran the second exposure study to BPA comparing hearts from only Control and 150 µg BPA/l treated fish, to determine the library preparation effectiveness. This gave a greater read number, and therefore a greater depth of

coverage. Given this, transcript expression changes in these samples could be more confidently detected. Although more differentially expressed genes were detected in this second exposure, differentially expressed genes were still low, with 130 genes found to be differentially expressed out of the 14396 genes detected. It is therefore possible that the low number of differentially expressed genes in the initial study are partly due to the lower depth of coverage and partially due to the fact that estrogenic chemical effects on the heart at this stage may not have a profound effect on gene regulation.

In the first study there were no significantly enriched GO terms for the differentially expressed genes observed in the BPA or MBP treated samples (Table 4). Indicating the differentially expressed transcripts do not group together in a way that disrupts specific processes. Though this is expected given the low numbers of differentially expressed genes, one commonly differentially expressed transcript between BPA and MBP treated fish was for the gene *brd4* where transcripts were up-regulated 1.6- and 2.0-fold respectively. BRD4 has been previously demonstrated as being a central regulator of ERα function in humans, where an increase has been observed in gene expression in ERα-positive cells under estrogen treatment (Nagarajan *et al.* 2014, Nagarajan *et al.* 2015). This may indicate that although there was no significant change in the expression of ERs under the treatment of these estrogenic chemicals, estrogen signalling is complex and alterations in the regulation of related genes could serve to alter the way ERs interact with EREs, changing expression of other related genes. Although *brd4* expression was not significantly altered in EE2 treated fish a similar up-regulation (1.2-fold) was observed, possibly suggesting that upregulation of *brd4* by these chemicals is estrogen dependent.

Although EE2 treated hearts were observed to have the highest number of differentially regulated genes the number was still low and only one GO term was found to be significantly over-represented (protein complex). Several genes involved in proteolysis were found to be differentially regulated only in this treatment group, including *sec11a, thop1, ubr5, cfd* and *wwp2*. Ovariectomised rats have previously been found to have an increase in gene expression mediating proteolysis in cardiac tissue, an observation that could be corrected with E2 treatment, indicating estrogen does play a role in proteolysis in the heart (Hamilton *et al.* 2008). However, genes that were differentially expressed in

hearts exposed in this study did not show a consistent pattern of up or down regulation, despite all chemicals being primarily estrogenic in action, therefore confounding the ability to draw clear conclusions on possible health outcomes.

There was only one common differentially expressed transcript in the first study; the solute carrier *slc4a1a* was significantly up-regulated compared to the control across all treatments. This may indicate that each chemical is targeting different pathways and that there is no conserved expression pathway for estrogenic chemicals in the heart. However, due to the fact that there was no expression for many genes across treatments, it could be that expression patterns are not seen due to poor coverage. This may have resulted due to the low levels of RNA as compared to traditional sequencing library preparations, or due to the methodology of the sequencing itself.

In the second BPA exposure study a greater number of reads was generated from the sequencing run, possibly as a consequence a greater number of differentially expressed genes and GO terms were also observed (Table 4). Ion transport was a significantly enriched GO biological process following exposure to 150 µg BPA/l, corresponding with a decrease in the expression of a number of transcripts encoding membrane transport proteins. A number of transcripts encoding ion transport proteins in the solute carrier family (*slc26a1, slc4a2a, slc4a4a, slc5a8, slc22a7b, slc4a4b, slc5a11l* and *slc13a3*) were down regulated (by 1.8-4.5 fold). Of these, *slc5a8l, slc5a11* and *slc13a3* were further associated with the significantly enriched GO term for sodium ion transport along with the downregulated carbonic anhydrase II *ca2* and upregulated *slc13a2*. The gene encoding for the potassium voltage-gated channel *kcnj13* was also significantly down-regulated by1.8 fold. Previous studies have demonstrated the ability of BPA to act rapidly, binding to ion channels to affect heart function (O'Reilly *et al.* 2012, Posnack *et al.* 2014). The mechanisms by which cardiac conduction may be impaired by BPA are complex, however reducing sodium and potassium ion transport have both been suggested as possible targets (Posnack *et al.* 2014). Both sodium and potassium are essential in maintaining cardiac contractions, therefore any downregulation in genes encoding for ion transport could potentially have effects on heart function such as arrhythmia as has been previously described for BPA (Gao and Wang 2014).

The majority of GO terms significantly enriched in the molecular function classification are also associated with transmembrane transport. Many of the same downregulated genes, in particular those belonging to the solute carrier family appear responsible for this enrichment. An additional significantly enriched molecular function GO term was structural molecule activity, consisting of transcripts largely encoding for claudin proteins, including *cldn7b, cldnb, cldnh* and *cldni*, all downregulated (by 2.5-3.0 fold). Claudin proteins are transmembrane proteins and the most important components of tight junctions, suggesting again that BPA may have some effects on cell transport. This is reflected in the cellular component classification where the majority of significantly enriched GO terms are again associated with cell junctions and membrane proteins. In addition to transcripts for claudin genes a significantly downregulated (2.8 fold) transcript was also identified for connexin 32.3 (*cx32.3*) another transmembrane gap junction protein.

Gap junctions mediate the cell-to-cell movement of ions and in the heart play an important role in impulse conduction, allowing heart muscle cells to contract in unison. In knockout mice where a cardiac specific gap junction (*cx40*) was silenced various phenotypes were observed including slowed conduction, partial atrioventricular conduction block and arrhythmia (Kirchhoff *et al.* 1998, Simon *et al.* 1998). Therefore, downregulation in the genes encoding for gap junctions in the heart could potentially have physiological consequences. A small number of studies have investigated the effects of BPA on gap junction proteins previously and they appear to concur an inhibitory effect (Lee and Rhee 2007, Salian *et al.* 2009, Li *et al.* 2010). However, mechanisms are less clear, it appears that BPA can act directly on connexin gap junctions. (Lee and Rhee 2007)determined that during exposure there was no effect on Cx43 mRNA level, suggesting that BPA (at concentrations as low as 91.3 µg/l) inhibits gap junction-mediated intercellular communication through modulation of the gap junction gating as opposed to through genomic modulation. However, in these studies exposure time was short, up to a maximum of 24 hrs. A long term study on male rats fed 400 µg BPA/kg BW during postnatal days 1-5 and sampled during adulthood did find a significant reduction in the expression of cx43 mRNA (Salian *et al.* 2009).

The significantly enriched GO terms in the second BPA exposure study were not seen in the original study for BPA exposed fish, or for MBP and EE2.

Furthermore, there are actually very few similar differentially expressed genes. The reasons for this could possibly be due to the reduced coverage observed in the original study, resulting from the sequencing methodology. In addition, the TotalScript library preparation technique requires particularly high RNA integrity, due to the lower RNA concentrations than conventional library preparations and the fact that it is not rRNA depleted. In the first exposure study average RIN score across all samples was 8.1±0.25, whereas average RIN score in the repeat study was 9.2±0.08. This indicates that although the original study passed the minimum RIN score necessary for the use of TotalScript library preparation (RIN >7) the RNA quality was higher in the repeat study, which may have resulted in a more robust library for sequencing.

Our findings demonstrate BPA has the ability to affect gene signalling in the heart of larval zebrafish, albeit only covering a relatively small number of functions. The main genes that appear to be affected are those associated with membrane transport, in particular ion transport and gap junction communication, where the majority of genes are downregulated. These functions play an integral part in cardiac function, in particular maintaining regular contraction. Despite the strongest fluorescent signal in our estrogen responsive transgenic fish being in the atrioventricular valves and bulbus arteriosus there was no significant effect on genes involved in the function or development of these tissues.

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CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5: General Discussion

A wide range of screening and testing systems have been used to further elucidate both the effects and mechanisms of action for BPA, and other environmental estrogens, including both *in vitro* and *in vivo* systems. The different available systems have their different strengths and weaknesses. *In vitro* systems are high throughput, can be transfected with a range of receptors/reporter genes whilst being cheap and easy to maintain. However, *in vitro* systems have their limitations. Several factors may be overlooked including the ability of a test compound to bioconcentrate. Certain cells will also express different co factors and metabolism may alter between species or even between tissue types. Transgenic fish have emerged in recent years in the field of ecotoxicology and they provide integrative systems that allow for comprehensive effects assessment across multiple tissues *in vivo* (Lee *et al.* 2015). The aims of this thesis were to utilise the zebrafish as a model organism, including a novel ERE-TG transgenic zebrafish line, to determine the tissue targets, health effects and estrogenic mechanisms of action of BPA, the BPA alternatives BPS, BPF and BPAF and the BPA metabolite, MBP.

I shall now discuss the key findings from the research presented in the thesis chapters and what the collective contribution of these findings are, followed by a discussion on the limitations and challenges of the work and possible future research directions. Through my thesis work the following has been established:

(1) The estrogen responsive target tissues for all bisphenolic chemicals tested appear to be the same, though concentration necessary to induce a response can vary (Chapter 2 and Chapter 3). In chapter 2 I demonstrated that BPA and its commercial alternatives BPF, BPS and BPAF all target the heart tissue, and this was confirmed in Chapter 3 to be the atrioventricular valves and bulbus arteriosus. In Chapter 3 I also demonstrated that the BPA metabolite MBP similarly targeted the heart valves. This would indicate that these bisphenolic chemicals have a similar mechanism of action, likely binding via ERs. However, the reason for the difference in potency is less clear, it is likely that substituents on the phenolic rings or the bridge between them affect interactions with the ER(s). *In silico* modelling of MBP suggests that the stronger response is due to

possessing a longer bridge between the phenolic rings (Baker and Chandsawangbhuwana 2012).

(2) In Chapter 2 I also showed that BPA, BPF, BPS and BPAF exerted teratogenic effects at concentrations higher than those needed to induce any estrogenic response. Although estrogenic response appears to possess similar targets and there were some similar teratogenic responses between the bisphenolic chemicals, namely cardiac edema and craniofacial abnormalities, there were many distinct phenotypes. This may suggest that the different chemicals can interact with different signalling pathways in addition to the ER(s). For example, it was suggested that the observed inhibition of pigment formation in fish exposed to BPF could be associated with ability to affect thyroid signalling. It has recently been shown that BPF does affect thyroid signalling in zebrafish from as low as 20 µg/l by altering concentrations of TSH, T3 and T4 (Huang *et al.* 2016). Characterising how different 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.

(3) Although receptor binding is likely to cause the differences in response, the uptake experiments conducted through this thesis also indicate that bioavailability may play an important role in estrogenic potency *in vivo*. In Chapter 2 I demonstrated that some of the alternative bisphenols have different capacities for accumulating in body tissues, possessing different BCFs. Particularly interesting was BPS, where, at a dosing concentration 50-fold higher than for BPA, the measured internal concentrations were similar. This may suggest that once in cells BPS has a similar affinity to BPA, as seen in various *in vitro* studies, and it is the lower bioavailability that limits its effect. In Chapter 3 I also showed that MBP had a greater potential to bioconcentrate compared with BPA, by approximately 10-fold. However, the estrogenic potency as measured by GFP increase was, 400 to 1000- fold higher indicating receptor binding is the main reason for the observed differences in potency here.

(4) Differences in the tissue targets (GFP expression) for the different chemicals in ERE-TG fish seen here in Chapter 3 and previously in (Lee *et al.* 2012) is likely

to relate to preferential binding to certain ERs that show tissue specific expression. Through morpholino knockdown I was able to illustrate that esr1 is essential for inducing a GFP signal in the heart. It has previously been observed using WISH (Gorelick *et al.* 2014), and I confirmed with RNA-Seq in Chapter 4 that the dominant ER expressed in the heart is esr1. From this work, it would appear that bisphenolic chemicals have a stronger ability to bind to and activate esr1 *in vivo*.

(5) Transgenic fish provide a powerful tool in combination with molecular manipulation techniques for determining signalling pathways. In Chapter 2 I illustrated that the bisphenolic chemicals induced responses through the classic ER signalling pathway. In Chapter 3 through combining chemical exposure and injection with morpholinos for specific ER(s) I was able to illustrate the specific ER(s) responsible for signalling in the different tissues. Specifically, esr1 was found to be essential in heart signalling, esr2a and esr2b essential for signalling in tail myotubes and all ERs play a role in liver signalling.

(6) BPA may have health effects in the heart. Work in Chapter 3 and Chapter 4 indicates BPA can exert a measurable effect on heart function and gene expression. In Chapter 3 live imaging of exposed zebrafish larvae indicated that exposure to BPA affected the ability of the heart to contract effectively (5 dpf and 14 dpf). Specifically at 5 dpf the A:V beat ratio was disrupted and at 14 dpf heart rate was lowered. It should be noted however, that these effects were only observed at a concentration of 2 500 µg/l, far exceeding even the highest environmental dose by 100-fold. Testing at a much lower concentration of 150 µg/l (that still exceeds environmental concentrations by at least 7-fold), I later showed (in Chapter 4 through RNA-seq analysis) that several genes involved in ion transport and cell-to-cell signalling were downregulated. These processes are essential for maintaining regular and consistent contraction. It is therefore feasible that downregulation of these genes may be having a more subtle effect on heart function not detected in my work. Much longer (including lifelong) exposure should be explored to assess for the possibility of more subtle health effects and thus provide greater re-assurance for a lack of health effects of BPA on heart function.

5.1 Collective contribution of this thesis

This thesis has presented a range of novel findings as stated above, which make a valuable contribution to the current understanding in this field. For example, despite the increasingly frequent use of alternative bisphenols in BPA-free plastics prior to my thesis there was very little research on *in vivo* health effects of these alternatives. Several *in vitro* studies have shown conflicting evidence as to the potency of the most commonly used alternative BPS (Masuno *et al.* 2005, Grignard *et al.* 2012). The data in this thesis does demonstrate that *in vivo* BPS does appear to be up to 50-fold weaker as an estrogen than BPA, making it more suitable as a replacement. However, we also show for the first time that it does display similarities in tissue targets, that would also suggest there are still concerns for both aquatic species and human health. The fact our research also determined that BPF and BPAF were found to be as potent and 10-fold more potent *in vivo* than BPA respectively should be cause for increased concern. These compounds are currently used in replacement plastics and resins without the scrutiny BPA receives and are found in environmental and human samples (Liao *et al.* 2012, Liao and Kannan 2013, Yang *et al.* 2014). This data presents the first findings comparing *in vivo* estrogenic potency and tissue targets of these bisphenols in an aquatic vertebrate. The data in this thesis, therefore contributes valuably to the current knowledge on the health effects of bisphenolic chemicals and as such may be used to aid policy and decision makers on the improvement of both environmental quality standards and human health protection.

Although water chemistry measurements are now common in toxicity testing (as advised in OECD guidelines Test, 236), measurements of chemical uptake and bioconcentration are much rarer in routine dose response studies (Yamaguchi *et al.* 2005, Rochester and Bolden 2015, Tisler *et al.* 2016). The data presented in this thesis demonstrates the importance of taking such measurements. This is the first study that has compared the uptake of various bisphenolic chemicals to their *in vivo* estrogenic activity. Previous data has relied on receptor affinity to determine possible health effects (Rosenmai *et al.* 2014, Rochester and Bolden 2015). Uptake data is important as it indicates that bioavailability in combination with receptor affinity plays an important role in the activity of a chemical. This further emphasises the relevance of *in vivo* studies to gain a greater understanding of the mechanisms of effect for any given chemical.

Although there are now several transgenic fish lines developed for use in ecotoxicity testing, few have progressed to the stage where they are commonly employed in empirical research, instead being reported in papers where their generation is the focus (reviewed in Lee it al. 2015). This thesis provides evidence that the ERE-TG zebrafish line can be employed to ask relevant research questions which is essential if these models are to ever gain a place in regulatory testing. We have shown that they can be used to compare *in vivo* estrogenic potency across a family of chemicals and in combination with receptor knockdown to determine tissue specific effect mechanism. Previously specific ligand receptor interactions were determined by *in vitro* binding assays or by changes in receptor expression both of which have limitations (Kitamura *et al.* 2005, Li *et al.* 2013, Delfosse *et al.* 2014 Rochester and Bolden 2015). This is the first study that has utilised receptor knockdown with an estrogen responsive transgenic fish to determine specific receptor signalling pathways, proving that it is a viable method useful to the field of ecotoxicology research.

In chapter 3 we determined that the heart valves were a key target for bisphenolic compounds. This finding demonstrates the importance of transgenic fish models 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, Peterson *et al.* 2013). Despite the observed signal, BPA did not appear to have any specific functional health effects relating to these structures, that we were able to identify. Therefore, although this thesis presents some of the first data using transgenic fish in novel ecotoxicology research, it also demonstrates the need for caution in interpreting results. It must be emphasised that although extremely useful in identifying specific tissue targets and determining signalling pathways, a fluorescent response cannot be assumed to result in health effects in these tissues. Moving forward with these models, with this in view, they should form a basis for further experimental work investigating the potential health effects in larvae, but also other life stages.

This thesis provides new evidence on the practical uses of RNA-seq for determining global transcriptome changes in specific larval tissues following chemical exposure. In previous studies when assessing alterations to gene transcription in larvae DNA has been extracted from whole larvae, likely due to the difficulties with isolating individual organs (of 33 studies reviewed by Schuttler

et al. 2017). However, the need to understand gene changes in specific organs is vital, particularly at this early stage as organogenesis is in progress (Martin and Bartman 2009, Staudt and Stainier 2012). The data presented in Chapter 4 demonstrates, again for the first time, that it is possible to extract high quality RNA from specific larval tissues and generate libraries suitable for sequencing. This information is valuable to those planning RNA-seq experiments in larval stages and moving forward investigators should look to examine the specific gene responses across organs in larvae as is frequently done in adult exposures.

5.2 Challenges and Limitations Encountered

Transgenic fish line

The use of the transgenic ERE-TG zebrafish line was an integral part of this thesis, consistently used throughout each research chapter. However, maintenance of the line proved to be time consuming. In particular, screening to ensure homozygosity of both transgenes (ERE:Gal4ff)/(UAS:GFP) that needed to be undertaken on several occasions. A further limitation in the line I used was that zebrafish natural pigmentation formation occluded the fluorescence signal at later life stages. This was a major driver for conducting all work in larval stages. Larval stages however were especially useful for much of this work, including for the application of morpholino knockdown to establish chemical effect mechanisms. Larval stages are practically easier also for conducting exposures and considering space and resources generally. However, embryonic and early life stage responses may not always be representative of those seen in later life and may only be used to infer possible health outcomes for older life stages. The issue with pigmentation has been addressed during the course of this PhD and a further line has been developed in the pigment free casper zebrafish line (Green *et al.* 2016). However, even in this line thickness of skin and depth of internal organs only allows for practical quantitation of fluorescence up to approximately 30 dpf. A further possible limitation of using a transgenic line is that there has currently been very little research into the comparable responses between transgenic and wildtype animals. Also as to how the nature of the promoter differs from natural promoters. In the ERE-TG fish used in this study a 3x repeat synthetic ERE promoter was used as this was determined to produce the strongest response and bind all ER-subtypes, capturing responses via all ER

mediated pathways. However natural EREs are likely to interact with differing efficiencies - an area where future research would be beneficial.

Timing of exposure and sampling

Calculating the timing of sampling frequently presented a challenge in several chapters. In Chapter 3 I exposed morpholino injected larvae. Morpholinos are known to be time sensitive, with effectiveness only lasting into the first few days post injection. A preliminary study indicated that at 96 hpf GFP could be induced in the heart of morpholino injected fish (against all ERs) by BPA, whereas at 72 hpf no induction was observed. Therefore, fish were sampled for microscopic imaging at 72 hpf to ensure we were observing effective knockdown by the morpholino. This was an earlier time point than for other studies in this thesis (largely 96-120 hpf). However, the fact that esr1 was the dominant ER expressed in the heart at 96 hpf in Chapter 4 would suggest we can be confident in the results obtained by morpholino injection that indicated estrogen signalling in the heart is dependent on esr1.

Timing of sampling also had to be decided for RNA-seq in Chapter 4. As valve formation has been reported to occur between 72 hpf and 120 hpf, I chose the midpoint of 96 hpf, in an attempt to capture specific gene pathways involved in this process. Preliminary work also indicated that 96 hpf was the most appropriate age to optimise the heart extraction technique, which was necessary to obtain sufficient RNA (discussed below).

In both sequencing experiments I did not detect any alterations in genes involved in valve/bulbus arteriosus formation. It may be the case that despite responding to estrogen (producing a fluorescent signal in these tissues) there is no direct effect on development of these structures. The GFP protein is relatively stable and without UV exposure may remain stable for several days, with a half-life of approximately 26 h in one system (Corish and Tyler-Smith 1999). GFP may therefore only indicate where there has recently been transcription. Further work should seek to investigate whether there is a time-related response to genes involved in valve formation on exposure to these chemicals.

RNA-seq RNA concentration and methodology

Although RNA-seq offered many advantages I encountered significant challenges in obtaining enough mRNA to perform RNA-seq. Larval zebrafish hearts contain a very low concentration of RNA, I calculated an average heart to have RNA present at approximately 36pg/µl. A significant period of time was therefore spent optimising the heart extraction and isolation technique and comparing the different methods of RNA extraction, namely using Trizol or Qiagen column methods. Qiagen columns were found to be most effective. As total RNA concentrations were still low (ranging from 1 497 to 3 279 pg/µl), even when pooling hearts of approximately 50 larvae it was necessary to investigate library preparation techniques that were optimized for this. The TruSeq library preparation method used frequently by Exeter Sequencing Service request a minimum of 200ng total RNA, TotalScript was therefore selected which is optimised to work with concentrations as low as 5ng total RNA. Further issues encountered with sequencing were discussed in Chapter 3, particularly with regards to the first sequencing experiment. It is believed these issues were possibly related to the sequencing run as the libraries for this experiment were multiplexed on a sequencing lane with a separate experiment. This resulted in a smaller number of reads per sample and could have affected downstream analysis. Another reason suggested was the lower quality of RNA in the first experiment. Unfortunately, due to the low levels of RNA it was not possible to perform a second sequencing run on the previous samples and a second experiment was instead performed.

Use of microscopy to quantify fluorescence

Another common theme throughout this thesis is the use of fluorescence microscopy and subsequent image analysis to measure GFP intensity, to infer differences in potency and target tissues between chemicals. There are some inherent limitations with carrying out this process. Firstly ,the number of observable fish per treatment is limited by workload constraints. Although microscope parameters (exposure length, light intensity, microscope aperture etc.) were maintained throughout an experiment it is necessary to orientate the larvae in an identical manner for every photograph. This can be difficult to judge by eye. Some of the problems with the nature of this work can be overcome with the use of automated systems, recently applied to rapidly scan and analyse responses in transgenic fish (Green *et al.* 2016). However, sensitivity is lost in

automated systems currently available, for example the lowest observed effect concentration for BPA was reported to be 1000 μg/l as opposed to 100 μg/l as observed using conventional microscopy. Also the inability to manually orientate larvae increases the difficulty of identifying specific tissue targets. It is likely therefore that although these high-throughput methods may be very useful in identifying chemicals of interest for future research they will be used in combination with more traditional microscopy techniques. Though it should be noted that the increased use of transgenic fish and the need for higher throughput screening, particularly necessary if intended as a tool for regulatory testing, is likely to drive rapid improvements in the field of automated detection. These improvements, for example use of automated insertion of larvae into a capillary tube allowing orientation to be manipulated, should overcome these issues.

Test Concentrations of Bisphenolic chemicals

Test concentrations for bisphenolic compounds were generally high and well above those found in the natural environment. This needs to be considered against the biological target and effects seen. The work presented nevertheless provides important information in establishing effect mechanisms of action and target tissues for potential health effects for these compounds. As MBP has never been detected in environmental samples, which may be because it has not been screened for, it was not possible to establish what an environmentally relevant dose was for the exposure studies. However it is likely that concentrations tested in Chapter 3 are unlikely to be found in water samples, due to the fact that production of MBP is not a major metabolic pathway in mammals (details on MBP production in other taxa are currently unknown). This work still allowed us to demonstrate that a known metabolite of BPA (in mammals) is more potent as an estrogen *in vivo* than BPA itself. More work is necessary to determine the potential in fish to produce similar metabolites, whether these metabolites circulate freely and whether they are present at concentrations likely to cause health effects. I did detect an estrogenic response in the heart valves at 100 μg BPA/l and changes in gene transcription at 150 μg/l, these concentrations are much closer to the highest environmental samples, but still 5 to 7-fold higher. Further work should aim to establish if similar genetic targets are affected at more environmentally relevant concentrations.

5.3 Future Considerations

Transgenic fish models

This thesis has demonstrated that transgenic fish may be employed to determine target tissues and effect mechanisms of bisphenolic chemicals. These data can then be used to further investigate possible health outcomes. As previously discussed this can be taken forward with high-throughput screening to rapidly screen and easily identify chemicals of interest (Green *et al.* 2016). However one of the most effective uses for transgenic fish is that receptor subtype effect pathways may be investigated relatively easily using silencing methods. In this thesis we used a generic drug targeted inhibition (Chapter 2) and a targeted morpholino knockdown approach (Chapter 3) to determine ER pathways specific to subtype. However new methods in gene silencing have been developed in recent years that may also prove effective. Future studies could utilise CRISPR technology, as one such method, that may prove useful in combination with transgenic fish species. This allows for simple and efficient targeting of the genome itself, as opposed to morpholinos that target mRNA (Wei *et al.* 2013). Advantages suggested in using CRISPR models are that possible side effects sometime apparent with morpholinos are reduced. Also changes are longer lasting, essentially creating a mutant organism, which would allow for observations at later life stages than with morpholinos.

A further method by which the use of ERE-TG fish could be improved in the future includes the use of different reporters and promoters. For example, currently in development is an ERE-TG fish where the reporter instead uses the kaede protein, which converts from green to red fluorescence after UV treatment (Lee *et al.* 2015). This offers opportunities to identify responses for sequential exposures, for either the same or different chemicals. Combining different reporters in one animal such as the ERE and cyp19a1b gene promoters (with separate fluorescent reporters) could also help to better understand the roles of estrogenic chemicals in fish.

Bisphenolic chemicals in plastics

During the course of my PhD there has been increased regulation on the use of BPA, with several regulatory bodies banning the use of the chemical, particularly

in baby bottles (Barraza 2013). This is a step forward, indicating that sound scientific evidence in combination with public pressure (itself brought about by increasing the public awareness of scientific research and its implications for environmental and human health) can have a positive effect on changing legislation. It is essential that research, industry and policy makers work in collaboration to thoroughly progress the understanding of these chemicals. However, there is now growing evidence, which this thesis contributes to, on the comparative toxicities of all bisphenolic chemicals. As yet, however, there do not appear to be any moves to reduce the use of alternative bisphenols. Research such as this is therefore important to highlight the potentially similar activity of these commonly used alternatives to better inform policy makers. Although currently chemicals have to undergo safety testing this largely relates to toxicity. Transgenic fish, such as the ERE-TG zebrafish used throughout this thesis could form an extremely useful addition to standardised toxicity testing, to determine sub-lethal negative health effects, though they have yet to achieve regulatory acceptance.

In addition to the estrogenic effects of these alternatives, my research has demonstrated that additional effects may also occur through different pathways. To fully understand the mechanisms of action of these bisphenol chemicals further research is necessary to determine their effects on other pathways such as androgen and thyroid signalling. Certain bisphenol analogues, TBBPA, for example, have been well documented as having a stronger effect on thyroid signalling than estrogen (Kitamura *et al.* 2005). BPF has also recently been demonstrated to disrupt thyroid hormone concentration in fish (Huang *et al.* 2016), which may indicate a possible reason for some of the effects we observed in Chapter 2.

Possible health effects of long-term exposure

Most of the experimental work in this thesis was carried out on larval zebrafish <120 hpf, with functional effects measured up to 14 dpf only. Though these studies have merit as this is an important stage for organ development and any effects may have lifelong health consequences. This may also be seen as a limitation, as levels of estrogens differ during different life stages and there may be life stage specific effects for bisphenol exposures. Most epidemiological

studies that link BPA exposure in human populations to cardiovascular risk factors are likely to have resulted from a lifelong exposure of BPA. The small numbers of mammalian *in vivo* studies that have investigated the effects of BPA on the heart have also investigated chronic exposure (Patel *et al.* 2013, Chapalamadugu *et al.* 2014, Kim *et al.* 2014). In the future it would be beneficial to determine the effects of life long BPA exposure on these fish to better link the health effects we observed in this thesis to those seen in humans. Further work should seek to investigate the effect of lifelong exposure on valve formation/function, but also general cardiovascular health. It would be interesting to compare this to early life exposure with a subsequent depuration until maturity to assess if being exposed at this early stage, when the heart is first developing, can have effects in maturity.

Metabolic activation

Metabolism of BPA and its alternative replacements could be a key area for future research to focus on. As we have demonstrated a metabolite of BPA – MPB - is much more potent than BPA as an estrogen *in vivo*. However, there is very little known about the comparative metabolism of the alternative bisphenols discussed in this thesis. The group that originally identified MBP as an active metabolite of BPA in mammalian S9 liver fractions also identified a similarly structured and potent metabolite of BPB, a bisphenol not tested in this thesis (Yoshihara *et al.* 2004). However, although metabolite structures were not identified, incubation of BPF with S9 liver fraction did not increase estrogenicity as observed with BPA and BPB (Okuda *et al.* 2011). Preliminary investigations carried out on uptake in Chapter 2 also appeared to identify that BPS metabolism in our fish differ distinctly from that seen for BPA. I would suggest that there should be a focus in future research on mapping the metabolic pathways of these chemicals to determine if metabolic activation is a likely mechanism of action. As with BPA it is then necessary to determine if any potentially potent metabolites are produced *in vivo* at concentrations likely to induce health effects. A further focus on metabolic activation could investigate the ability of different life stages to metabolise BPA, as it has been suggested foetal and adult livers may possess distinct mechanisms (Takahashi and Oishi 2000).

5.4 Concluding remarks

The research conducted in this thesis has provided knowledge on BPA and its most commonly used alternatives, BPF, BPS and BPAF in terms of their biological effects on zebrafish during early life stages. It provides the first evidence that differences in estrogenic activity likely relate not only to how these chemicals interact with ERs but also to the uptake and bioavailability of the relevant compound. This thesis established the target tissues for the various bisphenols as well as determining pathways by which these effects may be mediated. Metabolic activation was suggested as a way that BPA may exert some of its effects *in vivo*. This may hopefully initiate the further exploration of the different metabolic pathways of BPA alternatives, to further understand the differences observed in toxicity. As these chemicals may possess hormonal activities similar to or even beyond that of BPA, this demonstrates the need for rigorous safety evaluation. It may even be prudent to suggest that safety evaluations of compounds for consumer use should consider entire classes instead of individual compounds.

Through establishing the heart as a key tissue target of BPA, further investigations found health effects associated with contractility and genes related to ion transport. This data is of concern to aquatic species as BPA concentrations have been measured consistently in environmental water samples and also to humans where BPA exposure is known to occur consistently.

Plastics and resins are extremely useful materials, with a wide range of applications, it may even be suggested they are essential for modern living. However, as demonstrated throughout this thesis and related research there does not appear to be any suitable bisphenolic chemicals to use as monomers in production of these materials, due to reported health effects. Future research would therefore be best focused on the design of chemical substitutes that do not possess biological or hormonal activity similar to those of BPA. This would require an integrative multi-disciplinary approach in collaboration with industrial partners, where efficient screening methods are employed prior to mass manufacture.

CHAPTER 6

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