THE EFFECTS OF ADVANCED TREATMENT ON THE BIOLOGICAL ACTIVITY OF RECYCLED WATER

A thesis submitted for the degree of Doctor of Philosophy

by

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DECLARATION

The work submitted in this thesis was conducted between 2011 and 2016 at Brunel University London. This work was carried out independently, except where otherwise specified, and has not been submitted for any other degree.

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ABSTRACT

The world's growing population is causing an ever increasing demand for clean safe drinking water. In some countries suitable sources of drinking water are becoming scarce and will not be able to satisfy future demand. Consequently, there is a need to find alternative sources of water that can be used for potable supply or to augment current sources. Advanced water treatment methods are now being examined to investigate whether treated domestic sewage effluent can be treated to drinking water standards and discharged upstream of a drinking water abstraction point; a process known as Indirect Potable Reuse (IPR). The aim of this project was to investigate biological activity associated with developmental exposure to IPR water at the various stages of treatment using zebrafish embryos. Embryos reared in water at different stages of the treatment process were observed for developmental abnormalities, and differences in gene expression (compared to an aquarium water control) were used to establish both the nature and persistence of these effects along the treatment process. In addition to the embryo assays, passive sampling devices, Pharmaceutical Polar Organic Integrative Sampler (Pharm-POCIS) were deployed over eight, four week periods to collect composite concentrated samples of some of the contaminants present in the effluent. These concentrated extracts were then used in an *in vitro* assay; an Enzyme Immunoassay (EIA) to measure the inhibition of prostaglandins (an indirect measure of inhibitors of cyclooxygenase activity). We compared our results of the bioassays with the large body of chemical analysis data recorded over a number of years from each of the treatments. The developmental exposures highlighted a low frequency of consistent abnormalities to the heart and spine, and also a lack of pigmentation. Gene expression analysis demonstrates the developmental stage of the embryo to have the greatest influence on global gene expression as opposed to the treatment. Single genes of interest included the two cytochrome P450s (cyp1a and cyp1b1) and somatolactin beta. Some of the pathways disrupted included steroid synthesis, retinol metabolism, tryptophan metabolism and melanogenesis. The latter was consistent with observations of some embryos devoid of pigment. Along the treatment process reverse osmosis seemed to cause the largest change to the gene expression. The extracts from less treated effluent inhibited prostaglandin production, however following reverse osmosis prostaglandin inhibition was greatly reduced. The chemical contaminantion is rgreatly reduced as the effluent progresses along the IPR treatment process, this is evident from both the chemical data and the biological assays. Reverse osmosis seems to have the greatest influence on the gene expression. The results have highlighted the importance of an appropriate control, to remove background noise.

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List of abbreviations

2,3,7,8-TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

ADME: absorption, distribution, metabolism, and excretion

AOP: Advanced Oxidation Process

ATSDR: Agency for Toxic Substances and Disease Registry

AWTPs: Advanced Water Treatment Plants

BAA: benz[a]anthracene

BaP: benzo(a)pyrene

BBP: butylbenzyl phthalate

BCF: Bioconcentration Factor

BNF: British National Formulary

BOD: biological oxygen demand

CEFAS: Centre for Environment Fisheries and Aquaculture Science

CNS: central nervous system

COD: chemical oxygen demand

COX: cyclooxygenase

CTFA: Cosmetic Toiletry and Fragrance Association Inc.

CTP: cytidine triphosphate

DBPs: disinfection by-product

DEET: N,N-diethyl-m-toluamide

DEHP: di(2-ethylhexyl)phthalate

DES: diethylstilbestrol

DGT: Diffusive Gradients in Thin Films

DHI: 5,6-dihydroxyindole

DHICA: 5,6-dihydroxyindole-2-carboxylic acid

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

DOPA: 3,4 dihydroxyphenylalanine

DPR: Direct Potable Reuse

DTA: direct toxicity assessment

DWI: Drinking Water Inspectorate

E1: estrone

E2: 17β-estradiol

EC: European Commission

EDA: effects-directed analysis

EDCs: Endocrine disrupting chemicals

EE2: 17 alpha-ethinylestradiol

EFSA: European Food Safety Authority

EHC: Environmental Health Criteria

EIA: Enzyme Immunoassy

EMA: European Medicines Agency

EPO: erythropoietin

EQSs: Environmental Quality Standards

EU: European Union

FDA: Food and Drug Administration

FDRs: False discovery rates

FET: Fish Embryo Toxicity

FSA: Food Standards Agency

GAC: granular activated carbon

GC: gas chromatography

GDP: global gross domestic product

HAAs: haloacetic acids

HBCDD: Hexabromocyclodidecane

HBSS: Hank's Balance Salt Solution

HERA: Human and Environmental Risk Assessment on ingredients of household cleaning products

HPA: Health Protection Agency

HSDB: Hazardous Substances Data Bank

HSE: Health and Safety Executive

IARC: International Agency for Research on Cancer

IPCS: International Programmes on Chemical Safety

IPR: indirect potable reuse

IRIS: Integrated Risk Information Systems

ITER: Internal Toxicity Estimated for Risk Assessment

IUCLID: International Uniform Chemical Information Database

JECFA: Joint Expert Committee on Food Additives

LC: liquid chromatography

LDH: lactate dehydrogenase

LLE: liquid-liquid extraction

LOD: limit of detection

LRAT: lecithin:retinol acyltransferase

MBBR: moving bed biofilm reactor

MBT: 2-mercaptobenxothiazole

MF: microfiltration

MOA Mode of Action

MOX: Methoxime

MS: mass spectrometry

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MX: 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone

NDMA: N-nitrosodimethylamine

NF: Nanofiltration

NLS: National Laboratory Services

NOMs: naturally occurring organic matters

NORMAN: Network of reference laboratories for monitoring of emerging environmental pollutants

NOEC: No Observable Effect Concentration

NOEL: No Observable Effect Level

NOAEL: No Observable Adverse Effect Level

NTP: National Toxicology Program

OECD: Organisation for Economic Co-operation and Development

OPP: o-phenylphenol

PAC: powdered activated carbon

PAN: Pesticide Action Network

PBT: persistency, bioaccumulation and toxicity

PCBs: polychlorinated byphenyls

PCDDs: polychlorinated dibenzon-p-dioxins

PCDFs: polychlorinated dibenzofurans

PCR: Polymerase Chain Reaction

PES: polyethersulfone

PFOS: Perfluorooctane sulfonic acid

PNEC: predicted no effect concentration

PPCP: Pharmaceuticals and Personal Care Products

POCIS: Polar Organic Integrative Sampler

POMC: proopiomelanocortin

POPs: Persistent Organic Pollutants

PTU: 1 phenyl-2-thiourea

PUBCRIS: Public Chemical Registration Systems

PVC: polyvinyl chloride

QSARs: quantitative structure-activity relationships

REH: retinyl ester hydrolase

RO: reverse osmosis

RUP: Restricted Use Pesticides

SAM: Significance Analysis of Microarrays

SAT: soil-aquifer treatment

SBSE: sorptive extraction

SCCS: Scientific Committees on Consumer Safety

SIDS: Screening Information Data Set

SMPs: soluble microbial products

SPE: solid-phase extraction

SPMD: Semi-Permeable Membrane Device

SPME: solid-phase microextraction

TBT: tributyltin

TCDD: 8 tetrachlorodibenzo-p-dioxin

TGA: Therapeutic Goods Administration

THMs: Trihalomethanes

TIE: toxicity identification evaluation

TOC: total organic carbon

TRP-1: tyrosinase-related protein 1

TRP-2: tyrosinase related protein 2

TSCA: Toxic Substances Control Act

TSS: total suspended solids

UF: ultrafiltration

UNEP: United Nations Environment Programme

UPLC MS/MS: ultra-performance liquid chromatography tandem mass spectrometry

US EPA: United States Environmental Protection Agency

UV: Ultraviolet

VEGF: vascular endothelial growth factor

VTG: vitellogenin

WEA: whole effluent assessment

WET: whole effluent testing

WFD: Water Framework Directive

WHO: World Health Organization

YES: Yeast Estrogen Screen

ZET: zebrafish embryotoxicity test

CHAPTER 1: Background

1.2 Research question

1.2.1 Main aim

The main aim of this project is to assess whether there is a reduction of biological activity in the product water from a recycled water plant, specifically from the indirect potable reuse (IPR) pilot plant that was being trialled at Deephams waste water treatment works, London UK

1.3 Importance and relevance

In 2010, the United Nations Resolution 64/292 recognised access to safe drinking water and sanitation as being essential human rights (United Nations, 2014). However, the world's population is growing and increased incidences of extreme climate related events for example drought, flood, fire and storms, changes in nutrient loading and decreased water quality (Brookes et al., 2014) are placing vast amounts of pressure on the available potable water sources. Water scarcity is an increasing problem, including in the UK. Consequently, there is a need to find alternative sources of water that can be used for potable supply or to augment current sources.

The water industries have for a long time relied upon surface and ground water sources, however increasing demand could cause these to be less sufficient for the growing needs of the population (Brookes et al., 2014).

1.4 Environmental contaminants

There are two main groups of environmental contaminants. Firstly, there are the ones known as legacy pollutants which include polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and furans and chlorinated pesticides, for example DDT. These pollutants have been present in the environment for many years, their risks are understood, and legislation is in place to prevent and stop their release. However, due to the persistent nature of these compounds they are still widely detected. The other group of pollutants are known as emerging chemicals of concern, these include compounds such as endocrine disrupting chemicals, pharmaceuticals, personal care products, antibiotics and flame retardant (Burgess et al., 2013). New sources of water present new risks related to water quality and recycled water is reported to increase the risk of disinfection by-products, pathogens, antibiotic resistant bacteria, potentially endocrine disrupting compounds and cyanobacterial toxins (Brookes et al., 2014).

There has been increased focus on the effect of chemicals present as a mixtures. For years the majority of research in toxicology and ecotoxicology has been investigating the potential

effects of single chemicals, and from these studies safe levels have been suggested and used by regulators to protect both human and environmental health. Evidence has arisen that effects can be observed from a mixture of chemicals whereby they are individually present at concentrations below that has been deemed safe, known as mixture effects (Kortenkamp, 2014). Consequently, the potential risks of mixture effects should be taken into account when considering media such as waste water effluents, especially if the end use of the effluent is for human consumption.

CHAPTER 2: General Introduction

2.1 Water scarcity

It is reported that, of the total volume of water on the planet, 97.5% of it is saline; 99.99% of which makes up the oceans, and the remaining volume is found in salt water lakes (Gray, 2010). This leaves only 2.5% of the world's water as non-saline, of which 75% forms the ice caps and glaciers, 24% resides as groundwater, with the remaining 1% being present in lakes, rivers and soil (Gray, 2010). Consequently, a relatively small amount of freshwater is available to supply all human needs and demands.

With the world's increasing population and the growing concentration of humans in urban areas, there is pressure on the availability of suitable potable water. The world's population is increasing by approximately 80 million people per year, and by 2050, the world's population is predicted to be 9.1 billion (United Nations, 2015). Of this global population, 50% of people live in cities, with this proportion expected to increase over the next 30 years (United Nations, 2015). Moreover, between 1960 and 2012, global gross domestic product (GDP – a measure of economic growth) rose by an average of 3.5% per year, putting increasing burdens on social and environmental health. With this increasing population and rising consumption, demand for fresh water will inevitably increase to meet society's needs for drinking water, adequate sanitation, industry, agriculture, and power generation. It is estimated that global water demand will increase by 55% by 2050 (United Nations, 2015) and that by 2050, agriculture will need to increase production by 60% and by 100% in developing countries. Additionally, manufacturing industry is estimated to have increased production by 400% (United Nations, 2015). However, water scarcity is not a future threat; there are many countries already facing water scarcity in 2013, as shown in Figure 2.1 below.

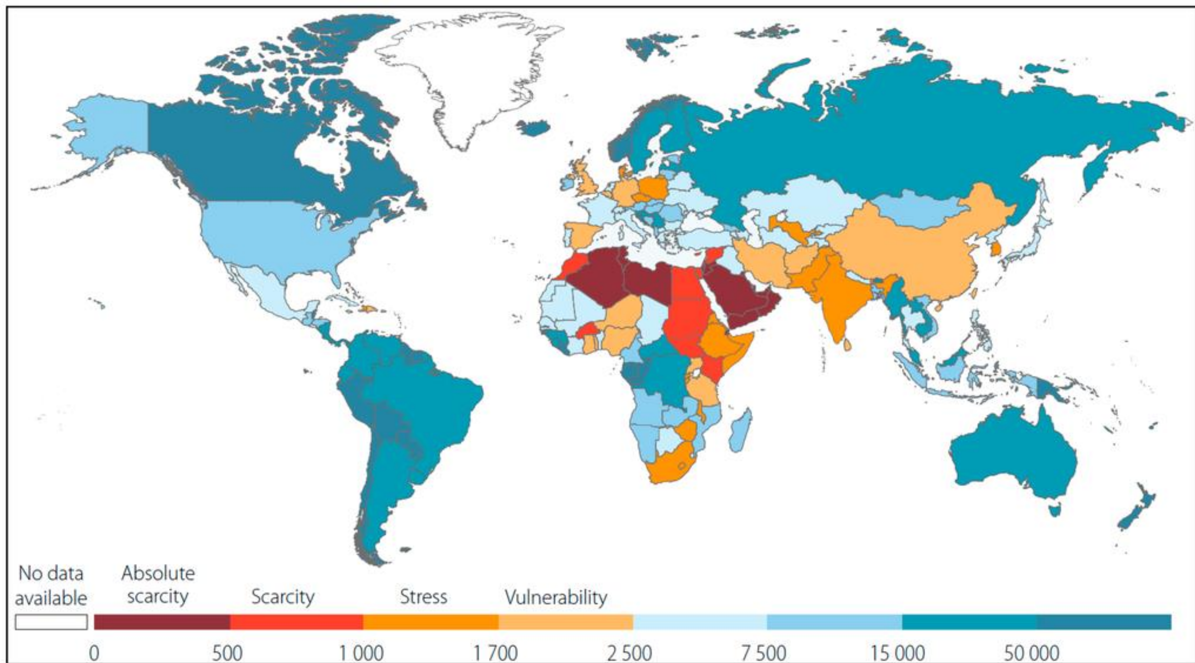

Figure 2.1. Total renewable water resource per capita in m^3 in 2013 (United Nations, 2015).

With global water demand predicted to increase, it is estimated that by 2030, worldwide, there will be a 40% water deficit if no action is taken (United Nations, 2015).

In addition to growing population, climate change is predicted to exacerbate the situation in certain regions and seasons, due to more frequent extreme weather events (e.g. drought) that will impact the availability of the water supply whilst also driving up water demand. Changes in rainfall patterns will alter local water cycles, meaning surface and ground water sources, usually relied upon as the main source of potable water, may not be recharged sufficiently. Increased temperatures will greatly affect already stressed ecological systems, and there would be increased need for the use of pesticides. Nutrient loading will also be altered, causing eutrophication and an increased burden on the water companies to combat these issues before treated waste water is released back into the environment (Brookes et al., 2014; United Nations, 2015).

The UK is certainly no exception; according to a report published by the English and Welsh Environment Agency in 2007, the southeast of England was classified as experiencing 'serious' levels of water stress (Environment Agency, 2007). This report was updated in 2013, and examined the current status of water stress in water company areas in England and Wales at present, and under four future scenarios. The report used three classifications of water stress; low, moderate and serious stress. The classification was based on abstraction, discharge and management and storage of water, and is a measure of stress on the water environment. It was highlighted that, even if an area is classified as experiencing

‘moderate’ or ‘low’ water stress now, future changes to population and/or climate could change this stress level to ‘serious, and therefore, the water users and water companies should not become complacent (Environment Agency and Natural Resources Wales, 2013). Out of the twenty four water company areas in England and Wales, nine of them (three areas of Affinity Water (formerly Veolia Water Central), East and South East, Anglian Water, Essex & Suffolk Water; South East Water; Southern Water; Sutton & East Surrey Water; and Thames Water) were classified by the report as currently experiencing “serious” water stress. Also, following the four future scenarios, all of these areas remain classified as experiencing “serious” water stress (Environment Agency and Natural Resources Wales, 2013). One other area (Portsmouth Water) changed classification from “moderate” water stress to “serious” water stress in two out of the four scenarios. Fourteen areas are classified by the report as currently experiencing “moderate” water stress (Bristol Water, Cambridge Water, Cholderton & District Water, Dee Valley Water, Dwr Cymru Welsh Water, Northumbrian Water, Portsmouth Water, Severn Trent Water, South Staffordshire Water, South West Water, United Utilities, Veolia Water Projects, Wessex Water and Yorkshire Water). Only one area out of the twenty four water company areas in England and Wales has been classified as currently experiencing “low” water stress (Sembcorp Bournemouth Water) (Environment Agency and Natural Resources Wales, 2013).

In a regional context, the UK is one of nine European countries that is considered to be suffering from water scarcity. The other countries include Cyprus, Bulgaria, Belgium, Spain, Malta, FYR Macedonia, Italy and Germany (European Environment Agency, 2008).

2.2 Chemical Contamination

There has been a global rise in chronic non-infectious diseases, including obesity, heightened blood pressure, diabetes, metabolic syndrome, and certain cancers (WHO/UNEP, 2013). Increases in human diseases have been coupled with observations made in wildlife that are associated with exposure to specific chemicals. For example, DNA methylation in the brain stem of polar bears in Greenland has been associated with exposure to mercury, and mercury exposure has been associated with observed neurological deficits in Inuit children (WHO/UNEP, 2013). Additionally, reproductive and developmental effects have been reported in male fish and amphibians in urban and agricultural areas, respectively. These diverse effects include genital deformities, decreases in semen quality and changes to sex hormones, and their occurrence is mirrored by similar increases in abnormalities in the human population (WHO/UNEP, 2013). Therefore, there has been increased attention focussed on the risk of chemical exposure and diseases in both the human and wildlife populations (WHO/UNEP, 2013).

Since 1970, the quality of Europe's surface waters has greatly improved as a result of a number of directives set by the European Union (Bueno et al., 2012). One of these was the Urban Waste Water Treatment Directive (91/271/EEC) which was adopted in May 1991. This directive had the objective of protecting the aquatic environment for biota, recreation, and its use for drinking water, sanitation, industry and commerce (DEFRA, 2012). Urban waste water, as referred to in this directive, is defined as the mixture of domestic waste water (household waste from kitchens, bathrooms and toilets), industrial waste water discharged to sewers, and rainwater run-off draining to sewers (DEFRA, 2012). In October 2000, another European directive was adopted, the Water Framework Directive (WFD) 2000/60/EC, and under this directive, chemical contamination is specifically dealt with under Decision 2455/2001/EC (Bueno et al., 2012; DEFRA, 2012). The WFD protects surface waters by considering all sources of pollution (point and diffuse sources) using a catchment based approach (DEFRA, 2012). In 2008, under Decision 2455/2001/EC, 33 substances were identified as priority substances, after which a further 8 other pollutants were added (Table 2.1 and Table 2.2, respectively). These all had Environmental Quality Standards (EQSs) applied to them to protect surfaces waters (rivers, lakes, transitional and coastal waters) (European Commission, n.d.-b). These 33 substances were decided upon based on their toxicity, persistence in the environment, bioaccumulation, and use, and consequently increased risk of them entering surface waters (Bueno et al., 2012). This list of priority substances is being continuously reviewed and amended, especially in light of new emerging chemicals of concern. In 2012, 15 additional priority substances were added, 6 of which were designated as priority hazardous substances, and previous EQSs were reviewed and revised. Additionally biota standards were set for several substances where it was deemed that due to a substance's bioaccumulation, a biota standard was more appropriate and protective than a standard for water, as the chemical would be present in the biota rather than the water column (European Commission, n.d.-b). The fifteen newly proposed substances were as follows; six plant protection substances - Aclonifen, Bifenox, Cypermethrin, Dicofol, Heptachlor and Quinoxyfen; three substances used as biocidal products - Cybutryne, Dichlorvos and Terbutryn; two industrial chemicals - Perfluorooctane sulfonic acid (PFOS) and Hexabromocyclodidecane (HBCDD); combustion by-products - dioxin and Dioxin-Like polychlorinated byphenyls (PCBs), and three pharmaceuticals - 17 alpha-ethinylestradiol (EE2), 17 beta-estradiol (E2) and Diclofenac (European Commission, 2015). The inclusion of the pharmaceuticals was not intended to cast doubt on their therapeutic value, but rather to acknowledge the potential adverse effects they have on the aquatic environment (European Commission, 2015).

Table 2.1 The 33 priority substances as identified in Annex II of Directive 2008/105/EC (European Commission, n.d.-a)

Number	Chemical name	CAS RN	EC number
1	Alachlor	15972-60-8	240-110-8
2	Anthracene[a]	120-12-7	204-371-1
3	Atrazine	1912-24-9	217-617-8
4	Benzene	71-43-2	200-753-7
5	Brominated diphenylether[a] Pentabromodiphenylether (congener numbers 28, 47, 99, 100, 153 and 154)	N/A 32534-81-9	N/A N/A
6	Cadmium and its compounds[a]	7440-43-9	231-152-8
7	Chloroalkanes, C10-13[a]	85535-84-8	287-476-5
8	Chlorfenvinphos	470-90-6	207-432-0
9	Chlorpyrifos (Chlorpyrifos-ethyl)	2921-88-2	220-864-4
10	1,2-Dichloroethane	107-06-2	203-458-1
11	Dichloromethane	75-09-2	200-838-9
12	Di(2-ethylhexyl)phthalate (DEHP)	117-81-7	204-211-0
13	Diuron	330-54-1	206-354-4
14	Endosulfan[a]	115-29-7	204-079-4
15	Fluoranthene	206-44-0	205-912-4
16	Hexachlorobenzene[a]	118-74-1	204-273-9
17	Hexachlorobutadiene[a]	87-68-3	201-765-5
18	Hexachlorocyclohexane[a]	608-73-1	210-158-9
19	Isoproturon	34123-59-6	251-835-4
20	Lead and its compounds	7439-92-1	231-100-4
21	Mercury and its compounds[a]	7439-97-6	231-106-7
22	Naphthalene	91-20-3	202-049-5
23	Nickel and its compounds	7440-02-0	231-111-4
24	Nonylphenols[a] (4-nonylphenol)[a]	25154-52-3 104-40-5	246-672-0 203-199-4
25	Octylphenols (4-(1,1',3,3'-tetramethylbutyl)-phenol)	1806-26-4 140-66-9	217-302-5 N/A
26	Pentachlorobenzene[a]	608-93-5	210-172-5
27	Pentachlorophenol	87-86-5	201-778-6
28	Polyaromatic hydrocarbons[a] (Benzo(a)pyrene)[a] (Benzo(b)fluoranthene)[a] (Benzo(g,h,i)perylene)[a] (Benzo(k)fluoranthene)[a] (Indeno(1,2,3-cd)pyrene)[a]	N/A 50-32-8 205-99-2 191-24-2 207-08-9 193-39-5	N/A 200-028-5 205-911-9 205-883-8 205-916-6 205-893-2
29	Simazine	122-34-9	204-535-2
30	Tributyltin compounds[a] (Tributyltin-cation)[a]	N/A 36643-28-4	N/A N/A
31	Trichlorobenzenes	12002-48-1	234-413-4
32	Trichloromethane (chloroform)	67-66-3	200-663-8
33	Trifluralin	1582-09-8	216-428-8

a: Identified as priority hazardous substance

Table 2.2 The list of 8 additional other pollutants amended by Directive 88/347/EEC and 90/415/EEC (European Commission, n.d.-a)

Number	Chemical name	CAS RN
6a	Carbon-tetrachloride[a]	56-23-5
9b	DDT total[a, b]	not applicable
	para-para-DDT[a]	50-29-3
9a	Cyclodiene pesticides	
	Aldrin[a]	309-00-2
	Dieldrin[a]	60-57-1
	Endrin[a]	72-20-8
	Isodrin[a]	465-73-6
29a	Tetrachloro-ethylene[a]	127-18-4
29b	Trichloro-ethylene[a]	79-01-6

a: This substance is not a priority substance, but one of the other pollutants for which the EQS are identical to those laid down in the legislation that applied prior to 13 January 2009

b: DDT total comprises the sum of the isomers listed in the reference European Commission, n.d.

In 2001, an international treaty known as the Stockholm Convention, calling for the elimination and restriction of Persistent Organic Pollutants (POPs), was signed and brought into force in 2004. Initially, there were 12 chemicals nominated (nicknamed the “dirty dozen”), but more recently this has risen to a total of 23 chemicals (Table 2.3) classified as priority POPs that are either intentionally or unintentionally produced (European Union, 2015).

The United Nations Environment Programme (UNEP) defined POPs as “chemical substances that remain in the environment, are transported over large distances, bioaccumulate through the food web, and pose a risk, causing effects to the environment and human health” (UNEP, n.d.).

Table 2.3 The 23 priority Persistent Organic Pollutants (POPs) as nominated under the Stockholm Convention which states their production and use must be restricted or eliminated (Stockholm Convention, n.d.)

Compound	Use	Annex
Aldrin	Pesticide	A
Chlordane	Pesticide	A
Chlordecone	Pesticide	A
Dichlorodiphenyltrichlorethane (DDT)	Pesticide	B
Dieldrin	Pesticide	A
Endrin	Pesticide	A
Heptachlor	Pesticide	A
Hexabromobiphenyl	Industrial chemical	A
Hexabromocyclododecane (HBCD)	Industrial chemical	A
Hexabromodiphenyl ether and Heptabromodiphenyl ether	Industrial chemicals	A
Hexachlorobenzene (HCB)	Pesticide, Industrial chemical and Unintentional production	C
Alpha-Hexachlorocyclohexane	Pesticide	A
Beta-Hexachlorocyclohexane	Pesticide	A
Lindane	Pesticide	A
Mirex	Pesticide	A
Pentachlorobenzene	Pesticide, Industrial chemical and Unintentional production	C
Perfluorooctane sulfonic acid and its salts and Perfluorooctane sulfonyl fluoride	Industrial chemicals	B
Polychlorinated dibenzo-p-dioxins (PCDD)	Unintentional production	C
Polychlorinated dibenzofurans (PCDF)	Unintentional production	C
Polychlorobiphenyls (PCBs)	Industrial chemical and Unintentional production	C
Technical endosulfan and its related isomers	Pesticide	A
Tetrabromodiphenyl ether and pentabromodiphenyl ether	Industrial chemical	A
Toxaphene	Pesticide	A

Annex A: Elimination of the chemical’s use and production

Annex B: Restriction of the production and use of the chemical

Annex C: Unintentional production, therefore parties need to reduce the release of the chemical and where feasible ultimately eliminate its release.

There are a vast number of chemicals in production and in use worldwide, and the exact figure is not known. However, in Europe there are estimated to be more than 140 000 chemicals on the market (UNEP, 2013). It is predicted that under European legislation REACH (REACH-Registration, Evaluation, Authorisation & restriction of CHemicals) the registered number of chemical substances produced in a volume greater than one tonne will exceed 30 000 substances before 2018 (UNEP, 2013). This number of chemicals is increasing every year. To further illustrate this point, the US Environmental Protection Agency (USEPA) adds, on average, 700 new chemicals every year to its Toxic Substances Control Act (TSCA) inventory (UNEP, 2013).

Despite the increasing demand for chemicals, there is a lack of toxicity and ecotoxicity data for approximately 95% of the chemical substances on the worldwide market (Lammer et al., 2009). Knowledge regarding the nature or extent of the effects of the majority of chemicals that are present in the environment is, therefore, limited or non-existent.

In recent years there has been increasing research on anthropogenic organic contaminants known as emerging contaminants, and there is growing fear that these substances could pose a threat to the environment, including the aquatic system, and consequently, human health. Emerging contaminants include the alkylphenols, flame retardants, hormones, pharmaceuticals and personal care products (PPCPs), steroids and pesticides. These contaminants can enter the environment via the domestic wastewater system via bathing, cleaning, laundry, use of toilets and improper disposal of unused pharmaceuticals (US Environmental Protection Agency, 2010).

2.2.1 Chemical contaminants in the environment

Trace amounts of various chemical contaminants and their biotransformation products remain in waste water effluent following treatment and are discharged into the aquatic environment. These can include inorganic compounds, metals, persistent organic pollutants (including chemicals that exhibit endocrine disrupting properties), pharmaceuticals and their metabolites, disinfection by-product (DBPs) and others (Fatta-Kassinos et al., 2011). Whether they pose a threat to aquatic systems will depend on many factors, including their concentration in water, their ability to bioaccumulate in aquatic organisms, their biological potency and their mode of action. The main classes of chemical contaminants present in the environment are described below.

Pharmaceuticals Personal Care Products (PPCPs) and their metabolites

PPCPs include prescription and over-the counter therapeutic drugs, veterinary drugs, illicit drugs, diagnostic agents, cosmetics, fragrances, sun-screen products, drug metabolites and transformation products (Fatta-Kassinos et al., 2011).

Due to incomplete removal during standard waste water treatment processes, PPCPs enter the environment via waste water treatment effluent. However, they can also get into the environment from the disposal of waste water treatment sludge to landfill, and direct application of veterinary medicines in aquaculture and agriculture, which would be added directly to the aquatic environment or enter via runoff (Brooks et al., 2010). Even if the input of PPCPs is relatively low, and even if they do not persist, the fact that they are being continuously used by the population (and therefore continuously discharged into the environment) makes them 'pseudo persistent' (Fatta-Kassinos et al., 2011). The pharmaceuticals which have been commonly detected in treated waste water effluents and also drinking water, include antibiotics, lipid regulators, anti-inflammatories, beta-blockers, cancer therapeutics and contraceptives (Fatta-Kassinos et al., 2011). Examples of some of these PPCPs are discussed below.

Antibiotics are widely used in both human and veterinary medicine. The main classes of antibiotics include: Aminoglycosides (e.g. Lincomycin); β-Lactams (e.g. Amoxicillin); 2,4-Diaminopyrimidines (e.g. Trimethoprim); Macrolides (e.g. Erythromycin); Pleuromutilins (e.g. Tiamulin); Quinolones (e.g. Ciprofloxacin); Sulfonamides (e.g. Sulfamethoxazole); and Tetracyclines (e.g. Oxytetracycline) (Brooks et al., 2010). Antimicrobials include chemicals such as triclosan and triclocarbon, that are used in personal care products such as antibacterial hand gels, soaps and toothpastes.

The fate and behaviour of antibiotics varies depending on their class. Quinolones are reported to be highly soluble in water and unlikely to adsorb to soil, sediment and suspended solids, whereas fluoroquinolones and macrolides are strongly adsorbed to soil, sediment and suspended sediment, and are therefore unlikely to be present in surface and groundwaters, unless directly discharged. Once bound to soil and sediment, it is reported that antibiotics persist. For example, in chicken manure and soil, chlortetracycline had a half-life of more than 30 days, and in marine sediment oxytetracyline's half-life was 150 days. Fluoroquinolones, sulfadiazine, sulfamethoxazole and sulfadimethoxine in sediment had half-lives greater than 30 days. Photodegradation is considered to be the main degradation pathway for several classes of antibiotics, with half-lives of only a few hours reported for fluoroquniolones and tetracyclines. However, once the compounds have bound to soil and

sediment it is likely that photodegradation will be less important as a means of removal, meaning in turbid waters there could be increased half-lives (Brooks et al., 2010).

One of the concerns surrounding antibiotic and antimicrobial substances entering the environment is that they target beneficial microorganisms. That is, upon entering the environment, they will adversely affect non-target microorganisms responsible for decomposition and nutrient cycling (Brooks et al., 2010) and may contribute to antibiotic resistance. The build-up of antibiotic resistant bacteria in the environment can occur due to direct release of antibiotics into the waste water system from patients prescribed antibiotics, and can also be gained from horizontal gene transfer, whereby resistant determinants are taken up through conjugation (whereby bacteria exchange DNA (plasmids) between individuals), transduction (virus introducing DNA) and transformation (genetic material is taken up from an external source for example from a lysed cell) (Fatta-Kassinos et al., 2011).

The presence of illicit drugs, or more notably, their metabolites, has been used to estimate a community's drug usage. An example of this was an investigation that analysed waste water from treatment plants in cities in Italy, Switzerland and United Kingdom (UK). Residues of cocaine, opiates, cannabis and amphetamines were measured, and the results enabled the researchers to gain a better understanding of the different communities' drug use. For example, the use of cocaine in Milan, Italy, was observed to markedly increase at weekends (Zuccato et al., 2008).

The metabolites and biotransformation products of pharmaceuticals are also found to be present in both the influent and effluent of waste water treatment processes, and some of these are still biologically active. Following administration of a drug product either orally, parenterally and/or topically, it undergoes absorption, distribution, metabolism, and excretion (ADME). During the metabolism stage, to allow excretion, the compound can be made reactive. The degree of metabolism is also very much dependent on the compound itself; for example the antiepileptic drug carbamazepine, and the anti-anxiety drug diazepam, both undergo complete metabolisation, whereas iopromide and diatrizoate both leave the body unchanged, having undergone no metabolisation (Fatta-Kassinos et al., 2011). Metabolism occurs via two stages, phase 1 and 2: phase 1 metabolism involves the addition of a functional group, causing it to be more reactive. Phase 1 reactions include oxidation, hydroxylation, epoxidation, reduction and hydrolysis. The products from the phase 1 reactions can then undergo metabolisation via phase 2 processes, including sulphation, glucuronidation, glutathione conjugation, acetylation and amino conjugation. Phase 2

processes produce highly polar conjugates that can be excreted in the urine or bile, and subsequently enter the waste water system (Fatta-Kassinos et al., 2011; Timbrell, 2001).

Endocrine Disrupting Chemicals (EDCs)

It is now known that trace organic chemical contaminants found in waste water effluent have endocrine disrupting properties. These include natural and synthetic steroidal hormones which have been reported to elicit effects on organisms at extremely low concentrations, including concentrations that have been detected in the environment. A number of these effects have been observed in wildlife. Endocrine disrupting chemicals (EDCs) are described as being exogenous chemicals, which have a secondary, unintentional effect whereby following absorption to an organism they either mimic or block hormones and disrupt the normal functioning of the organism (Diamanti-Kandarakis et al., 2009; Schug et al., 2011). The disruption to hormones is a consequence of the EDCs altering normal hormone levels, inhibiting or stimulating the production of hormones or changing the process by which the hormones are transported around the body (Schug et al., 2011). Initially it was understood that EDCs only acted upon nuclear hormone receptors which include estrogen receptors, androgen receptors, progesterone receptors, thyroid receptors, retinoid receptors and others (Diamanti-Kandarakis et al., 2009; Schug et al., 2011). More recently it has been discovered that the mode of action of EDCs is much wider. In addition to interfering with nuclear receptor signalling, EDCs can act via nonsteroidal hormone receptors, nonsteroidal receptors, orphan receptors, transcriptional coactivators, enzymatic pathways involved in steroid biosynthesis and/or metabolism, and many other mechanisms that converge upon endocrine and reproductive systems (Diamanti-Kandarakis et al., 2009; Schug et al., 2011). Other mechanisms that EDCs act upon include direct action on the genes and epigenetic effects (Schug et al., 2011). The group of molecules which have been identified as having endocrine disrupting properties are varied in nature. They include chemicals used as industrial solvent/lubricants and their by-products (polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins), plastics (BPA), plasticisers (phthalates), pesticides (methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)), fungicides (vinclozolin) and pharmaceuticals agents (diethylstilbestrol (DES)). There are also a number of natural chemicals found in both human and animal food, these include phytoestrogens, including genistein and coumestrol, that also act as endocrine disruptors (Diamanti-Kandarakis et al., 2009).

Oestrogenic substances are regularly detected in waste water effluents and the effects of these oestrogenic substances on non-target species, such as the wild fish population, are well documented. Effects include the induction of the egg yolk protein, vitellogenin (VTG) and intersex in fish (Jobling et al., 1998). VTG production in male fish is used as a biomarker

for oestrogenic effects of chemicals (Waller & Allen, 2010). It is reported that natural and synthetic oestrogens present in the waste water effluent are mainly responsible for the effluent's oestrogenicity (Servos et al., 2005). Natural oestrogens include 17β-estradiol (E2) and estrone (E1) and the synthetic oestrogen, 17α-ethynylestradiol (EE2). EE2 is used in birth control pills and other oestrogen therapies. This use as a pharmaceutical constitutes its main route into waste water (Waller & Allen, 2010). In a survey of 18 municipal waste water treatment effluents in Canada, E2 and E1 were reportedly detected at concentrations of 2.4 to 26.0 ng/l (mean 15.6 ng/l) and 19 to 96 ng/l (mean 49 ng/l), respectively, in the influent. In the effluent, in the same survey, the concentrations of E2 and E1 were reported to range from 0.2 to 14.7 ng/l (mean 1.8 ng/l) and 1 to 96 ng/l (mean 17 ng/l), respectively. This monitoring study was conducted at treatment sites with varying treatment regimes (Servos et al., 2005). Another survey taking effluent samples from a number of UK waste water treatment plants, again with a variety of levels of treatment, but mostly receiving domestic waste water, were found to have E1 and E2 concentrations ranging from 1 to 80 ng/l and 1 to 50 ng/l, respectively. In the effluent, EE2 was detected at between 0.2 and 7.0 ng/l (Desbrow et al., 1998). Concentrations of these compounds that induce VTG production in male fish were tested. In the tests, Rainbow trout (*Oncorhynchus mykiss*) were observed to have increased VTG levels at E2 threshold concentration of between 1 and 10 ng/l and for E1 the threshold concentration was between 25 and 50 ng/l (Routledge et al., 1998). In other studies reported by Routledge et al., (1998), EE2 at 10 ng/l was reported to produce effects in trout and concentrations as low as 0.1 ng EE2/l were observed to increase VTG production in male trout. Thus, the environmentally relevant concentrations are within the range that have been found to induce VTG production in male fish.

There are other chemicals that exhibit similar effects on the endocrine system, but often at higher concentrations, including plasticisers, pesticides and detergent degradation products (Fatta-Kassinos et al., 2011). Alkylphenols, used as precursor for detergents and as an additive for a number of compounds, are reported to be oestrogenic (Routledge & Sumpter, 1997). Male Rainbow trout (*O. mykiss*) were exposed to an alkylphenol, 4-tert-octylphenol, at concentrations of 10 and 100 µg/l, and was observed to significantly increase the production of VTG and the threshold concentration for this compound was reported to be between 1 and 10 µg/l in the trout and between 10 and 100 µg/l in roach (*Rutilus rutilus*) (Routledge et al., 1998). Bisphenol-A has been classified as a non-steroidal estrogenic compound. It is widely used particularly in the plastic industry for the production of polycarbonate, epoxy resins, unsaturated polyester-styrene resin and flame retardants (Al-Rifai et al., 2007; Fromme et al., 2002) and in consumer products such as coatings of cans, powder paints, additive in thermal paper, dental fillings and antioxidants in plastics (Fromme et al., 2002). It

is commonly found in waste water influent and effluent (Al-Rifai et al., 2007). A sampling survey conducted in 1997 in Germany, measured bisphenol-A and three phthalates in surface waters (lakes, rivers and channels), sediment (from the lakes, rivers and channels), sewage effluent and sewage sludge. Bisphenol-A was measured at concentrations ranging from 0.0005 to 0.41 µg/l, 0.018 to 0.702 µg/l, 0.01 to 0.19 mg/kg and 0.004 to 1.363 mg/kg dry weight (dw) in surface water, sewage effluents, sediments, and sewage sludge, respectively (Fromme et al., 2002). Phthalates are another group of compounds which have been found to exhibit endocrine disrupting properties, with estrogenic and/or anti-androgenic activity (Oehlmann et al., 2008). They are widely used in the manufacture of polyvinyl chloride (PVC) and other resins and plasticisers. Many of the phthalates, along with bisphenol A, are classed as high production plasticisers, due to the large quantities being produced and wide-scale use; their release into the environment is continuous and they are regularly detected in environmental sampling. Phthalates are listed by the EU as priority pollutants; they are not specifically persistent, but because of their continuous release, they are always present (Oehlmann et al., 2008). They have a higher affinity to suspended solids and sediment as a consequence of their low water solubility (Fromme et al., 2002). Three of these phthalates, namely di(2-ethylhexyl)phthalate (DEHP), dibutyl phthalate and butylbenzyl phthalate (BBP), were surveyed, along with bisphenol A, in the 1997 German survey discussed above. DEHP was the most abundant of the three phthalates, with concentrations ranging from 0.33 to 97.8 µg/l, 1.74 to 182 µg/l, 27.9 to 154 mg/kg dw and 0.21 to 8.44 mg/kg in surface water, sewage effluent, sewage sludge and sediment, respectively. dibutyl phthalate was detected at much lower concentrations compared to DEHP, and BBP was only detected in a few samples at very low concentrations (Fromme et al., 2002). Briefly, predicted no effect concentrations (PNECs) of 10 µg/l in water and 3.1 mg/kg dw sediment have been derived for dibutyl phthalate. It is reported that endocrine disruption has been reported in embryos of Japanese medaka (*Oryzias latipes*) at DEHP concentration as low as 0.01 µg/l (Chikae et al., 2004). These values are low and, therefore, effects could have been elicited at some of the sites from the German study.

Persistent Organic Pollutants (POPs)

Combustion by-products, PCBs, dioxin and dioxin-like compounds, are amongst the 15 newly proposed priority chemicals (European Commission, 2015) and are listed as priority POPs by the Stockholm Convention (European Union, 2015; Stockholm Convention, n.d.). Dioxins are made up of two groups; polychlorinated dibenzon-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). There are 75 structurally different chemicals in the PCDDs (dioxins) group, and 135 PCDFs (furans) (O'Neill, 1998; Wenning & Martello, 2010), and these are either impurities in the production of chlorinated organic compounds or they

are formed when chlorinated compounds are combusted at temperatures less than 1000°C (O'Neill, 1998). Together, the two different groups of dioxins are known as dioxin congeners. The number of chlorine atoms and the position of them on the phenyl rings determines the individual compound's behaviour in the environment and its toxicity (O'Neill, 1998; Wenning & Martello, 2010). The most toxic of the congeners is 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD). Coplanar PCBs share common structural similarities to PCDDs and PCDFs, have associated mechanisms of toxicities, therefore are sometimes called dioxin-like PCBs (Wenning & Martello, 2010).

PCDDs and PCDFs are detected widely in the environment; they are present both naturally and as a combustion by-product, and although they are not intentionally produced, have no known uses. PCBs are not natural, but PCDF is found as a large contaminate (WHO, 2010b). The manufacture of PCBs is banned worldwide, but their release to the environment is still occurring via the disposal of large scale electrical equipment and waste. Due to dioxins' persistence and bioaccumulation, the main human exposure route is via contaminated food (WHO, 2010b). Concentrations of dioxins in drinking and surface waters are generally reported to be low, as they are poorly water soluble, and therefore, are more likely to be found in soils and sediments, which can lead to contamination of food and biota, with a typical half-life in fatty tissues in humans of greater than 7 years (WHO, 2010b).

Dioxins and dioxin-like substances have been reported to be associated with a large range of endpoints, including immunotoxicity, developmental and neurodevelopmental effects, and alteration to the thyroid and steroid hormones and reproductive function. Consequently, developing infants and children are particularly at risk to the adverse effects of these compounds (WHO, 2010b). In mammalian studies they have also been found to be carcinogenic (O'Neill, 1998). Epidemiological studies have found evidence of carcinogenicity in humans, and the International Agency for Research on Cancer (IARC) has classified TCDD as within Group 1, meaning it is carcinogenic to humans. PCBs as a group have been classified by IARC as within Group 2A, meaning that they are probably carcinogenic to humans (WHO, 2010b).

Metals

Human health concerns associated with exposure to metals, including lead, cadmium, mercury and arsenic, are widely known (Järup, 2003). Lead, cadmium and mercury are all listed by the EU as priority pollutants (Table 2.1). Cadmium compounds are used in re-chargeable nickel-cadmium batteries and are often not properly recycled and go straight

in the household waste, leading to release from landfill leachates (Järup, 2003). It is also used as an anti-corrosive (electroplated onto steel), as pigments in plastics and electronic components and nuclear reactors (WHO, 2011a). In the environment it is more likely to be associated with suspended particles and sediment; in environmental water it is found at levels <1 µg/l. In humans, cadmium is particularly toxic to the kidneys and has been linked to osteoporosis (Järup, 2003; WHO, 2011a). In laboratory mammalian studies, oral exposure has caused fertility effects, testicular changes, tumours of the prostrate, testes and haematopoietic system (WHO, 2011a). Increased risk of cancer has also been observed from epidemiological studies (WHO, 2011a).

Lead is the most abundant heavy element on the planet. It is used for lead acid batteries, solder, alloys, cable sheathing, pigment, rust inhibitors, ammunition, glazes and plastic stabilisers. The use of lead compounds in petrol has been phased out in North America and Western Europe. Lead is also used in plumbing fittings and solder in water distribution systems. Lead pipes are still common in older properties in the UK. With the phase-out of leaded petrol, atmospheric levels have declined, and subsequently, so have inputs into surface waters. However, it is often detected in drinking water, due to its use in plumbing. Lead solder on drinking water pipes can lead to levels of between 210-390 µg/l, which is sufficient to cause intoxication in children (WHO, 2011b). Children are especially susceptible to lead exposure as they absorb up to 4-5 times as much as adults, who absorb 10% of what is present in food. Lead is also transferred to the foetus via the placenta in humans from 12 weeks of gestation. Lead in young primates resulted in significant behavioural and cognitive deficits, including impaired activity, attention, adaptability, learning ability and memory as well as increased distractibility. In humans, the threat posed by chronic lead exposure is highest in infants, children up to 6 years of age and the unborn child. Epidemiological studies have found that lead exposure is associated with kidney disease, gonadal dysfunction (decreased sperm counts and effects in females), increased risk of preterm delivery and minor developmental malformations. Other endpoints associated with lead exposure include evidence to indicate that it is mutagenic, with proven neurological effects on infants and children (WHO, 2011b).

Mercury is used as a cathode in the electrolytic production of chlorine and caustic soda, in electrical appliances, in industrial and control instruments, laboratory apparatus and as a raw material in fungicides, antiseptics, preservatives, pharmaceuticals, electrodes and reagents. However, over recent years, its industrial use has been reduced due to increasing environmental concerns. It is reported that in rainfall, levels of mercury range from 5 to 100 ng/l (mean 1 ng/l). Mercury is naturally present in groundwater and surface waters at concentrations <0.5 µg/l, but sometimes higher in groundwaters with naturally high mercury

deposits. In the US and Japan, levels in wells have been recorded as high as 2 µg/l and 5.5 µg/l, respectively. However, food is reported to be the main source of human exposure via fish and fish products. As with cadmium and lead, exposure to mercury can cause adverse effects to the kidney, but more importantly, it causes neurological disturbances (WHO, 2005).

Considering ecological concern, in the UK, copper has been ranked as being of greatest concern (Donnachie et al., 2014). Copper is reported to be detected in UK rivers at concentrations between 0.02 and 133 µg/l with the median of 4.7 µg/l, this is with lowest adverse effects in freshwater organisms being reported at 2.5 µg/l and 2.8 µg/l in periphyton (algae) and rainbow trout (*O. mykiss*), respectively (Donnachie et al., 2014).

Disinfection by-products

There are reported to be over 500 disinfection by-products (DBPs). These DBPs are formed following reaction between the disinfectants routinely used during waste water and drinking water treatment (i.e. chlorine, chloramine, ozone, ultra violet radiation and chlorine dioxide), and naturally occurring organic matters (NOMs), inorganic matter (bromide and iodide) and chemical contaminants (Richardson et al., 2007). Consequently, the higher the concentration of NOMs in the water prior to disinfection, the greater the level of DBPs formed. Therefore, with increasing pressure on the already stressed water sources, there will be an increased tendency to use water with higher natural levels of NOMs, bromide and/or iodide which, under less stressed circumstances, would not be used as a potable source. In the UK, the Drinking Water Inspectorate (DWI) state that water treatment companies must take measures to minimise the production of DBPs, by removing DBP pre-cursors and taking steps not to create conditions that are specifically favourable for their formation (DWI, 2012). However, in the UK there are only drinking water regulations for a handful of DBPs. Trihalomethanes (THMs) as a group have a guideline level of 100 µg/l (this includes chloroform, bromoform, dibromochloromethane and bromodichloromethane) and 10 µg/l for bromate (DWI, 2012). Other DBPs include the group known as haloacetic acids (HAAs), formaldehyde, acetaldehyde, 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone (MX) and N-nitrosodimethylamine (NDMA).

THMs in drinking water have been associated with increased occurrence of bladder cancer. Risk of cancer, mutagenicity and genotoxicity are the main focus surrounding the toxicological research in DBPs. There is evidence that emerging DBPs are more genotoxic than some of the regulated ones (Richardson et al., 2007). A review carried out in the US found that brominated DBPs were more genotoxic and carcinogenic than the chlorinated

DBPs and iodinated DBPs were found to have the greatest genotoxic potency (Richardson et al., 2007), however there were still many data gaps.

Pesticides

Pesticides are designed to kill targeted pests (weeds, insects, fungi etc.) and they are released into environment in large volumes. They are heavily regulated and their occurrence in the environment is usually carefully monitored. However, research on the environmental risks posed by chemicals including pesticides is on-going, and as has been discussed previously, bans and restrictions on use can be put in place if new information comes to light to indicate that they pose an undue risk to human health and/or the environment. One such pesticide is atrazine, which is widely used globally to control annual broadleaf and grassy weeds. Atrazine belongs to the class of herbicides known as chlorotriazines and is a selective systemic herbicide (WHO, 2010a). It is now listed as an EC priority substance (Table 2.1) and since 2004 has been banned in the UK and the rest of Europe. However, due to its unusually high persistence, it is still being detected in UK and European waters. It is reported to cause intersex in frog and immunosuppression in amphibians and fish (WHO/UNEP, 2013). Atrazine altered the hypothalamic control of pituitary-ovarian function, which included the decrease of levels of prolactin and luteinising hormone (Birnbaum & Fenton, 2003).

Another pesticide of interest is metaldehyde, which is the active ingredient in slug pellets. These are widely used both in agriculture and by amateur gardeners and are then washed into rivers and waste water treatment system. It has recently gained interest because it is often detected in UK drinking water at a concentration above the standard of 0.1 µg/l for pesticides and it is not removed by conventional treatment processes. The European Food Safety Authority (EFSA) reported that following repeat exposure in dogs, metaldehyde targeted the testes and prostate, and the liver in rats and mice. Some neurological effects were observed following acute exposure in rats, dogs and rabbits. However, it has not been found to be genotoxic or carcinogenic and mammalian tests have shown it to have no foetotoxicity or teratogenic effects (EFSA, 2010). Its high usage and poor removal from water treatment processes does raise its level of concern.

Glyphosate is a broad spectrum herbicide, and is reported to have the highest production volume out of all herbicides currently on the market. It is detected in the air during spraying, surface waters and in food (Guyton et al., 2015). Glyphosate is most commonly used as a formulation known as Roundup, which contains glyphosate in the form of isoproylamine salt, along with various adjuvants to enhance glyphosate's herbicidal properties, including polyethoxylated tallow amine (POEA) (Uren Webster et al., 2014). Glyphosate and Roundup

are both extensively used in agriculture and as domestic and urban-area weed killers (Uren Webster et al., 2014). The use of genetically modified crops which are engineered to be resistant to glyphosate has increased its global usage (Al-Rajab & Hakami, 2014). Glyphosate is highly water soluble (10500 mg/l) with a half-life of 6 to 10 weeks in water and 1 to 9 weeks in soil (Al-Rajab & Hakami, 2014). The International Agency for Research on Cancer (IARC) has classified glyphosate as a group 2A, meaning it is probably carcinogenic to humans. This classification is based on evidence in humans that it increased the risk of non-Hodgkin lymphomas, increases the incidence of renal tubule carcinoma in CD-1 male mice, increased incidence of haemangiosarcoma in male mice, increased incidence of pancreatic islet-cell adenoma in male rats and promoted skin tumours in an initiation-promotion study in mice (Guyton et al., 2015). Glyphosate has also been reported to induce DNA and chromosomal damage in mammals *in vivo* and in human and animal cell *in vitro* assays, and it causes oxidative stress in studies in rodents and *in vitro* studies (Guyton et al., 2015). In the environment, glyphosate is degraded to aminomethylphosphoric acid (AMPA), both of these are detected in blood and urine samples taken from agricultural workers, as well as in the wider environment (Guyton et al., 2015). Worldwide concentrations of glyphosate in rivers have been reported up to approximately 10-15 µg/l, with much higher concentrations being reported following direct application to the water body (Uren Webster et al., 2014). In laboratory studies, adverse effects in fish including DNA damage have been reported at environmentally relevant concentrations (Uren Webster et al., 2014).

Chlorpyrifos is another pesticide which is widely used worldwide and in the UK. It is a broad-spectrum chlorinated organophosphate insecticide, nematicide and acaricide used for the control of pests on numerous crops as well as lawns and ornamental plants (John & Shaike, 2015). It is reported to be toxic, but it is not persistent, which has allowed its continued use and registration. However, even though it is reported not to persist its persistence in soil varies from a few days to four years, this variability is dependent upon the climate and soil microorganisms (John & Shaike, 2015). Following application chlorpyrifos rapidly binds to soil and plants (Eaton et al., 2008). Due to this high affinity to soils, it has a low tendency to leach, but can reach surface waters when bound to suspended solids and particulates, and once in the water it will bind to sediments, with unbound residues undergoing volatilisation (Eaton et al., 2008; John & Shaike, 2015). Chlorpyrifos is reported to be still to be present in water following eight weeks and in sediments under anaerobic conditions the half-life is between 100 to 200 days (John & Shaike, 2015). The main target of chlorpyrifos is the central and peripheral nervous system, due to it being aacetylchlolinesterase (AChE) inhibitor (Eaton et al., 2008). It is reported that there is limited evidence to indicate that chlorpyrifos has toxicological effects in tissues other

than the nervous system (Eaton et al., 2008). However, there are mammalian studies that have indicated that chlorpyrifos caused adverse effects on brain development, is reproductive toxicant, feototoxic, developmental neurotoxin and endocrine disruptor (John & Shaike, 2015). Chlorpyrifos is toxic to fish, it is reported to cause developmental, behavioural, neurological, oxidative, histopathological and endocrine effects in aquatic life. The lowest LC50 is reported to be 1.8 µg/l in bluegill sunfish (*Lepomis macrochiru*) (John & Shaike, 2015).

2.3 Mixture/combination effects

The health risk of all the above chemicals, chemical groups and classifications cannot be fully determined on the basis of their concentrations in the environment alone. Data are now available reporting that mixtures of chemicals (each individually present at concentrations below their predicted no effect concentration (PNEC) and therefore deemed to be safe), can cause adverse effects when in combination. It is considered, and has been found, that chemicals together in a mixture can have synergistic or antagonist effects. There are a number of approaches to test and predict these effects. The approaches include; concentration addition, toxicity equivalency factor, independent action, and effect summation. The theory behind concentration addition assumes that the components of a mixture behave in a similar way, and that if one of the components is removed, it can be replaced with another at a concentration that has the equivalent effectiveness of the one removed, and the overall effect of the mixture will remain unchanged. This means that every component in the mixture contributes a proportion of the overall effect. Therefore, when dealing with a mixture of components of similar effects, the concentration addition approach can be applied (Silva et al., 2002). Independent action is stated to be more applicable to mixtures where the components have differing mode of actions. Effect summation assumes that the mixture effect is caused by the arithmetic sum of the effects of the individual components of the mixture (Silva et al., 2002). However, as reviewed by Silva et al. (2002), there are doubts as to the reliability of this method. Tests that combined a number of xenoestrogens (including hydroxylated PCBs, benzophenones, parabens, bisphenol A and genistein) at concentrations below their individual NOECs or EC10s were carried out using the Yeast Estrogen Screen (YES). It was reported that concentration addition and toxicity equivalency factor were the most relevant approaches to calculate the additive mixture effects of these chemicals. From this it was concluded that the estrogenic agents tested at the concentration below the derived NOECs or EC10s were capable of acting together to cause a significant biological effect (Silva et al., 2002).

In addition to mixtures of chemicals, in the environment, organisms can be exposed to chemicals as well as alterations conditions such as temperature, dissolved oxygen etc. This was investigated by Fitzgerald et al. (2016), whereby zebrafish embryos were exposed to low oxygen conditions and copper. It was observed that the during early development low oxygen levels reduced the effect of copper toxicity, however after hatching, at low oxygen concentrations, copper toxicity was increased due to increased up-take of the copper (Fitzgerald et al., 2016).

2.4 Technical Solutions/Water treatment

Waste water treatment plants are not specifically designed to remove the emerging contaminants discussed earlier, although many contaminants are removed or partly removed by waste water treatment systems, greatly reducing the environmental burden of chemical contaminants. Nevertheless, many chemical contaminants and chemicals of emerging concern are being increasingly detected in drinking water supplies. As with waste water treatment, drinking water treatment is not specifically designed to remove chemicals of emerging concern. Standard drinking water treatment systems typically consist of coagulation/flocculation and granular filtration which are designed to remove colloidal and suspended solids. Following this the water is then disinfected to inactivate and/or remove pathogens (US Environmental Protection Agency, 2010).

The discharge of untreated waste water into the aquatic environment can cause environmental harm and adverse effects on the human population using the waterways. These effects include oxygen depletion, eutrophication, water-borne pathogens, sewage litter and sewage solids (DEFRA, 2012). Therefore, effective treatment processes have to be utilised. In the UK the sewer network consists of over 624 200 km of pipeline, which manages over 11 billion litres of waste water from domestic and industrial sources each day (DEFRA, 2012). Waste water treatment consists of five stages; preliminary treatment, primary (sedimentation) treatment, secondary (biological) treatment, tertiary treatment, and sludge treatment (DEFRA, 2012; Gray, 2010). The level of treatment given is dependent on a number of factors, including the size of the community the treatment plant serves, or more accurately, the population equivalent. This factor is a measure of the oxygen demand of the organic load, therefore it is specific to loading, rather than just a measure of the population in the catchment area (DEFRA, 2012). The level of treatment is also dependent on the discharge point, whether that is inland, estuarine or coastal waters, and if the place of discharge has been classified as sensitive (eutrophic or at risk of becoming eutrophic or used as an abstraction source, and if there is further protection on the area, such as bathing waters, shellfish waters or freshwater fishing waters) (DEFRA, 2012).

2.4.1 Preliminary treatment

Preliminary treatment entails the removal of large solids and grit, separation of storm water and, when present in large amounts, the removal of oil and grease (Gray, 2010). Discharge of effluent into freshwater or estuarine systems with a population equivalent of <2000 (or coastal discharge when the population equivalent <10 000) is permitted under the Urban Waste Water Directive following only preliminary treatment (DEFRA, 2012). This level of treatment would remove very few, if any, chemical contaminants and pathogens.

2.4.2 Primary (sedimentation) treatment

The purpose of primary treatment is to remove settleable solids; the waste water enters the sedimentation tank at a speed that allows the fine solids to settle-out via gravity. This stage of the process produces a sludge (containing the settled solids) known as primary sludge (Gray, 2010). As well as gravity causing the suspended solids to settle out, floatation allows suspended solids which are less dense than water (e.g. oil and grease) to rise to the surface, where they can be skimmed off using blades (Gray, 2010). Primary treatment reportedly removes between 30 and 40% of the biological oxygen demand (BOD), 40 to 70% of suspended solids, and up to 50% of faecal coliforms (Gray, 2010). The removal of suspended solids removes some chemical contaminants, in particular the ones adsorbed to the surfaces of the particles. Under the Urban Waste Water Directive, waste water with a population equivalent of between 2000 and 1000 and >10 000 can be discharged to estuarine and coastal water, respectively, if the receiving water has been classified as a "less sensitive area". In the UK this was practiced up until the 1990's, when the impact of this practice was realised. Consequently, from the late 1990s and early 2000s these "less sensitive area" designations were phased out and withdrawn completely. This means that secondary treatment is now the minimum requirement for all these cases (DEFRA, 2012).

2.4.3 Secondary (biological) treatment

Following primary treatment, the settled wastewater undergoes secondary treatment. This is a biological treatment whereby dissolved and colloidal organic matter is oxidised by microorganisms (Gray, 2010). It acts by treating the BOD and total suspended solids (TSS) (US Environmental Protection Agency, 2010). The microorganisms act under both aerobic and anaerobic conditions, and following treatment the wastewater has to be separated from the microbial biomass. This is achieved by secondary sedimentation, and produces an effluent known as clarified effluent (Gray, 2010). There are a number of different biological treatment methods, but the main ones used in the UK are the activated sludge process and filter trickling (DEFRA, 2012). Activated sludge treatment is designed to remove organic material responsible for the BOD. It is a two stage process; the initial stage is carried out in

an aerated reactor, where a mixed microbial population utilises the biological load, removing the organic material, thus resulting in a reduced BOD load. The second stage consists of a settling tank, or clarifier, whereby the solids (activated sludge) are removed from the wastewater process. Some of this activated sludge is discarded, but a portion of it is recycled back into the first stage of the process (US Environmental Protection Agency, 2010). Alternatively, the waste water is trickled over filter beds consisting of media with a large surface area, for example gravel. The large surface area of the media and the feed of organic matter from the waste water together with the addition of aeration, provides ideal conditions for the growth of biofilms on the surface of the media. The chemical nutrients in the water are consequently broken down by the microbial community in the biofilm, resulting in microorganism growth. Following the treatment, as with the activated sludge process, there is the production of sludge waste. Contaminants can therefore be removed during this process via biodegradation and/or adsorption on to solids. However, adsorption of chemicals to the sludge can become a concern when disposing of the activated sludge. Trickling filter systems have been found to be less effective at removing natural oestrogens (E1 and E2) compared to the activated sludge processes. However, plants which utilised a tertiary treatment process had much improved removal rates compared to that of secondary treatment process alone (Servos et al., 2005).

A requirement of the Urban Waste Water Directive was that catchments with a population equivalent greater than 15000 had to be carrying out secondary treatment. In the UK, by the end of the year 2000 there was 90% compliance with this requirement. At the end of 2007, the UK was 99.9% compliant, there was just one catchment with a population equivalent of >15 000 without secondary treatment in Brighton and Hove (DEFRA, 2012).

2.4.4 Tertiary/advanced treatment

Due to the secondary treatment process only reducing the biological loading of waste water and reducing contaminants, in specific circumstances (such as when the discharge is into a sensitive area), a tertiary process can be required. There is an increasing demand for improved water quality, and this treatment step is designed to further treat the biologically treated effluent, to remove more of the BOD, suspended solids, bacteria, known chemical contaminants or nutrients (Gray, 2010). Tertiary treatments include prolonged settlement in lagoons, irrigation on to grassland or via percolation areas, straining through a fine mesh, sand or gravel filtration, membrane filtration, and disinfection using chlorination, ultraviolet and ozone treatments (Gray, 2010). In the UK, tertiary treatment is rarely used to treat waste water effluent, only in specific areas during summer months to protect bathing waters.

Activated Carbon

A tertiary treatment used for waste water treatment and drinking water treatment is Granular activated carbon (GAC). Contaminants in the water being treated are adsorbed on to the GAC, and held on to the surface by chemical and physical bonding (US Environmental Protection Agency, 2010). GAC used for waste water treatment is produced in granular or powdered forms. Granular form is used as a fixed-bed column and the water being treated is passed through the bed (US Environmental Protection Agency, 2010). GAC treatment is effectively a final 'polishing treatment' step which will remove organic chemicals present at low concentrations. GAC is used as an advanced treatment step in wastewater treatment and in drinking water treatment (US Environmental Protection Agency, 2010). Powdered activated carbon (PAC) can be added at various stages of the treatment process and intermittently, making it a flexible treatment method. PAC is added directly into the treated water as a slurry and is removed from the treated water in the sludge so cannot be reused like a GAC bed (Gray, 2010). The efficiency of GAC at removing a number of pharmaceuticals was tested in lab scale and pilot plant drinking water treatment processes, and was found to be highly effective at removing carbamazepine and diclofenac. GAC was used in this study as a final treatment step following ozonation, and it was found to remove all the pharmaceuticals tested (e.g. carbamazepine, diclofenac, bezafibrate and primidone), including clofibric acid, which had proved resistant to ozonation (Ternes et al., 2002).

Disinfection

Disinfection of treated water, whether treated effluent from a waste water treatment process, or abstracted water entering a drinking water treatment process, does not result in sterile water. However, it reduces the risk of infection by reducing the number of bacteria and viruses to an acceptable level (Gray, 2010).

Chlorination

In the US, disinfection using chlorination is used as both an advanced waste water treatment step and in drinking water treatment, in the UK chlorination is used only in drinking water treatment. Its purpose is the same in both waste water and drinking water treatments; to inactivate and/or remove pathogens from the water (US Environmental Protection Agency, 2010). Worldwide, chlorination is the most commonly used disinfection treatment for drinking water systems, but only widely used for effluent treatment in the USA; ultraviolet and ozone are more commonly used treatments as a tertiary process in Europe (Gray, 2010). Chlorine reacts with water to form hypochlorous acid and hydrochloric acid (Gray, 2010). The chlorine is used as either a gas or as concentrated hypochlorite liquid (US Environmental Protection Agency, 2010). However, when used as an advanced treatment step for waste water treatment, the water has to be dechlorinated before being discharged to the environment. Not only does the chlorine act on the pathogens, as a disinfectant, it can also act on

removing chemical contaminants via oxidation and chlorination. However, this reaction is known in certain instances to form potentially harmful disinfection by-products (DBPs), such as chloroform (US Environmental Protection Agency, 2010).

Ultraviolet radiation

Ultraviolet (UV) radiation can also be used as a disinfectant to inactivate pathogens in drinking water, and as an advanced treatment for waste water. UV light breaks the bonds in organic molecules (particularly benzene rings) and it reacts with water molecules to produce highly reactive hydroxyl radicals which then react with the organic molecules (US Environmental Protection Agency, 2010). These reactions may inactivate pathogens and transform contaminants. The reaction efficiency can be increased by adding hydrogen peroxide, thus increasing the concentration of hydroxyl radicals (US Environmental Protection Agency, 2010). Unlike chlorine, UV radiation has no residual action, therefore if it is being used to disinfect drinking water, it is usually only used in smaller treatment plants, where the consumers are in very close vicinity to the treatment process so as to avoid re-contamination of the water (Gray, 2010).

Ozone

Ozone is a strong oxidant and disinfectant, which inactivates pathogens and reacts with contaminants. Ozone is reported to directly oxidise contaminants and, like the UV treatment, reacts with water molecules to produce hydroxyl radicals that, in turn, react with the contaminants (US Environmental Protection Agency, 2010). Like UV treatment, ozonation can be improved by adding hydrogen peroxide, or coupling with UV radiation (US Environmental Protection Agency, 2010). Ozone is a strong oxidiser, but has no residual treatment to protect the water in the distribution network (Gray, 2010). The efficiency of ozone at removing a number of pharmaceuticals was tested in lab scale and pilot plant drinking water treatment processes. Ozone at 0.5 mg/l removed diclofenac and carbamazepine by >90%, ozone at 1.0 and 1.5 mg/l removed 50% of primidone and bezafibrate, respectively. At 3.0 mg ozone/l, 10% and 20% of primidone and bezafibrate, respectively, remained. Although, even at the highest concentration of ozone treatment, namely 3 mg ozone/l, clofibric acid was only removed by ≤40% (Ternes et al., 2002).

Membrane filtration

Membrane filtration is used in water treatment and other sectors that require separation; for example the food industry. It includes microfiltration, ultrafiltration, nanofiltration and reverse osmosis, Figure 2.2.

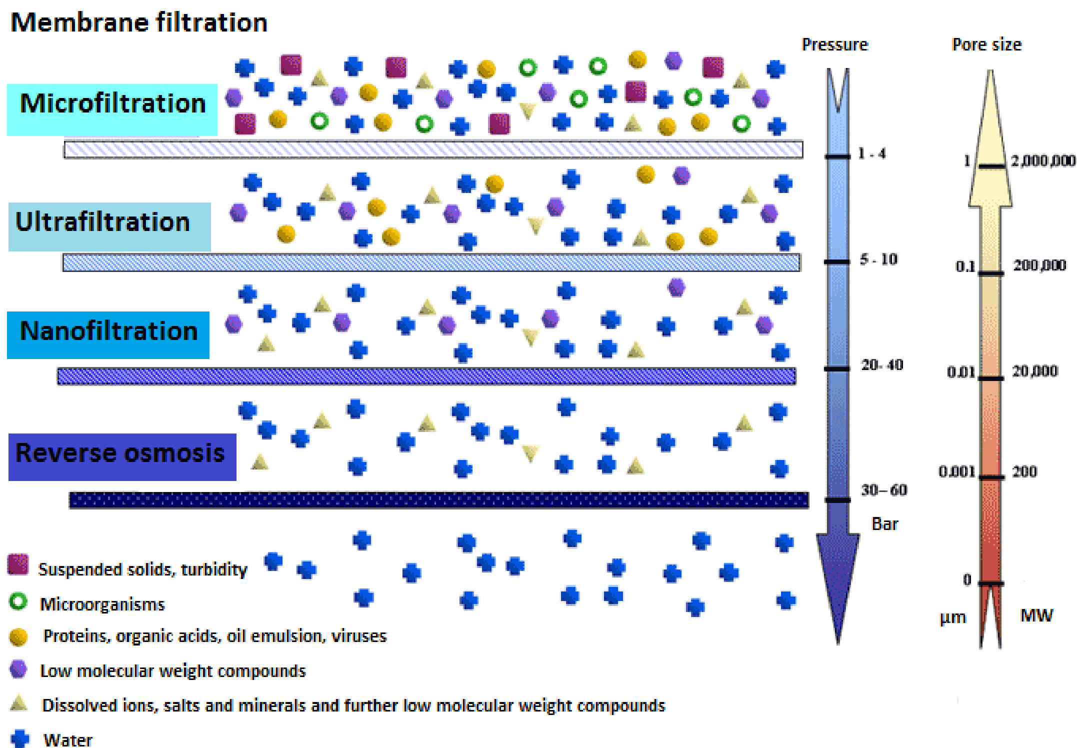

Figure 2.2 Illustration of the different membrane filtration processes, detailing the increasing pressure, decreasing pore size and increased removed efficiency of the different processes from microfiltration down to reverse osmosis (modified from Environmental Technology Centre, 2016).

Microfiltration

Microfiltration removes suspended or colloidal particles by a sieving technique, which is dependent on the size of the pores in membranes relative to that of the size of the particles in the particulate matter (US Environmental Protection Agency, 2010). Microfiltration removes particles between 0.1 and 5 µm in size (Figure 2.2). Membranes can be either tubular, capillary, hollow fibre, or spirally wound sheets.

Ultrafiltration

Similar to microfiltration, ultrafiltration (UF) removes suspended or colloidal particles using the same sieving technique explained above. However, the pores are much smaller, requiring the pressure to be much greater, up to 3000 kPa. The treated water is forced through the membrane and the concentrated particles are pumped to a waste tank. The size of the particles removed by UF, 0.1 to 0.01 µm (Figure 2.2), means that UF can remove bacteria, viruses and cellular fragments (pyrogens) (Gray, 2010).

Nanofiltration

Nanofiltration (NF) is driven by pressure and utilises membranes that both allow diffusion and filtration. NF can be operated at a lower pressure and has increased output of treated

water compared to that of reverse osmosis (RO, described below), but RO produces a higher quality of treatment. The particles size range that NF removes is between 0.01 and 0.001 µm (Figure 2.2), and is used to remove colour, total organic carbon (TOC), humic acid and organic molecules (Gray, 2010). A study investigating the removal of a number of pharmaceuticals from a groundwater source using both NF and RO processes found that the removal efficiencies of the two processes were similar, and highly effective at greatly reducing the concentrations of the majority of the pharmaceuticals tested. The pharmaceuticals hydrochlorothiazide, propyhenazone, carbamazepine and gilbendamide were removed by >90%; ketoprofen sulfamethoxazole and metoprolol were removed by >80%; acetaminophen was removed by >70%; gemfibrozil by >40% and mefenaminc acid by >30%. Dicolfenac and sotalol were not detectable following treatment with NF (Radjenović et al., 2008).

Reverse osmosis

Similar to the other membrane processes, reverse osmosis (RO) is operated under pressure and utilises a semi-permeable membrane (Gray, 2010; US Environmental Protection Agency, 2010). The movement of the water arises from different pressures (see Figure 2.3) and the movement will keep occurring until the pressure has equalised; this process is known as osmotic pressure (Gray, 2010). It has been designed to remove contaminants from water. The water is forced through the membrane, leaving the concentrate waste on the membrane, which then has to be treated and disposed of separately (US Environmental Protection Agency, 2010). Unlike MF and UF, the membranes on RO and nanofiltration remove contaminants that are dissolved (US Environmental Protection Agency, 2010). Reverse osmosis operates via diffusion-controlled transport, and allows water to pass through the membrane of an RO system, it leaves the concentrated salts on the untreated side of the membrane (Gray, 2010). The particles size range that RO removes is between 0.001 and 0.0001 µm (Figure 2.2), and removes both organic and inorganic compounds. It is reported to remove metals such as aluminium, copper, nickel, zinc and lead at an average removal rate of between 94% and 98% of the total dissolved solids. The organic compounds it has been reported to remove include trihalomethanes (THMs), polychlorinated biphenyl (PCBs), pesticides, benzene, as well as others, with removal rates of between 85 and 90% (Gray, 2010). In the same study described in the NF section, RO had similar removal efficiencies for the following pharmaceuticals hydrochlorothiazide, propyhenazone, carbamazepine and gilbendamide were removed by >90%; ketoprofen was removed by >80%; metoprolol and sotalol were removed by >70%; acetaminophen was removed by >50%; gemfibrozil by >40% and mefenaminc acid by >30%. Dicolfenac and sulfamethoxazole were not detectable following treatment with RO (Radjenović et al., 2008).

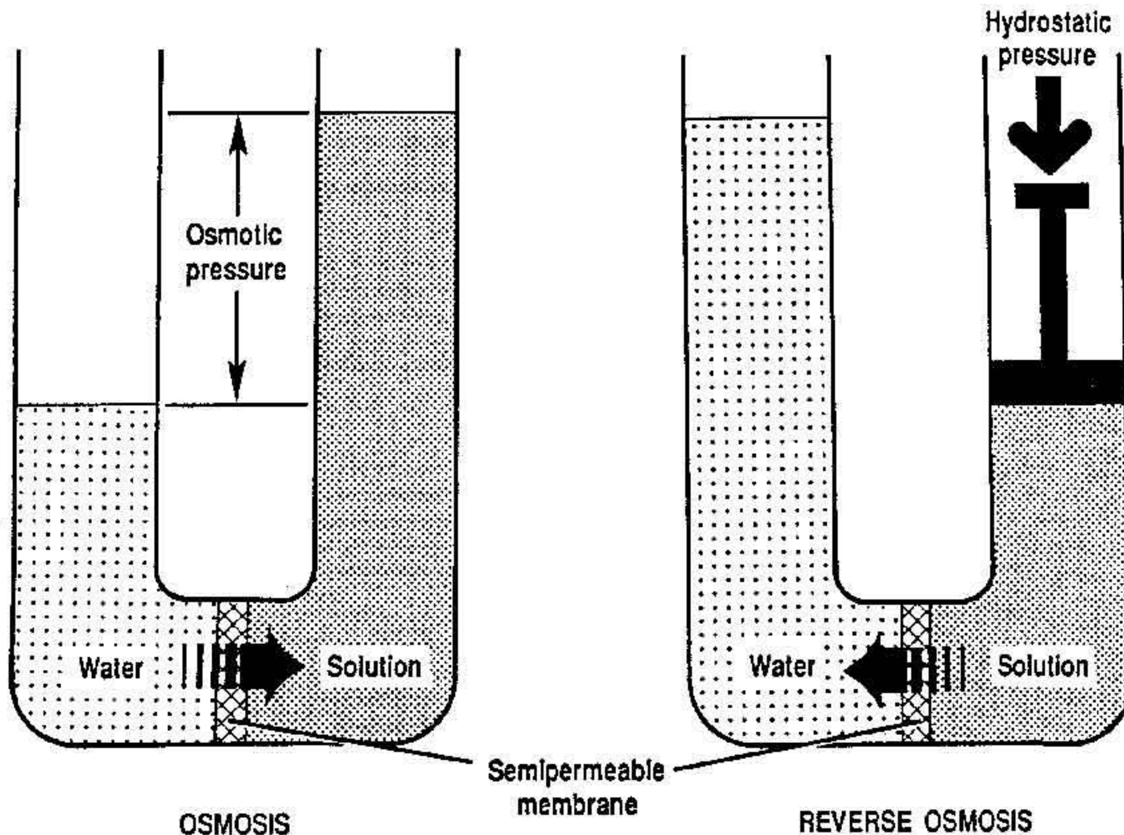

Figure 2.3 Schematic detailing the difference between osmosis and reverse osmosis (Aqua Technology Water Stores, 2015).

2.4.5 Sludge treatment

Sludge treatment simply consists of removing the water, then the stabilising and disposal of the sludge (Gray, 2010). Following primary treatment, the sludge consists of 95-96% water and, depending on the process, secondary sludge from an activate sludge process can consists of up to 98.5% water (Gray, 2010). Removal of water from the sludge can be achieved by addition of chemicals. The sludge needs to be stabilised to control the anaerobic degradation which takes place during storage. Stabilisation is reached via anaerobic digestion, aerobic digestion or lime addition (pH>11). Previously sludge was disposed of to surface waters or at sea, however at the end of 1998 this practice was ceased by the Urban Waste Water Directive (DEFRA, 2012). Now, the treated sludge is disposed of to land or incinerated (DEFRA, 2012; Gray, 2010). The sludge can also disposed of via anaerobic digestion; this process produces biogas which can be used as a renewable energy source (DEFRA, 2012).

2.5 Water reuse

Worldwide, the total volume of water remains unchanged, but fluctuations occur in terms of its quality, physical state and availability, due to the hydrological cycle and rate of use in society. Due to this continuous movement of water, potable water, having been used in domestic and industrial processes, is carried by the sewers to waste water treatment works for treatment, and then recycled back into the drinking water system.

It has been estimated that, globally, 20% of groundwater supplies are currently over-exploited (United Nations, 2015). Consequently, there is a need to find alternative sources of water that can be used for potable supply, or to augment current sources. Water recycling (or water reuse) is one of those alternatives. There are two main types of water potable reuse, known as direct and indirect reuse. These are detailed below and Figure 2.4 illustrates their general process.

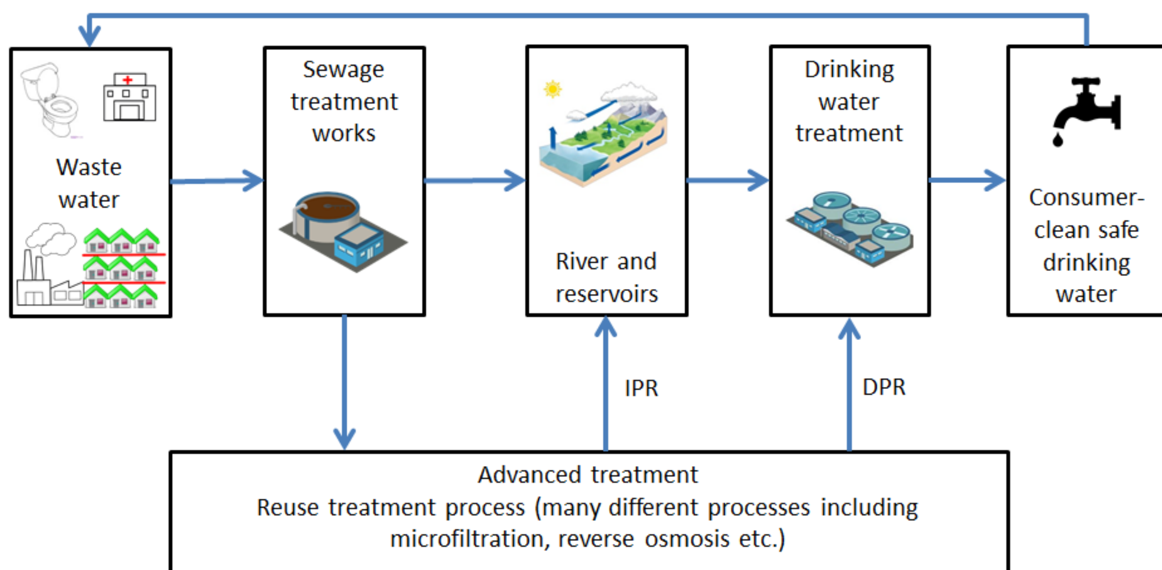

Figure 2.4 Schematic illustrating potable water reuse, including both direct (DPR) and indirect potable reuse (IPR) systems

2.5.1 Direct Potable Reuse (DPR)

Direct potable reuse describes the method that introduces waste water that has undergone waste water treatment, followed by a form of advanced treatment, directly into a drinking water distribution system. Direct reuse eliminates the intermediary ecological steps that would usually act upon the treated sewage or storm water in a standard treated waste water or indirect reuse scenario (NRC, 1998; NRMMC et al., 2008).

This method is only used in one place, Windhoek in Namibia (Dominguez-Chicas & Scrimshaw, 2010; Khan & Roser, 2007). The city of Windhoek relies on three reservoirs for 70% of its water supply (du Pisani, 2006). In 1969, the potable water treatment plant that supplied potable water to the city of Windhoek was converted to be able to treat water from the reservoirs and the final effluent from the city's wastewater treatment plant (du Pisani, 2006). The treatment plant has undergone many upgrades, but following independence in 1990, a new water recycling plant has been built, which now provides the city with 35% of its water requirements (du Pisani, 2006). The recycling project in Windhoek uses a multiple barrier system that includes: pre-ozonation, coagulation/flocculation, dissolved air flotation, dual media filtration, main ozonation, activated carbon filtration, ultrafiltration and finally chlorination (Lahnsteiner & Lempert, 2007, cited in Verstraete & Vlaeminck, 2011).

2.5.2 Indirect Potable Reuse (IPR)

There are two types of indirect potable reuse (IPR); unplanned (or incidental) and planned. This normal recycling process, known as unplanned or incidental IPR, occurs after the treated effluent is discharged upstream from a drinking water treatment abstraction point. This receiving water body acts as an ecological buffer over time (this can be weeks to years, but in UK rivers this would be only a few days), and works by natural degradation processes (biodegradation, photodegradation, adsorption etc.) and dilution (NRMMC et al., 2008). Planned IPR describes the reuse method where waste water that has undergone waste water treatment followed by advanced treatment is discharged to a water course. The treated water is then mixed with the receiving water and natural environmental processes occur, leading to further degradation of any contaminants and dilution. The mixture of recycled water and ambient water is then abstracted and added to the local drinking water treatment system (NRC, 1998).

Planned IPR differs from what is often the case in unplanned processes in that it is the deliberate augmentation of potable water supplies with highly treated recycled water from conventional sewage treated processes (Khan & Roser, 2007). The potable water sources that are augmented with recycled water can be both surface waters (e.g. rivers and reservoirs) as well as groundwater sources (Khan & Roser, 2007). When an aquifer is recharged using recycled water it can be indirect, by infiltration, or direct, via injection into the subsurface (Drewes et al., 2003). Since 1962, indirect potable reuse via ground water recharge has been used in Los Angeles, USA (NRC, 1998).

Singapore has been running a reuse scheme since 2003. Secondary treated effluent undergoes further treatment via micro- and ultra-filtration, reverse osmosis and ultraviolet disinfection. In Singapore there are reported to be five of these treatment facilities that

collectively produce 30% of the country's water requirements. However, the majority of this water is destined for non-potable uses (i.e. for industry), but 7.5% of this recycled water is now being used as planned indirect potable reuse through its addition to a reservoir and then going for further drinking water treatment, it now constitutes up to 2.5% of the city's potable water needs (Verstraete & Vlaeminck, 2011).

Australia boasts the largest water recycling facility in the Southern Hemisphere and the third largest in the world (Freeman et al., 2008; Lawrence et al., 2008; both cited by Hawker, Cumming, Neale, Bartkow, & Escher, 2011). This project uses treated wastewater from six existing wastewater treatment plants in the South East Queensland area (Hawker et al., 2011). The existing wastewater treatment plants that feed the water-recycling project in Queensland undertake biological nutrient removal, producing treated water of secondary standard (Hawker et al., 2011). This water then undergoes advanced water treatment, which includes a dual membrane approach, utilising microfiltration and reverse osmosis, followed by advance oxidation (hydrogen peroxide /Ultra Violet (UV)). The water is then stabilised and finally disinfected with chlorine (Hawker et al., 2011).

In this example from Queensland, it is reported that, at present, the recycled treated water from the Advanced Water Treatment Plants (AWTPs) is being used only by coal-fired power stations (Queensland Water Commission, 2008; cited in Hawker et al., 2011). The current government policy states that the recycled water can only be used to recharge the reservoir, Lake Wivenhoe, when the combined dam levels in South East Queensland fall below 40% (Rodriguez et al., 2009; cited in Hawker et al., 2011). Due to large amounts of rainfall since the recycling project has been in operation, this 40% threshold has not yet been reached.

Drewes et al. (2003) reported that in the USA indirect potable water reuse projects that have been established, or that have been proposed, used conventional secondary wastewater treatment, followed by tertiary filtration before soil-aquifer treatment (SAT). Before recycled water is injected into an aquifer or for surface water recycling projects in the USA, the water has to be treated using membrane technologies such as pre-treatment using microfiltration followed by nanofiltration or reverse osmosis (Drewes et al., 2003). In the project in California the recycling plant uses a treatment process that includes microfiltration, reverse osmosis, ultraviolet and hydrogen peroxide treatment. This highly treated water is used to recharge an aquifer, which also prevents saltwater intrusion (Verstraete & Vlaeminck, 2011).

2.5.3 Issues surrounding water reuse

Public perception

The Water Reuse Foundation conducted a survey in both the US and UK, and one of the criteria was public perception and public acceptance of water reuse. It was reported that almost all the respondents found the use of recycled water for non-potable applications such as irrigation and industrial use generally acceptable. However, when asked whether recycled should be used in indirect potable reuse situations the responses were negative in nature (Miller, 2006). It is considered that there are two beliefs held that prevent the full acceptance of water reuse, "naturalness" and "contagion". In the case of "naturalness", the general public consider natural to be best, and the belief held is that waste water is not natural, whereas river water can be considered to be natural, even if it contains waste water from an earlier discharge point (unplanned IPR). The theory of "contagion" is that once the water is contaminated then it will always be contaminated (Miller, 2006). However, unplanned reuse is a well operated system in many parts of the world, including the UK.

Lack of uniform regulations

Internationally, there are reported to be vast differences between the standards and guidelines set for recycled water in each country (Miller, 2006). There is also a lack of limits and standards for new and emerging contaminants (Fatta-Kassinos et al., 2011). The lack of consensus internationally is likely to hinder wider acceptance of what constitutes safe practice for recycled water within society.

Pricing of recycled water

Recycling water is energy intensive, and the costs of supply are generally higher. This will not be accepted publicly, thus it will be unprofitable for the water companies to invest in these technologies. Reuse systems are often more expensive than traditional potable systems, but water from reuse systems is often under-priced (subsidised) to encourage use and acceptance. However, this price discrepancy can be off-set later when the pressures on water sources increase. This in turn increases the value and positivity towards the reuse system (Miller, 2006).

Technologies

There are a large number of different technologies for water reuse available and being used and trialled around the world. However if these are technologies are not paired with the end-use of the water, or potential future use, then these schemes will be unsuccessful (Miller, 2006).

Economics

In the 1970s Perth, Australia, suffered with a reduction in rainfall of between 15 and 20%, which caused a 40% decrease in the levels of reservoirs, which was coupled with a rapid increase in the population. This resulted in Perth investing in two large and costly

desalination plants to meet the city's demands including the period of severe drought in 2010 (Brookes et al., 2014). The authorities in Sydney and Adelaide also invested in desalination plants following droughts. However, unlike the Perth example, once the plants were finished, rainfall levels increased, and additional water supply from the new desalination plants was not required. Another example in Australia saw the authorities in Brisbane investing in a water reuse system to implement an indirect reuse system, following long persistent droughts. Similarly to the Sydney and Adelaide examples, rainfall increased, and the decision has been made to reduce the reliance on water reuse (Brookes et al., 2014).

Environmental impact

The main purpose of water reuse is to provide an alternate water source or to augment existing sources. However, removing a treated waste water discharge from a river can have adverse environmental impact. It could cause low flows in rivers, especially during periods of reduced rainfall. This will impact the aquatic organisms and could, in turn, impact the recreational use of the water course, for example fishing.

Chemical and microbiological safety

The quality of recycled water is normally based on standard determinants such as BOD, chemical oxygen demand (COD), pH, total suspended solids, metals and microbiological load, which includes viruses, bacteria and protozoa (Fatta-Kassinos et al., 2011). However, as already discussed, standard wastewater treatment often leaves trace chemical contaminants and microorganisms still present in the effluent.

2.5.4 Trace chemical contaminants in recycled water

It is reported that the two main issues related to potable water reuse are pathogens and residual organic constituents that are not completely removed by the conventional and advanced wastewater treatment processes (Drewes et al., 2003). The residual organic constituents of recycled water arise from three main sources: anthropogenic organic compounds added by consumers, natural organic matter (NOM) already found in drinking water, and soluble microbial products (SMPs) produced during wastewater treatment from the decomposition of organic material (Drewes et al., 2003).

The number of potential contaminants in effluent is vast, and it is impractical, if not impossible, to screen for all known potential chemicals. Therefore, Total Organic Carbon (TOC) is often used as means of assessing the water quality. Drewes et al. (2003) reported that the State of California Department of Health Services proposed the use of TOC as relevant representative parameter for monitoring the quality of reclaimed water. The

Department of Health Services proposed a TOC concentration <0.5 mg/l for recycled water in groundwater abstracted for drinking water.

In the large water-recycling project in Queensland, Australia, the water entering and leaving the Advanced Water Treatment Plants (AWTPs) has undergone chemical analysis. Between May and November 2008 the recycled water from the AWPTs underwent analysis for a total of 113 organic chemicals; these included DBPs, hormones, PPCPs, pesticides and other organic micro-pollutants as well as inorganic microbial analytes that had been detected in source wastewaters (Queensland Water Commission 2009; cited in Hawker et al., 2011). Of the 113 chemicals analysed, 15 were detected and quantified at least once (described below in terms of the maximum concentration detected in treated recycled water). The DBPs detected and quantified included: bromodichloromethane (8 µg/l), dibromochloromethane (2 µg/l), chloroform (16 µg/l), dichloroacetic acid (0.9 µg/l) and N-nitrosodimethylamine (NDMA) (0.01 µg/l); the contaminants considered to be good indicators of industrial waste detected and quantified included: 4-t-octylphenol (0.04 µg/l), bisphenol A (0.022 µg/l) and 4-nonylphenol (0.069 µg/l for total nonylphenols); the PPCPs detected and quantified included: paracetamol (0.01 µg/l), salicylic acid (a breakdown product of aspirin) (0.01 µg/l) and N,N-diethyl-m-toluamide (DEET) (used in insect repellent) (0.01 µg/l); the pesticides detected and quantified included dalapon (0.02 µg/l) and triclopyr (0.03 µg/l); and the other contaminants detected and quantified included caffeine (consumer product) (0.03 µg/l) and cholesterol (natural product excreted by mammals including humans) (0.011 µg/l) (Hawker et al., 2011).

In this Queensland example, the planned destination for the recycled treated water was the reservoir, Lake Wivenhoe. Contaminants not removed by the advance treatment would undergo dilution upon entering the reservoir and also further attenuation will occur via volatilisation, sorption to suspended particles and sediment, hydrolysis, photolysis and biodegradation.

When used in ecotoxicity testing, effluents from some advanced treatments have been observed to still cause adverse effects, including advanced treatments such as ozonation. It is considered that is potentially because transformation products are formed during the treatment, which are more toxic than the parent compounds (Lundström et al., 2010). For example, a life cycle test using harpacticoid copepod, *Nitocra spinipes*, was conducted to investigate juvenile development and survival when the animals were exposed to differently treated effluents. Effluent having undergone conventional treatment was observed to cause adverse effects. No effects on early development were found, but there was increase in time taken to reach sexual maturity and decreased survival. Effluent that had undergone

conventional treatment with the addition of ozonation (the lowest dose) resulted in the least degree of negative effects overall, and decreased the amount of time it took the copepods to reach sexual maturity. The effluent that had undergone conventional treatment with the addition of a moving bed biofilm reactor treatment combined with ozone, did not improve the quality of the treatment compared with ozone alone. The effluent that had undergone conventional treatment with the addition of active carbon treatment was observed to have more negative effects than the effluent that had the addition of ozone treatment. The authors suggested that this was due to activated carbon removing essential metal ions from the water. The time taken to reach sexual maturity was increased, and survival was decreased. The effluent that had undergone further treatment with UV and hydrogen peroxide caused few developmental and survival effects on the copepods over time, but there was still a negative effect at the population level (Lundström, Björlenius, et al., 2010).

The effects of disinfection processes on the adverse effects effluents have on the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*) were studied. Fish were exposed for 28 days to the effluent that had undergone primary treatment and then before and after one of the following treatments, UV, ozonation and peracetic acid. Fish exposed to the effluent from the primary treatment and all three disinfectant processes were observed to have significantly decreased in their mass:length ratios. Exposure to primary treated effluent resulted in significant increase in macrophage-related phagocytosis, the disinfection step removed this effect. However, ozonation resulted in a decrease in unstimulated and nitrogen-stimulated T lymphocyte proliferation compared to fish exposed to primary-treated or to aquarium water. T lymphocytes proliferation were stimulated in the fish exposed to peracetic acid (Hébert et al., 2008).

Zebrafish embryos were exposed to a number of different effluents: embryos that were exposed to effluent that had undergone conventional treatment were observed to have significantly prolonged hatching times (in the 75% and 100% concentrations) compared to the control. In effluent that had undergone sand filtration, the hatching was prolonged in 12.5%, 75% and 100% effluent concentration groups. The advanced treatments removed this effect. Interestingly the embryos in the reference toxicant mixture group (50 µg/l musk ketone and 7.6 mg/l phenylthiourea in DMSO) were observed to have complete lack of pigment, which was considered to be due to the phenylthiourea. Heart rates of the fish embryo were decreased compared to the control in the conventional treated effluent. The sand filtration did not remove the concentration of pharmaceuticals or triclosan, although it did reduce the hormone levels (Lundström, Adolfsson-Erici, et al., 2010).

In another study, Rainbow trout were exposed to differently-treated effluents, and the metabolic profiles of the fish investigated. The metabolic profiles in the plasma from the fish exposed to the reference effluent were similar to those fish that had been exposed to tap water and the reference effluent that had been further treated with activated carbon. The authors suggested that this is an indication that the plasma profile of fish exposed to effluent treated in more advanced treatments such as in this case (the moving bed biofilm reactor (MBBR) technology and high dose ozone) was very different to "normal" water (Samuelsson et al., 2011).

In a different study, MBBR was found to be efficient at removing oestrogens. However, expression of oestrogen-responsive genes suggested that some estrogenic substances were still present in the effluent. Membrane bioreactor removed most of the measured oestrogens, and reduced the induction of oestrogen-responsive genes. However, fish exposed to this effluent had significantly enlarged livers (Gunnarsson et al., 2009).

Embryo larval development of Japanese medaka (*Oryzias latipes*) was examined following exposures to a number of differently-treated effluents and various dilutions; the LC50s were 33.7%, 62.9%, 60% and 64.4% from exposures to raw effluent, secondary treated effluent, with the addition of ultrafiltration and with the addition of microfiltration, respectively. No acute toxicity was observed in the exposure to effluent that had undergone further treatment with activated carbon or reverse osmosis. The sex ratio was altered in the raw effluent from 25%, secondary treated, ultrafiltration and microfiltration all at 50%, but no alteration was observed in the larvae exposed to the effluent that had undergone further treatment with activated carbon or reverse osmosis (Zha & Wang, 2005).

2.6 Effects monitoring

Monitoring the health of the environment, and the effects of chemicals on wildlife and human health, is a complex task, and new knowledge is continually being gained. Environmental risk of chemicals can be determined in a number of ways, and ultimately, requires a multidisciplinary approach. Sometimes an effect is observed, either in wildlife or in the laboratory, resulting in the requirement for further research to determine the chemical or chemical group responsible, or a chemical is detected in the environment, and research is undertaken to ensure that that chemical does not pose an undue risk to the environment as a whole. Examples of the former include the well documented case of bird populations seen to be declining because of decreased survival of chicks due to eggshell thinning. The ultimate cause of this was the insecticide DDT, and its breakdown product, DDE (Hickey & Anderson, 1968; Henry & Kaiser, 2009 and Blus 2003 cited by WHO/UNEP, 2013). In 1970, imposex was observed in dog whelks following a large population decline. This was found to

be a consequence of tributyltin (TBT) use as an antifoulant (Gibbs & Bryan, 1986 cited by WHO/UNEP, 2013). More recently, in 2003, the pharmaceutical, diclofenac was found to be the cause of the dramatic decline in vulture population which had been occurring since the 1990s (Oaks et al., 2004). As discussed earlier in Section 2.2.1, chemicals are assessed with regards to their toxicity, and fate and behaviour. These assessments require both chemical analysis and biological assessment.

2.6.1 Analytical chemistry

Current analytical methods available to determine the chemical contaminants present in environmental water samples usually include a pre-concentration step, which consists of solid-phase extraction (SPE), solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE), or liquid-liquid extraction (LLE) (Bueno et al., 2012). This is then followed by a separation and determination step which can include liquid (LC) or gas chromatography (GC), coupled with mass spectrometry (MS), therefore, LC-MS or GC-MS. These tandem mass spectrometry methods offer higher performance than that of single-quadrupole instruments. Triple-quadrupole ion trap or hybrid triple quadrupole-ion trap systems are most commonly used techniques to analyse organic compounds in complex matrices, with LC-MS/MS being utilised to analyse polar and semi-polar organic compounds, and GC-MS being used for non-polar analytes, which are relatively volatile and thermally stable (Bueno et al., 2012).

2.6.2 Environmental sampling

Chemical pollutants often exist in the environment at low concentration, which means measuring them can pose difficulties, hence large volumes of water are often necessary to enable the sample to be concentrated and to allow detection of a larger number of chemicals. These samples can either be taken as spot or composite samples. Spot sampling will take a sample from a specific time point, and, as a consequence, might not capture the entire range of chemicals present in the environment. Composite sampling can be taken using autosamplers, which continuously sample the water over a specific time period, for example for 24 hours. Also, passive samplers can be deployed in a body of water (and also used for air sampling) for days or weeks, and collect a composite concentrated sample of the chemicals in the water. However, once the sample is collected, a choice is made as to what specific chemicals to measure. This is because the analytical methods required vary, depending on the physicochemical properties of specific chemicals. Therefore, the chemistry is targeted to specific chemicals, potentially leading to chemicals of known or unknown concern being missed. Biological testing can then be used to determine the biological activity

of an environmental sample, but this is very often also targeted; for example, VTG expression in male fish.

2.6.3 Toxicity testing

Regulatory toxicity testing requirements for substances include; acute freshwater fish test (96 hour LC50), acute toxicity to daphnids (48 hour EC50), and the growth inhibition test in freshwater algae (growth rate and/or biomass 72 hour EC50). For the standard OECD 203 fish acute toxicity test, approximately 120 adult fish are required per chemical test (Schulte & Nagel, 1994). With increasing knowledge of the adverse effects that chemicals cause on the environment and human health, regulations and legislations concerning the testing of chemicals are becoming stricter. Consequently, the numbers of animals used in the process of registering chemicals is likely to increase, if the same standard testing scheme remains in place. There has, therefore, been pressure to develop alternative methods of testing new and existing chemicals. For fish, these alternatives include *in vitro* fish cell assays and Quantitative Structure-Activity Relationships (QSARs). Another alternative to the adult fish test is to use the early developmental stages of fish embryos.

Braunbeck & Lammer, (2006) report that in a 1997 review, following an evaluation of approximately 150 toxicological studies on different life-stages of fish, it was found that, in at least 80% of studies, chronic toxicity could be reliably estimated using the results from early life-stage studies. Braunbeck & Lammer (2006) also stated that this conclusion has been further confirmed by other reviews of the data.

2.6.4 Genomic approaches

Genomics or ‘omics’ approaches are explained in detailed in subsequent chapters, but briefly there are three strands to this approach: transcriptomics, proteomics and metabolomics. The process of main relevance to this project is transcriptomics, which measures the fluctuations in gene expression (Poynton et al., 2008). This approach has been used extensively as a tool for investigating human disease, but more recently has been utilised for ecotoxicology, whereby the gene expression between exposed and unexposed cells, tissues or whole organisms can be compared. This allows any differences in gene expression (RNA) between the two groups to be highlighted, and acts as an indication or confirmation of a specific biological response (Figure 2.5). Gene expression can be measured for individual genes, or as in the case with microarrays, groups of genes, or the test organism’s entire transcriptome are measured. When the entire transcriptome is examined, this can highlight genes and, therefore, biological responses that could be

overlooked in standard targeted approaches. This is especially useful when investigating the toxicological risk of environmental samples consisting of a complex mixture of contaminants.

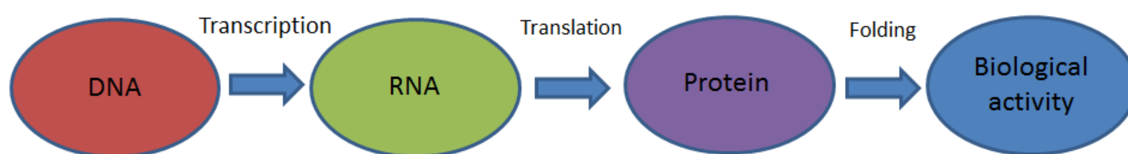

Figure 2.5 Gene expression (modified from Dale et al., 2012)

2.6.5 Whole effluent testing

Single substances are tested to determine their persistency, bioaccumulation and toxicity (PBT) on a routine basis. This is often a requirement under discharge consents, and for European legislation such as REACH. However, in the wider environment, organisms are rarely exposed to a single substance, bearing in mind the existence of an estimated 140 000 chemicals on the market worldwide (UNEP, 2013). In the real world, a single organism is likely to be exposed to a whole array of chemicals during its life. In the assessment of the PBT properties of an effluent from an industrial process, assumptions as to the composition of the effluent can be made from knowledge of the chemicals used in that specific industrial process. However, a study of the potential risks of this effluent to the environment will only consider the effects of individual chemicals in isolation rather than to the combination of all chemicals used/produced. This method of risk assessment has the potential to overlook the presence of other potentially harmful substances that are undetected, synthesised as by-products or as breakdown products, and may also miss the consequences of the mixture effects of multiple chemicals acting upon different points in a biological pathway. Domestic and industrial water treatment works receive wastewater from homes, industries, and businesses, making any assessment of the hazard of the plant's influent difficult. Additionally, using the standard single substance testing and assessment approach, the effects that substances may have when mixed together i.e. potential additive and/or synergistic effects, may be missed. Consequently, there has been increasing interest in the development of a PBT-test that can used to assess entire environmental samples, such as effluent, surface water or sediments (OSPAR Commission, 2007). It has been reported that this 'whole sample' approach has been used in a number of studies and, following analytical chemical analysis, only a small proportion of the adverse effects observed could be explained by the substances identified (OSPAR Commission, 2007). Therefore, it could be concluded that the remaining adverse effects observed in these studies were caused by

unidentified substances, and/or by mixture effects caused by exposure to a combination of substances (OSPAR Commission, 2007).

Testing every single conceivable combination of chemicals at varying concentrations is unrealistic, which is one of the reasons that the ‘whole sample’ approach is becoming increasingly recognised as a sensible approach to risk assessment. Whole Effluent Assessment (WEA) which is also referred to as Whole Effluent Testing (WET), and Direct Toxicity Assessment (DTA), is an assessment for ‘whole sample’ approach, but specifically for effluent (OSPAR Commission, 2007). In some countries including USA, Canada, Germany Ireland, Spain and Sweden, WEA is already used in national legislation and permitting (OSPAR Commission, 2007).

2.7 Model Organisms

A model organism is defined as “an organism suitable for studying a specific trait, disease, or phenomenon, due to its short generation time characterised genome, or similarity to humans; examples are a fly, fish, rodent or pig, whose biology is well known and accessible for laboratory studies” (Nature, 2014). Therefore, an appropriate model organism should provide both technical and practical advantages for studying the biological process, mechanism, endpoint etc., and the traits need to be generalised so that they are representative for a wider group of organisms (Segner, 2009). Zebrafish, as will be described below are both easy and cost-effective to use in studies, and they can provide theoretical data that can be used to answer specific questions surrounding vertebrate biology, genetics, toxicology and disease (Segner, 2009).

2.7.1 Zebrafish and zebrafish embryos

The chosen organism for the *in vivo* assays for this project was the zebrafish (*Danio rerio*) embryo. Adult zebrafish are a common laboratory fish, originally from India and Burma (they are thus tropical fish). They are a popular fish in laboratories because (i) they are a relatively small with an adult growing to approximately 5 cm, (ii) they can be housed at a relatively high stocking density, (iii) they are easy to care for and maintain, (iv) they have a life-span of approximately 3 years but with a short generation time of 3 months and are reproductively capable for approximately 1.5 years, (v) one female zebrafish can produce between 100 to 200 eggs on a weekly basis and these eggs are externally fertilised, (vi) their eggs are transparent, making the development easy to observe, and (vii) they rapidly develop and are free-swimming larvae at 5 days post fertilisation (Scholz et al., 2008). Due to the zebrafish’s popularity as laboratory fish (not only for ecotoxicological assessments, but also for human drug developmental, human diseases research, and for human health assessments), their

development has been extensively documented and staged, and their genome is sequenced and fully annotated with microarrays available. Due to the development and genome sequence of the zebrafish being well understood, any abnormalities can easily be identified, making them the ideal test organism for this project.

As zebrafish are vertebrate organisms, they share many similarities with mammals, based on organs and cell types present. Zebrafish have been described as the "canonical vertebrate", because of the similarities they share with mammalian biology. In combination with the similarities they share in mammalian biology, and because of the advantages listed above, zebrafish have become an important test organism and zebrafish models are used to study human diseases with a known genetic cause, induced by chemical exposure and bacterial pathogenesis (Rubinstein, 2003).

There is significant demand from the public to reduce (or stop all together) the use of animal testing. There is also a demand from industry to replace animal experimentation with a less costly, and less time consuming method of testing the environmental and health effects of substances (Scholz et al., 2008). There are a number of alternative testing methods to animal testing; these include predictive modelling, using structural activity relationships, toxicity testing using cell based systems, and fish embryos (Scholz et al., 2008). One of the most popular fish embryo models currently used in developmental toxicity, drug discovery, human disease research, genetics and ecotoxicological studies, is that of the zebrafish, *Danio rerio* (Scholz et al., 2008). Other relevant fish species which have also been suggested as relevant are medaka (*Oryzias latipes*), and the fathead minnow (*Pimephales promelas*) (Braunbeck & Lammer, 2005).

2.8 Sensitive window of exposure

Testing chemicals on zebrafish embryos results in exposure during development. Developmental exposure is known as a sensitive window of exposure; for example, hormones act during all stages of life, but the timing of action is important because it affects the potency. Once the hormone is removed, the action is halted, consequently during embryonic, foetal, and neonatal development, alterations to 'normal' hormone levels can cause permanent alternation to development. Additionally, once the action has occurred during this sensitive window, the changes will last a lifetime (WHO/UNEP, 2013). For example, thyroid hormone is essential for brain development during foetal and neonatal period of human development; insufficient supply of thyroid hormones causes mental retardation (WHO/UNEP, 2013).

2.9 Testing Unknowns

When determining the environmental risk posed by a chemical or a contaminated environmental sample (e.g. effluent entering receiving water), targeted testing has the potential of missing significant adverse and unknown biological effects, and can miss the compounds responsible. Two methods available to assess environmental risk are toxicity identification evaluation (TIE), and effects-directed analysis (EDA). TIEs are used to identify chemicals causing a toxic effect in effluents, receiving waters, interstitial waters, groundwater, and sediments. They use whole organism testing, resulting in ecological relevance. There are three stages of a TIE: characterisation, followed by identification and finally confirmation. Initially, the environmental sample is demonstrated to cause toxicity, with no assumption made about the cause of the toxicity. This is followed by assessment of classes of chemicals that could be responsible for the toxicity. Specific toxicants are identified and then finally the chemical responsible is confirmed by further whole organism tests using spiked environmental samples of the suspect chemical at a known concentration (Burgess et al., 2013). EDAs use mostly *in vitro* endpoints, with the focus on organic contaminants that have a known adverse effect. As with TIEs, EDA are used to test samples such as effluents, receiving waters, interstitial waters, groundwaters, sediments, but, unlike TIEs, EDAs can include tissues and biological fluids. In EDAs, chemical analysis is non-targeted, and they use biological effects as the method to identify the toxicant from the vast number of possible toxicants; this then enables the chemical analysis to be targeted on chemicals that have a significant contribution to the measured toxic effects. The steps involved in an EDA are: separation of organic chemicals from the sample matrix (this includes sample clean-up, preconcentration, and fractionation), followed by biological testing, then chemical analysis, and finally as with TIE, there is a confirmation step. The main steps of EDA are fractionation and toxicity testing, and as a consequence of there often being many fractions to test, high throughput *in vitro* assays are favoured. The fractions are created based on physicochemical properties of the components of the sample, for example hydrophobicity and polarity (Burgess et al., 2013).

2.10 Testing strategy

Based on the sum of this information, a multi-disciplinary approach was used for this project. Initially, toxicity testing was carried out on zebrafish embryos, using a method based on the OECD testing methods. This was following by microarrays to determine any genetic markers of concern, and further confirmation was carried out using *in vitro* testing. Available chemical data was also used to compare and inform the results.

CHAPTER 3: General Materials and Methods

3.1 Deephams Indirect Potable reuse pilot plant

The reuse pilot project that was the subject of this study was trailed by Thames Water Utilities Ltd. The 600 m^3 d^{-1} IPR pilot plant was designed to generate data for any future plans to operate a full scale project (M. Raffin et al., 2011). The pilot IPR plant was fed final effluent from a conventional activated sludge plant (M Raffin et al., 2012).

The final effluent from the waste water treatment plant enters the IPR plant and undergoes a 500 µm pre-filtration step (M. Raffin et al., 2011). Once the effluent has been pre-filtered it is filtered by a submerged continuous membrane filtration process providing finer filtration, known as microfiltration (MF) (M Raffin et al., 2012). Following MF, one stream of product water is treated with anti-scalant and sulphuric acid to control scaling after which it is passed through a reverse osmosis (RO) process (Marie Raffin et al., 2012). The resulting water from the RO process is then treated by an Advanced Oxidation Process (AOP) consisting of ultraviolet disinfection and hydrogen peroxide dosing (M. Raffin et al., 2011). The final stage of the treatment process consists of the water being pH corrected and fed through a degassing tower (M Raffin et al., 2012).

A second stream of product water from the MF process is directed straight into a separate AOP system, where it does not undergo treatment via reverse osmosis or anti-scalant. The product from this AOP system will be referred to as AOP1, and the product water which has undergone both MF and RO treatment followed by AOP will be referred to as AOP2. There are three points along the IPR treatment process where chloramine can be dosed, at pre-filter, pre-MF and pre-RO (Figure 3.1); the chloramine is used to minimise biofouling in the pipework and equipment (M. Raffin et al., 2011; M Raffin et al., 2012).

Figure 3.1 Figure 3.1 illustrates the IPR treatment plant processes that were in operation. The IPR plant was fully automated, with an advanced water sampling instrumentation that monitors and controls the process, and an on-site supervisory control and data acquisition (SCADA) system to record trends and collect data on quality at various stages of the process for analysis (M. Raffin et al., 2011; M Raffin et al., 2012).

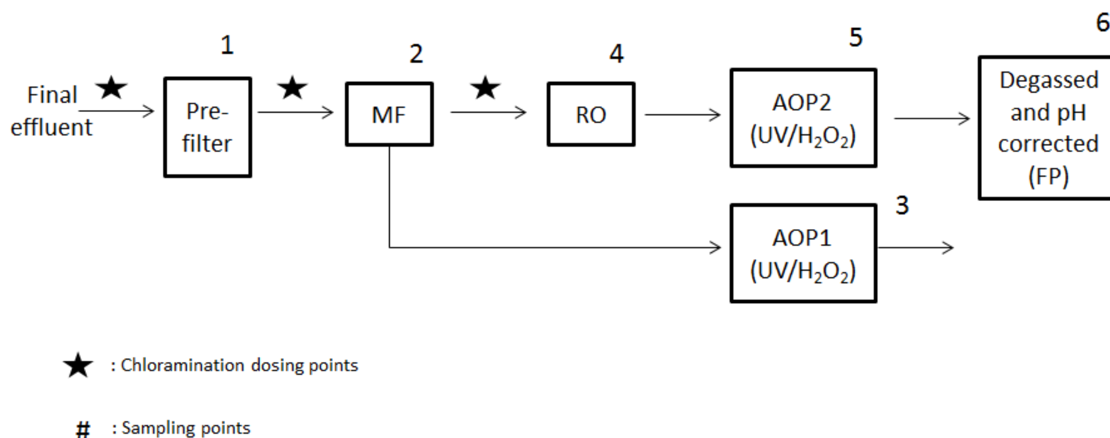

Figure 3.1 The IPR treatment process that was in operation at the Deephams waste water treatment plant. MF: Microfiltration; RO: Reverse Osmosis; AOP: Advanced Oxidation Process; and FP: Final Product.

As previously stated, the main aim of this project is to identify any potential risks to health from exposure to recycled water and, more specifically, from the pilot IPR plant that was being trailed at Deephams waste water treatment works. The effluent/product water from each treatment process within the IPR plant was examined for potential biological activities. This was accomplished using a number of steps: collation and interpretation of the chemical analysis data provided by Thames Water, *in vivo* bioassays in zebrafish and their embryos, deployment of passive samplers, gene expression analysis using microarray technology, and *in vitro* mammalian cell confirmatory tests.

3.2 Analytical Chemistry (Historical Thames Water data)

Between 2008 to 2012, Thames Water Ltd analysed the water at the different treatment stages of the IPR plant to determine the presence or absence and concentration of over 400 compounds. Given the extent of the analytical chemistry data, and as a way to initially assess the chemical make-up of the different product waters, the chemicals detected in the water at each stage of the treatment process were analysed, categorised and some basic physicochemical, fate and behaviour, aquatic toxicological and mammalian toxicological data were gathered. Once these data were gathered, the known effects of chemicals that occurred above the limit of detection (>LoD) were noted.

The information collected for each contaminant present above the LoD was as follows:

- *Identification and use*: chemical name and CAS registration number; and the use.
- *Physical chemical properties and fate and behaviour*: the compound's molecular weight; dissociation constant (pKa); volatility (Henry's Law constant and vapour

pressure); solubility in water; octanol water coefficient (Log Kow); Bioconcentration Factor (BCF); and biodegradation within 28 days (%) and half-life (days).

- *Ecotoxicology*: aquatic ecotoxicology (lowest acute and chronic L(E)C50/NOEC).
- *Potential target organs and/or toxicological endpoint from mammalian data*: skin and eye irritation and skin sensitisation; effects to sensory organs; effects on the gastrointestinal tract and/or liver; effects on the brain and/or the central nervous system (CNS); effects on the immune system; effects on the kidney; effects to the cardiovascular system and/or blood; contaminant reported to be carcinogenic; contaminant reported to be genotoxic; contaminant reported to cause developmental toxicity; contaminant reported to be a reproductive toxicant; and toxicant reported to be endocrine disrupting.
- *Other*: this section includes compound approvals. For example, if it is a pesticide, has it been approved for use as a pesticide in the UK, also any health standards set by authoritative bodies such as the World Health Organization (WHO), the European Food Safety Authority (EFSA) etc. and any other pieces of information which may be useful in the risk assessment of the specific contaminant.

The literature search for each compound was restricted to a specific number of sources. These sources being used were considered to be authoritative and reliable, the sources are listed in Table 3.1. A wider search was conducted on the compounds for which had no or very limited data were available.

Table 3.1 The table below details all the sources used to search for relevant toxicity, ecotoxicity, physical chemical properties, fate and behaviour data. The majority of the searching was carried out in the years 2011 and 2012

Sources	Address
Classification	
PAN	http://www.pesticideinfo.org/Search_Chemicals.jsp
ChemID	http://chem.sis.nlm.nih.gov/chemidplus/
EC Priority Pollutants	http://ec.europa.eu/environment/water/water-framework/priority_substances.htm
NORMAN	http://www.norman-network.net/index_php.php?module=public/about_us/emerging&menu2=public/about_us/about_us
Physical properties/Fate & Behaviour/Uses	
ChemID	http://chem.sis.nlm.nih.gov/chemidplus/
SRC PhyProp	http://www.syrres.com/what-we-do/databaseforms.aspx?id=386
SRC Biodeg	http://www.syrres.com/what-we-do/databaseforms.aspx?id=382
HSDB	http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
IUCLID	http://esis.jrc.ec.europa.eu/index.php?PGM=dat
WHO EHC	http://www.who.int/ipcs/publications/ehc/ehc_alphabetical/en/index.html
Human Health/Toxicity	
IUCLID	http://esis.jrc.ec.europa.eu/index.php?PGM=dat
IPCS InCHEM	http://www.inchem.org/
ATSDR	http://www.atsdr.cdc.gov/substances/indexAZ.asp#A
IARC	http://monographs.iarc.fr/ENG/Classification/index.php
US EPA IRIS	http://cfpub.epa.gov/ncea/iris/index.cfm?fuseaction=iris.showSubstanceList
TOXNET	http://toxnet.nlm.nih.gov/
NTP	http://ntp.niehs.nih.gov/
WHO Background documents	http://www.who.int/water_sanitation_health/dwq/chemicals/en/index.html#E
ITER	http://www.tera.org/iter/
HPA Compendium of Chemical Hazards	http://www.hpa.org.uk/Topics/ChemicalsAndPoisons/CompendiumOfChemicalHazards/
OECD SIDS	http://www.chem.unep.ch/irptc/sids/OECDSIDS/sidspub.html
Ecological	

Sources	Address
US EPA ECOTOX	http://cfpub.epa.gov/ecotox/
HSDB	http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
IUCLID	http://esis.jrc.ec.europa.eu/index.php?PGM=dat
OECD SIDS	http://www.chem.unep.ch/irptc/sids/OECDSIDS/sidspub.html
WHO EHC	http://www.who.int/ipcs/publications/ehc/ehc_alphabetical/en/index.html
Pesticides	
HSE	http://www.pesticides.gov.uk/guidance/industries/pesticides/topics/publications/guide-to-pesticides
Australian PUBCRIS	http://services.apvma.gov.au/PubcrisWebClient/welcome.do
US EPA RUP	http://www.epa.gov/opprd001/rup/rup6mols.htm
WHO Classification	http://www.who.int/ipcs/publications/pesticides_hazard/en/
EU Pesticide Database	http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection
EFSA	http://www.efsa.europa.eu/
Pharmaceuticals	
FDA	http://www.accessdata.fda.gov/scripts/cder/ob/default.cfm
BNF	http://www.bnf.org/bnf/index.htm
Australian TGA	http://www.tga.gov.au/index.htm
EMA	http://www.ema.europa.eu/ema/
Cosmetics and personal care products	
CTFA	http://www.ctfa.org.nz/
SCCS	http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm
HERA	http://www.heraproject.com/
Food additives	
FSA	http://www.food.gov.uk/
EFSA	http://www.efsa.europa.eu/
JECFA	http://www.fao.org/ag/agn/jecfa-additives/search.html
Veterinary medicines	
EMA	http://www.ema.europa.eu/ema/

ATSDR: Agency for Toxic Substances and Disease Registry
BNF: British National Formulary
CTFA: Cosmetic Toiletry and Fragrance Association Inc.
EC: European Commission
EFSA: European Food Safety Authority
EHC: Environmental Health Criteria
EMA: European Medicines Agency
EU: European Union
FDA: Food and Drug Administration
FSA: Food Standards Agency
HERA: Human and Environmental Risk Assessment on ingredients of household cleaning products
HPA: Health Protection Agency
HSDB: Hazardous Substances Data Bank
HSE: Health and Safety Executive
IARC: International Agency for Research on Cancer
IPCS: International Programmes on Chemical Safety
IRIS: Integrated Risk Information Systems
ITER: Internal Toxicity Estimated for Risk Assessment
IUCLID: International Uniform Chemical Information Database
JECFA: Joint Expert Committee on Food Additives
NORMAN: Network of reference laboratories for monitoring of emerging environmental pollutants
NTP: National Toxicology Program
OECD: Organisation for Economic Co-operation and Development
PAN: Pesticide Action Network
PUBCRIS: Public Chemical Registration Systems
RUP: Restricted Use Pesticides
SCCS: Scientific Committees on Consumer Safety
SIDS: Screening Information Data Set
TGA: Therapeutic Goods Administration
WHO: World Health Organization
US EPA: United States Environmental Protection Agency

3.3 *In vivo* studies

3.3.1 Fish Husbandry

A group of 30 adult male and 30 female zebrafish (*Danio rerio*) (Tübingen strain) of breeding age were sourced from University College London (UCL) in July 2011. Upon arrival at Brunel University the fish were kept in quarantine, in static renewal tanks for a two weeks period prior to being sexed and placed into flow-through tanks. The fish were separated based on sex and placed into 10 litre tanks. The physical difference between the male and female zebrafish is illustrated in Figure 3.2. Throughout the quarantine period and the following breeding period, the fish were fed 3 times a day; twice with flake food (King British Tropical flake food, Lillicos, Surrey) and once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated). They were kept at a temperature of 26-28°C and with a photoperiod of 14 hours of light and 10 hours of dark, which are considered to be optimal husbandry conditions (Westerfield, 2007). The water was dechlorinated carbon-filtered (5 and 10 μm) tap water from header tanks at a flow rate of 20 litres/hour. All tubing (flow-line, air-lines, siphon and attached to the grids) was made of medical grade silicon (VWR, UK).

Figure 3.2 Male and female zebrafish. The female (bottom of the picture) has a larger abdomen whereas the male (top of the picture) has a sleeker body shape and has a darker yellow pigmentation. Differences in behaviour can also be observed, with males chasing the females. The photo is from "Zebrafish Change Color for Sex," n.d. with the photo being credited to Vetmeduni Vienna/Zala.

3.4 Fish Breeding and Embryo Collection

In the wild, zebrafish breed in the morning shortly after the sun rises. In laboratory conditions this is soon after the artificial light is switched on (Westerfield, 2007).

Male and female zebrafish were kept separately until the day before embryos were needed. In the afternoon, 4 females to 2 males were grouped together in narrow breeding tanks with a volume of 9.5 litres (15 cm wide and 40 cm deep with a height of 25 cm) (Figure 3.3). Each tank was fitted with a metal grid, allowing the eggs to fall to the bottom of the tank and preventing them from being eaten by the adults (Figure 3.3). Egg collection took place the following day approximately 1 hour after dawn (the lights were switched on at 7.30 am, and collection of embryos commenced at 9 am).

Figure 3.3 The photograph on the left illustrates the narrow breeding tank containing the breeding fish (four females and two males) metal grid, flow-line and air-line. The photograph on the right illustrates five breeding tanks and three holding tanks (one for males and two for females), to enable the breeding females to be rotated to ensure adequate rest days between breeding events.

Once it had been confirmed visually that the breeding groups had produced eggs, the adult fish were returned to their relevant holding tanks and the grids were then removed. Once the eggs had settled to the bottom of the tanks a glass siphon was used to collect the eggs into a fine mesh net and placed into 300 ml glass crystallising dishes containing fresh water from the header tank. From these dishes a plastic 3 ml Pasteur pipette was used to move some batches of the embryos into a glass petri dish containing fresh water from the header tank. A Leica MZ FL III fluorescence stereomicroscope with a maximum magnification of 800x was then used to sort the embryos, removing debris, dead and unfertilised eggs. The pre-cent fertilisation was not recorded. The healthy looking fertilised embryos were then transferred to

a clean crystallising dish containing water from the header tank. The collected, sorted and unsorted embryos were kept in the SANYO incubator to monitor them at the desired temperature.

3.4.1 Effluent collection

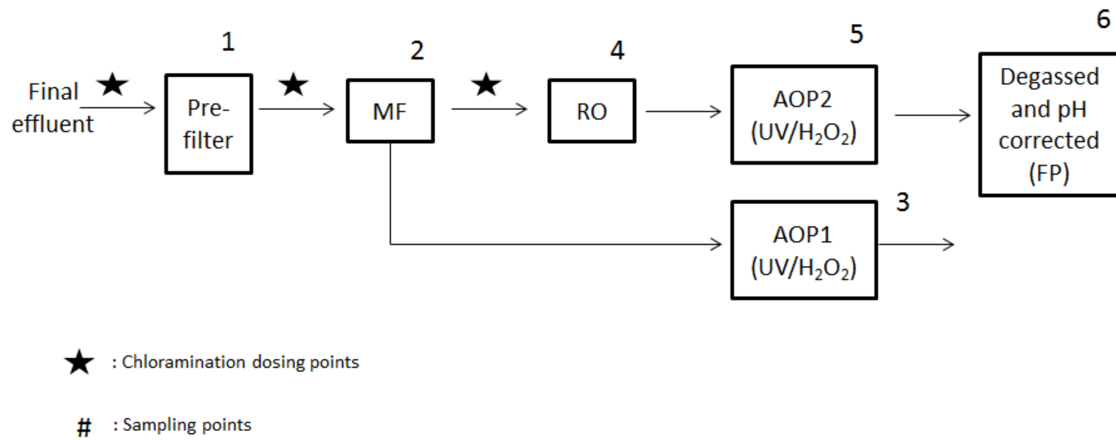

Figure 3.4 Schematic of the IPR plant with the positions (1-6) of the where the composite samples or point samples were taken

Twenty-four hour composite effluent samples were collected from Deephams IPR the day before an before an effluent exposure was due to start if possible. Composite samplers were held at the IPR plant (the IPR plant (

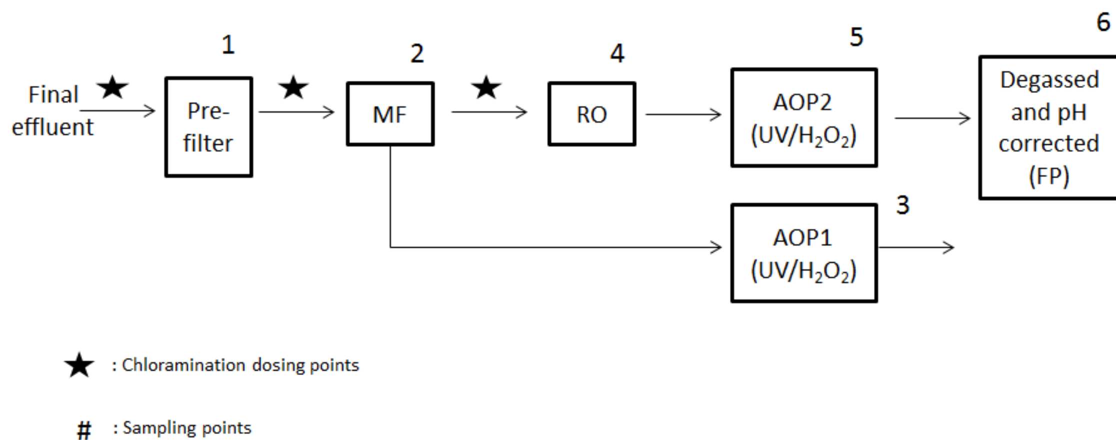

Figure 3.4), and the staff at the site would program them to start sampling the day before water samples were due to be collected (Figure 3.5). The treatment water was collected in acid washed and silanised 2.5 litre brown glass Winchester bottles. The collection chamber of the sampler was chilled to 4°C, and they were transported back to Brunel University in cool-boxes containing ice blocks. On return to Brunel they were immediately transferred to the fridge or cold storage room to be kept at 4°C.

Figure 3.5 Some of the composite samplers positioned along the IPR treatment process They had to placed close to an outlet for the specific treatment process, but must not impede access to the containers; therefore, some had to be placed outside the container (as is seen in the far right picture) whereas there was sufficient space for others to be left inside (the left and middle pictures). The middle picture illustrates the collection of the effluent within a glass Winchester.

3.4.2 Zebrafish development exposures (48 hours)

For each of the 48 hour zebrafish developmental exposures, embryos were collected as described above described above and, if possible, composite samples were collected from IPR plant the day before the before the exposure was due to start. If composite samples were not available, a grab sample was sample was collected instead. Each sample bottle containing treatment water was vigorously shaken shaken after which 2 ml (or the relevant control; aquarium header tank water or tap water) was pipetted was pipetted into a well of a 24 well plate. The plates were then labelled and placed in the incubator. The incubator. The configuration of the plates is detailed in Figure 3.6

Figure 3.7. While the plates were equilibrated to the correct temperature within the incubator, the embryos were collected and sorted as detailed above. Once the healthy looking and fertilised embryos had been isolated and were free of other debris, one embryo was gently placed into each well (containing 2 ml of the exposure water) of the 24 well plates, which were then returned to the incubator. The embryos were collected shortly after fertilisation; they were placed in the exposure between the 2-cell and 64-cell stages (0.75 hours to 2 hours). Basic physical chemical readings were taken at the start of every exposure; these

included dissolved oxygen (HACH oxygen and temperature probe), pH, general hardness, carbonate, nitrate and nitrite (API 5 in 1 test strips), and ammonia (API ammonia test strips) (Table 3.2). The temperature within the incubator was recorded with a thermometer to check on-the-spot and continuous readings were also taken using the two Tiny Tag data loggers (Figure 3.7).

Table 3.2 The range of general physio-chemical properties of the control and exposure waters

Treatment	Range of physico-chemical properties					
	pH	Nitrate (mg/l)	Nitrite (mg/l)	Ammonia (mg/l)	General hardness (calcium and magnesium) (mg/l)	Carbonate hardness (mg/l)
Control (header tank water)	7.5	0-20	0-0.5	0	180	240
Tap water	8	0	0	0	180	240
Final product	6.5	0-20	0	0-1.0	0-30	0
AOP2	6.0	0-20	0-0.5	0-3	0	0
Reverse osmosis	6-7	0	0	0-1.0	0-30	0-40
AOP1	7.5-8.5	40	0-1	0-3	180	180-240
Microfiltration	7.5-8	40-80	0-3	0-3	180	240
Final effluent	7.5-8	40-80	0-3	0-3	180	240

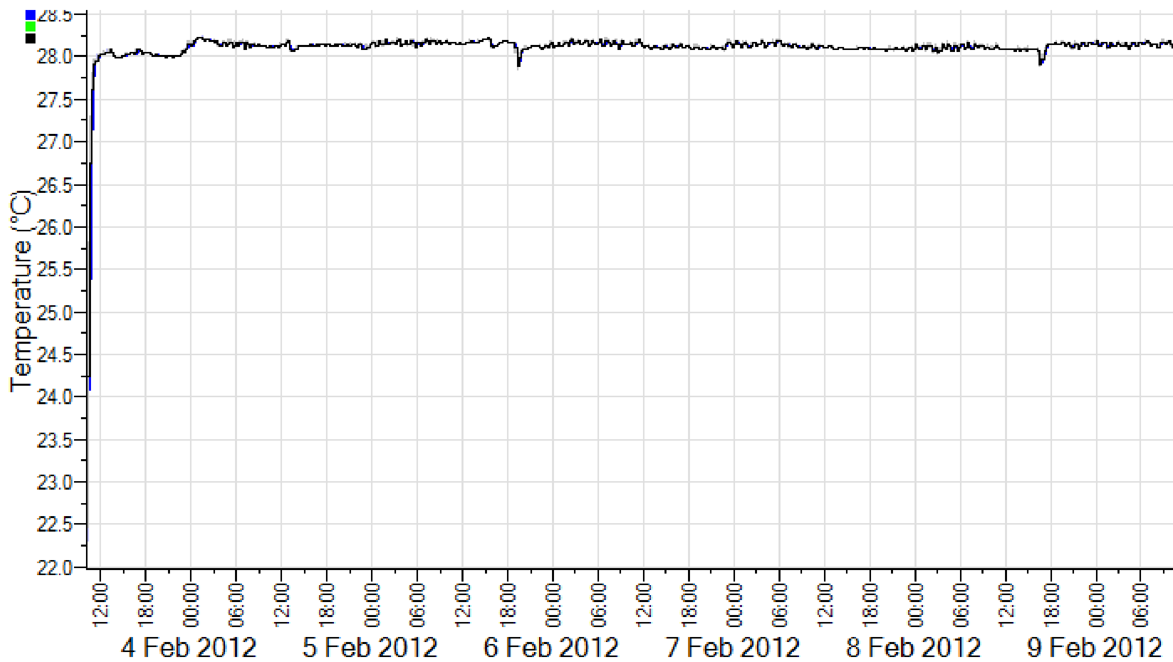

Figure 3.6 The temperature within the incubator was measured using TingTags, showing the temperature remained relatively constant over an extended time period.

Plate 1		1	2	3	4	5	6
	A	CONTROL	CONTROL	CONTROL	AOP2	AOP2	AOP2
	B	TAP	TAP	TAP	RO	RO	RO
	C	MF	MF	MF	AOP1	AOP1	AOP1
	D	FE	FE	FE	FP	FP	FP

		1	2	3	4	5	6
Plate 2	A	AOP2	AOP2	AOP2	CONTROL	CONTROL	CONTROL
	B	RO	RO	RO	TAP	TAP	TAP
	C	AOP1	AOP1	AOP1	MF	MF	MF
	D	FP	FP	FP	FE	FE	FE

		1	2	3	4	5	6
Plate 3	A	FE	FE	FE	FP	FP	FP
	B	CONTROL	CONTROL	CONTROL	AOP2	AOP2	AOP2
	C	TAP	TAP	TAP	RO	RO	RO
	D	MF	MF	MF	AOP1	AOP1	AOP1

		1	2	3	4	5	6
Plate 4	A	FP	FP	FP	FE	FE	FE
	B	AOP2	AOP2	AOP2	CONTROL	CONTROL	CONTROL
	C	RO	RO	RO	TAP	TAP	TAP
	D	AOP1	AOP1	AOP1	MF	MF	MF

		1	2	3	4	5	6
Plate 5	A	MF	MF	MF	AOP1	AOP1	AOP1
	B	FE	FE	FE	FP	FP	FP
	C	CONTROL	CONTROL	CONTROL	AOP2	AOP2	AOP2
	D	TAP	TAP	TAP	RO	RO	RO

		1	2	3	4	5	6
Plate 6	A	AOP1	AOP1	AOP1	MF	MF	MF
	B	FP	FP	FP	FE	FE	FE
	C	AOP2	AOP2	AOP2	CONTROL	CONTROL	CONTROL
	D	RO	RO	RO	TAP	TAP	TAP

		1	2	3	4	5	6
Plate 7	A	TAP	TAP	TAP	RO	RO	RO
	B	MF	MF	MF	AOP1	AOP1	AOP1
	C	FE	FE	FE	FP	FP	FP
	D	CONTROL	CONTROL	CONTROL	AOP2	AOP2	AOP2

		1	2	3	4	5	6
Plate 8	A	RO	RO	RO	TAP	TAP	TAP
	B	AOP1	AOP1	AOP1	MF	MF	MF
	C	FP	FP	FP	FE	FE	FE
	D	AOP2	AOP2	AOP2	CONTROL	CONTROL	CONTROL

Figure 3.7 Distribution of the treatments across the well plates used for both the 48 hour observation (Experiments 3 and 4). CONTROL: water from the header tank; TAP: water from Brunel University drinking taps; FP: IPR final product water; AOP2: water following the advanced oxidation process

following reverse osmosis treatment; RO: water from the reverse osmosis treatment process; AOP1: water from the advanced oxidation process following microfiltration treatment; MF: water following the microfiltration treatment stage; and FE: water following secondary sewage treatment and a pre filtration step.

Above both shelves of the incubator, aquarium lights were attached and a timer was set to ensure the embryos had a photoperiod of 14 hours of light and 10 hours of dark. Observations using the Leica MZ FL III fluorescence stereomicroscope and the Leica DC 300F digital image recording system were taken at 4, 8, 12, 16, 24, 36 and 48 hours post fertilisation and specific endpoints/developmental endpoints were noted to have occurred or not. The well plates had lids, so limited evaporation would have taken place. Due to the volumes used in the study (2 ml per well/per embryo) it was not possible to conduct chemical analysis on exposure water, however, chemical analysis had been undertaken on the product waters from the IPR site and chemical analysis data was recorded for the tap and control water (Appendix).

Table 3.3 shows the toxicity endpoints/developmental endpoints, which were examined in each embryo at each time point. The endpoints displayed in Table 3.3 are sourced from a number of references, including the OECD background document for the fish embryo toxicity tests (Braunbeck & Lammer, 2005; OECD, 2006; Schulte & Nagel, 1994).

Table 3.3 Toxicological and developmental endpoints visible in zebrafish embryos at specified time points

Development stage	Hours post fertilisation						
	4	8	12	16	24	36	48
Coagulated egg*	+	+	+	+	+	+	+
Termination of blastula formation	+						
Start of epiboly		+					
Termination of epiboly			+				
Formation of somites*				+	+	+	+
Eye development				+	+	+	+
Tail detachment*					+	+	+
Spontaneous body movement					+	+	+
Malformation of head					+	+	+
Malformation of sacculi/otoliths					+	+	+
Malformation of tail					+	+	+
Malformation of heart					+	+	+
Modified chorda structure					+	+	+
Scoliosis					+	+	+
Yolk deformation					+	+	+
General growth retardation					+	+	+
Presence of heart-beat/blood circulation*						+	+
Heart-beat frequency							+
Formation of edemata							+
Pigmentation							+

*Endpoints used by the German DIN in their whole effluent testing regime.
+This developmental should be visible at this time-point or the specified malformation could be visible at this time-point.

At 48 hours heart rates were recorded by counting the number of beats over a period of 30 seconds. The embryos were then transferred into RNAlater® stabilisation solution (Life Technologies, UK), kept at 4°C for 24 hours to allow the RNAlater® stabilisation solution to penetrate the tissue, and then stored at -80°C.

3.4.3 Extended zebrafish embryo exposure

The zebrafish breeding, embryo collection, and sorting and effluent collection for this set of exposures were the same as for the 48 hour observation described above. However, instead of using 24 well plates, 300 ml crystallising dishes were used to begin with, containing 200 ml IPR product water, header tank water or tap water. IPR product water was pre-warmed in a water bath before being put into the crystallising dishes. Approximately 50 sorted embryos were placed in to each crystallising dish. There were 3 replicates, and the IPR product water, control or tap water was partially renewed, by replacing a third of the treatment water every other day, until feeding began on 5 days post-fertilisation, after which it was renewed on a daily basis to prevent the build–up of uneaten food. On day 9-10 post-fertilisation the zebrafish larvae were moved to larger (1 litre capacity) glass beakers. As these were too large to sit in the incubator, the beakers were placed in fish tanks and water flowed from the header tanks and acted as large water baths; this is illustrated in Figure 3.8.

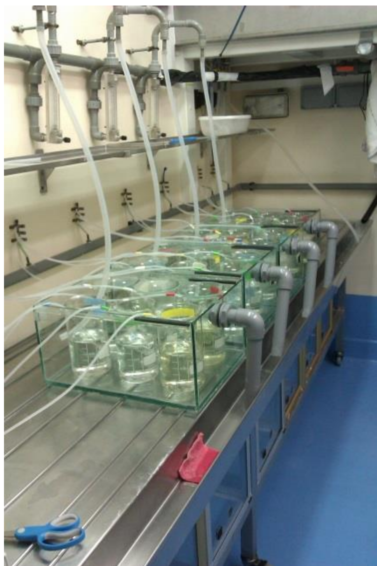

Figure 3.8 Photograph of the exposure beakers in the tanks which acted like large water baths to keep the water within the beakers at a constant temperature, optimum for zebrafish development. The air-lines can also be seen, the air-lines as in previous studies were medical grade silicone tubing and on the end of each air-line there was a glass Pasteur pipette which allowed a gentle air flow to be bubbled through.

Air-lines were also added, with a very gentle air flow so as not to damage the delicate larvae, but to prevent the dissolved oxygen level from dropping (Table 3.4)

Table 3.4 Average dissolved oxygen levels in an extended exposure study over a 24 hour period prior to adding airlines

Treatment	Dissolved oxygen (mg/l) at 0 hours	Dissolved oxygen (mg/l) at 24 hours
Control	9.20	7.74
Tap	8.71	7.78
Final product	9.52	7.84
AOP2	8.50	7.63
Reverse osmosis	9.80	8.04
AOP1	9.60	7.61
Microfiltration	8.93	7.67
Final effluent	5.03	5.51

The zebrafish (now free swimming larvae) were observed on a daily basis, so that any abnormalities could be noted and photographed, and dead ones removed. Once in the beakers, the larvae were fed twice daily; once with ZM-0 flake food and once with live artemia. This exposure was terminated on day 15.

3.4.4 Zebrafish embryo exposures for gene expression (48 hours)

For each 48-hour zebrafish embryo exposures for gene expression, the same breeding regime was followed as is detailed in Section 3.4 and composite samples (when available) were collected from the IPR plant the day before the exposure was due to start.

Once the IPR product waters arrived back from the pilot plant, they were emptied out into labelled acid washed 300 ml glass crystallising dishes; 200 ml was placed into each dish. A glass petri dish was then used as a lid, and they were placed in large fish tanks which received a flow of heated water from the header tank to keep them at the optimum temperature of 28°C. They were left overnight to allow them to reach the desired temperature. There were two replicates (i.e. two crystallising dishes) for each of the exposure water, i.e. the six product waters collected from the IPR plant, the tap water and control water from the header tank. There were also two replicates (two crystalising dishes) for each time-point (six in total). Therefore there were 96 dishes in each experiment. At the start of the exposure, the day after effluent collection, the embryos were sorted as previously described and 50 individual embryos were placed into each of the 96 dishes.

Figure 3.9 The photographs illustrate the 96 dishes in the large tanks acting as water baths. There were four large tanks in total and the dishes were randomly placed within the tanks. Within the large tanks there were a number of smaller up-turned tanks to prevent header tank water intrusion.

Observations and sampling were carried out at the six time-points, 8, 12, 16, 24, 36 and 48 hour post fertilisation. At each time-point the relevant dishes were removed i.e. a group of 50 embryos that had been exposed to either header tank water, tap water or one of the six IPR product waters along with the corresponding replicate. Consequently, at each sampling/time-point, 16 dishes were removed from the tanks. At each sampling point photos were taken of one individual from each dish, dead ones were counted and removed, and any abnormal embryos were removed and preserved separately. The 'normal' looking embryos remaining were pooled (due to the survival rate there was always >20 embryos in each pool) and preserved together in RNAlater®. These exposures were repeated 3 times over a period of approximately a month.

3.5 Genomics

The embryos from the 48 hour exposures for gene expression (detailed in Section 3.4.4) were transported to Liverpool University on dry ice where the RNA was extracted and microarrays were conducted.

3.5.1 RNA Extraction

RNA from the preserved embryos (stored in RNAlater® at -80°C transported on dry ice) was extracted using RNeasy Mini Kit provided by Qiagen (Qiagen, 2012). The kit enables 100 µg of RNA longer than 200 bases to bind to the silica membrane provided in the kit. Broadly, the embryos were lysed and homogenised in a buffer which inactivates the RNases to ensure purification of intact RNA. Ethanol is added to precipitate RNA ; the sample is then added to a spin column. In the column the RNA binds to the membrane where the contaminants can be washed away, the RNA can then be eluted from the membrane (Qiagen, 2012).

The protocols supplied with the RNeasy Mini Kit are not specific to fish embryos so were slightly adapted and are detailed as follows.

β-Mercaptoethanol was added to the Buffer RLT before use, 10 µl of β-mercaptoethanol was added per 1 ml of Buffer RLT, this was then be stored at room temperature (15-25°C) for up to 1 month (Qiagen, 2012). Ethanol (100%) was added to the Buffer RPE as indicated on the bottle (Qiagen, 2012).

Lyse and homogenise

The embryos were removed from the -80°C freezer and defrosted on ice, the RNAlater® was removed using a pipette. Then 350 µl Buffer RLT (with the added β-mercaptoethanol) was added, then, using a 1 ml syringe, the mix was drawn up to enable homogenisation within the syringe. The lysate was passed 10 times (5 times as stated in the Qiagen protocol was insufficient and blocked the filter) through a blunt 20-gauge needle (0.9 mm diameter). Then 350 µl of 70% ethanol was added to the homogenised lysate and mixed well via pipetting and syringe the homogenate (700 µl) into each RNeasy spin column placed in a 2 ml collection tube. The lid was closed and gently centrifuged for 15 seconds at ≥8000 x g (≥10 000 rpm) and then the flow-through was discarded.

DNase digestion and binding total RNA

The next series of steps were optional "On-Column DNase Digestion with the RNase –Free DNase Set". The RNase-Free set (cat. No. 79254) is reported to give on-column digestion of DNA during RNA purification. The DNase is then removed in the following wash steps.

For the first time the kit was used, DNase I stock solution was prepared by dissolving the lyophilized DNase I (1500 Kunitz units) in 550 µl RNase-free water. Qiagen advise to do this via injecting the water into the vial using a syringe, and then gently mixing by inverting, not with use of the vortex. The DNase I can be divided into single use aliquots and stored at -20°C for up to 9 months.

Once the DNase I solution is prepared, 350 µl Buffer RW1 was added to the RNeasy spin column. The lid was closed and centrifuged for 15 seconds at ≥8000 x g (≥10 000 rpm) to wash the spin column and membrane and then the flow-through was discarded. The collection tube is kept for the last step.

Then 10 µl DNase I stock solution is added to 70 µl Buffer RDD. This was then gently mixed by inverting the tube, and then briefly centrifuged to collect any residual liquid from the sides

of the tube. DNase I is reported to be very sensitive to physical denaturation, this is why gentle inversion should be used to mix rather than a vortex.

The DNase I mix (80 µl) was then added to the RNeasy spin column membrane and placed on the benchtop (20-30°C) to incubate for 15 minutes. It was ensured that the DNase I mix was added directly to the membrane as, if left to stick to the walls of the tube or O-ring of the column, the removal of DNA will be incomplete.

In this final stage of this optional DNase digestion step 350 µl of Buffer RW1 was added to the RNeasy spin column. The lid was closed and it was centrifuged for 15 seconds at ≥8000 x g (≥10 000 rpm) and then the flow-through was discarded.

Wash

Then 500 µl Buffer RPE was added to the RNeasy spin column, the lid closed, and it was centrifuged for 15 seconds at ≥8000 x g (≥10 000 rpm) to wash the spin column membrane and then the flow-through was discarded.

Another 500 µl Buffer RPE was added to the RNeasy spin column, the lid closed and it was centrifuged for 2 minutes at ≥8000 x g (≥10 000 rpm) to wash the spin column membrane. This longer time in the centrifuge dries the spin column membrane to ensure there is no ethanol left following the RNA elution. Residual ethanol has the potential to affect with subsequent reactions.

The RNeasy spin column is then added to a new 2 ml collection tube and the old collection tube with containing the flow-through is discarded. The lid was closed and centrifuge at full speed for 1 minute. This step removes the chance of carryover of Buffer RPE or residual flow-through remains on the outside of the spin column.

Elution

The spin column was then placed in a new 1.5 ml collection tube, and 36 µl RNase-free water was directly added to the spin column membrane, the lid was closed and this was allowed to soak into the membrane for 1 minute. Then it was centrifuged for 1 minute at >8000 x g (≥10 000 rpm) to elute the RNA.

The eluate from the previous step was placed back on to the spin column membrane and left for 1 minute before centrifuging again for 1 minute at >8000 x g (≥10 000 rpm).

3.5.2 Quantify RNA

The Thermo Scientific NanoDrop™ 1000 Spectrophotometer is a full spectrum (220-750 nm) spectrophotometer. The sample was pipetted directly onto the end of the fibre optic cable, and then the second fibre optic cable was brought into contact with the sample, consequently the liquid sample acts as a bridge between the two fibre optic ends. A pulsed xenon flash lamp provides the light source (Thermo Scientific, 2008). The manufacturer's instructions were followed, the pedestal was cleaned prior to use, the Nucleic acid reading and RNA-40 measurement options chosen, and the instrument was blanked using RNase-free water with a volume of 1 µl. Following this each sample could be measured taking 1 µl from each sample. The quantity of RNA was recorded in ng/µl and the purity of the sample is recorded as the ratio of absorbance at 260 and 280 nm, 260/280 ratio. For RNA a ratio of 2.0 is stated to generally be accepted as "pure" RNA. If the ratio is considerably lower than this it can be an indication of the presence of protein, phenol or other contaminants that absorb strongly at or near to 280 nm (Thermo Scientific, 2008).

3.5.3 RNA quality

The extracted RNA was checked for contamination and quality using gel electrophoresis. The technique separates out the molecules dependent on their size. Both DNA and RNA are negatively charged, therefore with the gel having a positive charge at one end and a negative charge at the other, the molecules of interest in this instance the RNA, will move toward the positive charge. The smaller molecules will travel a greater distance than the larger molecules. A DNA ladder was used to estimate the size of the molecules. The RNA bands were visualised using ethidium bromide which fluoresces under UV illumination. A 1.5% standard agarose gel was used.

3.5.4 Microarray

One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling supplied by Agilent Technologies was used to conduct the microarray. RNA samples ranging between 10 and 200 ng of total RNA can be input in this kit. The kit produces fluorescent cRNA and uses T7 RNA polymerase, which amplifies target material and incorporates cyanine 3-labeled cytidine triphosphate (CTP) (Agilent Technologies, 2010). It is stated in the protocol that the kit produces an amplification of at least a 100-fold from total RNA to cRNA with this kit.

Broadly, the procedure includes the following steps; cDNA synthesis, cRNA synthesis and amplification, cRNA purification, preparation of hybridization sample, 17 hour hybridisation (65°C), wash, scan and feature extraction (Agilent Technologies, 2010).

Preparation of One-Colour Spike Mix

Heating blocks were equilibrated at 37°C, 65°C, 80°C, 40°C and 70°C.

RNase-free microfuge tubes and tips were used and volumes of at least 2 µl were used in the pipette to improve accuracy.

A dilution series of the One-colour Spike mix was then made and this was dependent on the starting amount of mRNA. A concentration of 75 ng total RNA was used.

The first dilution was prepared by labelling a new sterile 1.5 ml microcentrifuge tube "Spike Mix First Dilution". The One-Colour Spike Mix stock solution was vigorously mixed on the vortex mixer and heated at 37°C for 5 minutes, followed by a final mixing on the vortex. Then they were briefly spun on the centrifuge to drive the contents to the bottom of the tube. The 2 µl of the Spike Mix stock was added to the First Dilution tube and 38 µl of Dilution Buffer (provided in the Spike-In Kit) to a ratio of 1:20 (Agilent Technologies, 2010). This was then thoroughly mixed on the vortex and spun down quickly to collect all the liquid at the bottom of the tube. This tube then contained the First Dilution.

To make the second dilution, a new sterile 1.5 ml microcentrifuge tube was labelled "Spike Mix Second Dilution". Then 2 µl of the First Dilution is added to this tube and then 48 µl of Dilution Buffer to make a ratio of 1:25 as stated in protocol provided by Agilent Technologies. This is then thoroughly mixed on a vortex mixer and spun down quickly to collect all of the liquid at the bottom of the tube. This tube now contained the Second Dilution.

To make the third dilution, a new sterile 1.5 ml microcentrifuge tube "Spike Mix Third Dilution" was used. Then 2 µl of the second Dilution was added and then 38 µl of the Dilution Buffer to the ratio of 1:20. This is then thoroughly mixed on a vortex mixer and spun down quickly to collect all of the liquid at the bottom of the tube. This tube now contained the Third Dilution.

When this assay was conducted for the this project, batches of 24 and 48 samples were prepared, which meant an increased volume was needed as 2 µl of the Spike Dilution was needed for each labelling reaction, thus volumes of 48 and 96 µl were needed, rather than 38 µl. Therefore, final volumes of the Third Dilution of 60 and 120 µl were made. Instead of 2 µl of the second dilution and 38 µl of the Dilution Buffer (final volume 40 µl), for 24 samples, a volume of 3 µl of the second dilution and 57 µl of the Dilution Buffer (final volume of 60 µl) in the correct ratio of 1:20. For 48 samples a volume of 6 µl of the second dilution and 114 µl of the Dilution Buffer (final volume of 120 µl) in the correct ratio of 1:20.

The fourth dilution, as referred to in the protocol was not made up, because the starting total RNA was 75 ng and the fourth dilution pf Spike Mix is only required for $\geq$50 ng. A total RNA

amount of 50 ng was initially tried, but the labelling of cyanine 3 dye was poor; better results were gained using the amount of 75 ng.

The final step was to add 2 μl of the Third Dilution to 75 ng of sample total RNA, and to continue with the cyanine 3 labelling.

Preparation of labelling reaction

For each assay with the total RNA and diluted RNA spike in control cannot exceed 3.5 μl. Each batch of assays included 24 samples so the master mix was made up to 30 μl. The kit allows total RNA amount of between 10 to 200 ng. We started at 50 ng but the labelling was poor, so we increased the concentration of total RNA to 75 ng.

Total RNA of 75 ng was added to a 1.5 ml microcentrifuge tube in a final volume of 1.5 μl. The RNA samples were diluted with RNase free water. Then 2 μl of the diluted Spike Mix was added to each tube, resulting in each tube containing 3.5 μl.

This was followed by the preparation of the T7 Promoter Primer, the T7 Promoter Primer was mixed with water, the volumes of which were determined depending on the number of reactions been completed. In this instance 24 reactions were being carried out each time, therefore 24 μl T7 Promoter Primer was added to 30 μl of nuclease free water giving a final volume of the mixture of 54 μl. From this T7 Promoter Primer, 1.8 μl was added to each tube containing the RNA, giving a final volume of 5.3 μl. The primer and template were then denatured by incubating at 65°C for 10 minutes. Followed by 5 minute incubation on ice. Then the 5X first strand buffer was warmed at 80°C for 3 to 4 minutes to ensure that it was fully re-suspended, mixed using a vortex and spun down in the microcentrifuge, this was then kept at room temperature until required.

These steps were followed by the preparation of the cDNA Master Mix, the components of the master mix were mixed just prior to use, gently mixed using a pipette and kept at room temperature. The volumes used of the components of the master mix were again dependent on the number of reaction being performed. As 24 were being carried out as standard the volumes of each components added were as follows: 5X First Strand Buffer 60 μl, 0.1M DTT 30 μl, 10mM dNTP mix 15 μl and AffinityScript RNase Block Mix 36 μl (this is a blend of enzymes so had to mixed on ice). Before the addition of the cDNA Master Mix was added to the template and primer mix, these were spun down in a microcentrifuge to ensure the contents was at the bottom of the tube. Following on from this 4.7 μl of the cDNA Master Mix was added to each sample tube resulting in a final volume of 10 μl. These sample tubes

were then incubated at 40°C for 2 hours, followed by 15 minutes at 70°C. The samples were then placed on ice for 5 minutes and then spun down in the microcentrifuge.

The next step is the addition of Transcription Master Mix. As with the cDNA master mix the components have to mixed together just prior to being used, and as like previous mixtures, the volumes are dependent on the number of reaction being prepared. For making up 24 reactions the following volumes of each component were mixed together: nuclease-free water 22.5 µl, 5x Transcription Buffer 96 µl, 0.1 M DTT 18 µl, NTP mix 30 µl, T7 RNA Polymerase blend 6.3 µl (kept on ice and added to the mix just prior to use) and cyanine 3-CTP 7.2 µl. These components were gently mixed by pipetting at room temperature, then 6 µl of the Transcription Mix was added to each sample tube, mixed by pipetting, resulting in a final volume of 16 µl. The samples were then incubated for 2 hours at 40°C.

Purification of the labelled/amplified RNA

The first stage of this step was to add 84 µl of nuclease-free water to the cRNA sample which results in a final volume of 100 µl following this 350 µl of the Buffer RLT is added and mixed by pipetting, then 250 µl of ethanol was added and mixed again by pipetting. The 700 µl cRNA sample was then transferred to an RNeasy mini column in 2 ml collection tube. This was then centrifuged at 4°C for 30 seconds at 13 000 rpm, the flow-through discarded. Then 500 µl of the buffer RPE was added to the column, placed in the centrifuge at 4°C for 30 seconds at 13 000 prm and the flow-through discarded again. Another 500 µl was then added and placed in the centrifuge for 4°C for 1 minute at 13 rpm, and the flow-through discarded. The RNeasy column was then transferred to a new 1.5 ml collection tube and placed in the centrifuge at 4°C for 30 seconds at 13 000 rpm to remove any trace of the buffer. The collection tube was then discarded and a fresh tube was used ready to elute the cleaned cRNA sample into. Then 30 µl of RNase-free water was added directly on to the RNeasy column's filter, the tube was left for minute and then placed into the centrifuge at 4°C for 30 seconds at 13 000 rpm. The column was then discarded and the 30 µl of purified labelled RNA was safely in the 1.5 ml tube.
Quantification of the cRNA

The quantification step was carried using the Nanodrop and using the Microarray Measurement tab, RNA-40 as the sample type. The standard procedure for using the Nanodrop was used and 1 µl of sample was loaded onto the pedestal and the measurements recorded, specifically cyanine 3 dye (pmol/µl), RNA absorbance ratio (260 nm/280 nm) and the cRNA concentration (ng/µl). These measurements were used to determine the yield and specific activity of each reaction. The calculations are as follows:

Firstly, the concentration of cRNA (ng/µl) was used to determine the cRNA yield (µg):

$$\mu g\ of\ cRNA = \frac{(Concentration\ of\ cRNA\ )x\ 30\ \mu l\ (elution\ volume)}{1000}$$

Secondly, the concentration of cRNA (ng/µl) and cyanine 3 (pmol/µl) was used to determine the specific activity:

$$\text{pmol Cyanine 3 per µg cRNA} = \frac{Concentration\ of\ cyanine\ 3}{Concentration\ of\ cRNA} x1000$$

The recommended yields and specific activity are dependent on the microarray format, 1-, 2-, 4- or 8-pack (Figure 3.10). The microarrays being used in this study were 8-pack format. Therefore the recommended yield and specific activity were 0.825 µg and 6 pmol Cy3/cRNA, respectively.

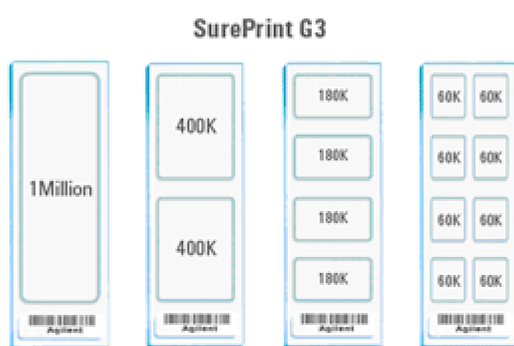

Figure 3.10 Illustration of the 1-, 2-, 4- and 8 pack Agilent microarray formats (Agilent Technologies, 2016).

Hybridisation

Following on from calculating the cRNA yield and the specific activity, the hybridisation step was started.

Preparation of x10 blocking agent

To prepare the x10 blocking agent, 500 µl of nuclease-free water was added to the vial containing lyophilised 10X Blocking Agent which was supplied with the Agilent Gene expression Hybridisation kit. This mix using the vortex and then placed in the centrifuge to drive the solution to the bottom of the tube, this can then be stored at -20°C for 2 months.

Preparation of hybridisation samples

For the 8-pack format microarray the following components were mixed together to make the fragmentation mix: 600 ng cyanine3-labeled, linearly amplified cRNA, 5 µl 10X Blocking Agent, make up to 24 µl with nuclease-free water and then add 1 µl of 25X Fragmentation Buffer to get the total volume of 25 µl. The volume needed to achieve 600 ng of cyanine3-labeled, linearly amplified cRNA is calculated using data collected in the previous step to determine the cRNA yield and specific activity. This mix was then incubated at 60°C for 30 minutes to fragment the RNA. It was then immediately placed on ice for 1 minute. Then a hybridisation mix is added, this is made up, again volumes of components are dependent on the microarray format, for the 8-pack microarray format, the following volumes of component were mixed together the 25 µl of cRNA from the Fragmentation mix and 25 µl 2x GEx Hybridisation Buffer HI-RPM, which will stop the fragmentation reaction. The two components should be mixed well using a pipette, but without introducing any bubbles. These were then spun in the centrifuge for 1 minute at 13 000 rpm, the sample was then kept on ice ready to load to load onto the array.

Preparation of the hybridisation assembly

A clean gasket slide was loaded into the Agilent SureHyb chamber base, then the 40 µl of the hybridisation mix was pipetted into one of the wells. Once all eight wells were filled the DNA microarray was placed array “active side” down onto the SureHyb gasket slide. Then the SureHyb chamber cover was placed onto the sandwiched slides and the chamber fastened was clamped on and the clamp tightened (Figure 3.11). Once assembled the slide chamber was placed in the chamber rotisserie in a hybridisation oven and was set at 65°C with the rotation set at 10 rpm. This was left to hybridise for 17 hours.

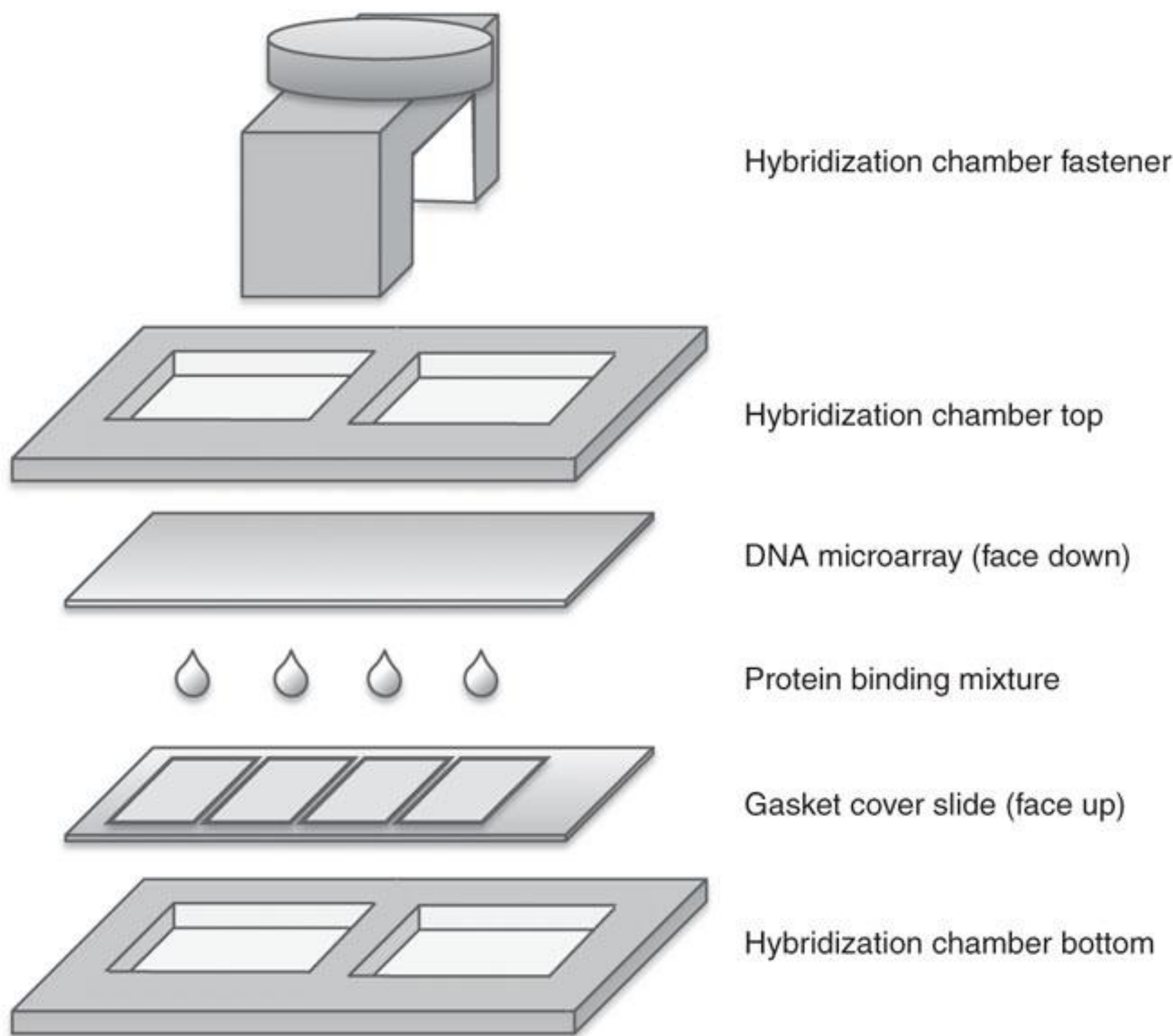

Figure 3.11 Schematic of Agilent SureHyb hybridisation chamber (Berger & Bulyk, 2009).

Microarray Wash

Once the hybridisation step is complete than the chambers need disassembling and the slides need to be washed. The wash procedure to prevent ozone degradation of the cyanine 3 was used. Firstly, the stabilisation and drying solution was warmed in water bath at 40°C, following complete dissolution of the precipitate the solution was allowed to stand at room temperature to equilibrate to room temperature. Secondly, the washes with stabilisation and drying solution was carried out, Table 3.5.

Table 3.5 Wash conditions as defined in the protocol (Agilent Technologies, 2010)

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	GE Wash Buffer 1	Room temperature	
1st Wash	2	GE Wash Buffer 1	Room temperature	1 minute

2nd Wash	3	GE Wash Buffer 2	37°C	1 minute
Acetonitrile wash	4	Acetonitrile	Room temperature	10 seconds
3rd Wash	5	Stabilisation and Drying solution	Room temperature	30 seconds

GE: Gene Expression

The wash steps were followed as per the protocol.

Scanning and Feature Extraction

Once the wash steps were completed the slides were scanned immediately as to minimise the impact of environmental oxidants on the signal intensities. The slides were place in holders which were then placed in the scanner carousel. The relevant settings were selected and once read the data were extracted. For each sample there was a QC Report which was checked to intensity reading, limited wash residue, that the QC spots are correct, that the data are normally distributed and that all the other parameters have been assessed as good or excellent.

The microarray data analysis is explained in detail in Chapter 6.

3.6 Passive Samplers

Polar Organic Integrative Sampler (POCIS) devices were deployed at the site. These were supplied by National Laboratory Services (NLS) part of the Environment Agency.

3.6.1 Deployment

A series of 8 back-to-back deployments were conducted. The first deployment started in mid-August 2011, and the last deployment was removed at the end of March 2012. Due to restrictions at the IPR pilot plant at Deephams, the passive samplers could only be deployed at the three stages of the treatment process: post final effluent (FE), post microfiltration (MF) and post reverse osmosis (RO), Figure 3.12 represents where, along the treatment process, the passive samplers were deployed, highlighted in red.

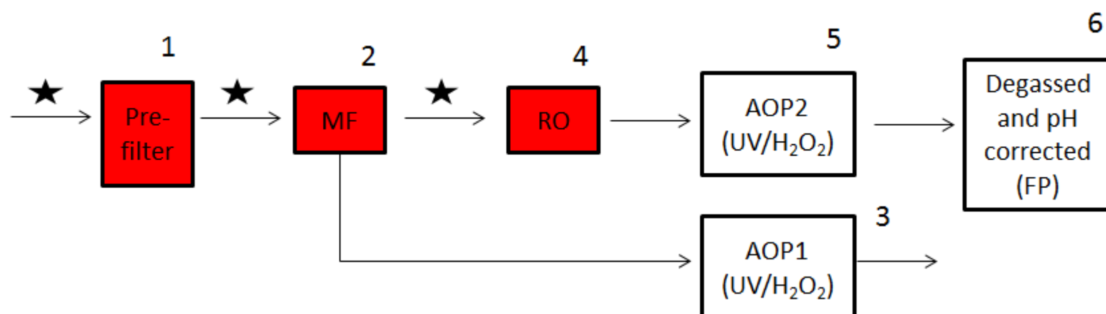

Figure 3.12 A diagram representing the IPR treatment process, the processes highlighted in red indicate where the passive samplers were deployed.

Ideally, samplers would have been placed after each part of the treatment process. Each deployment lasted approximately 4 weeks Table 3.6 indicates the schedule of the deployments.

Table 3.6 Dates, lengths and types of passive sampling deployments

Deployments	**Date deployed**	**Date removed**	**No. of days deployed**	**No. of weeks deployed**	**No. of locations**
1	17/08/2011	13/09/2011	27	3.9	3
2	13/09/2011	14/10/2011	31	4.4	3
3	14/10/2011	14/11/2011	31	4.4	3
4	14/11/2011	06/12/2011	22	3.1	3
5	06/12/2011	18/01/2012	43	6.1	3
6	18/01/2012	15/02/2012	28	4	3
7	31/01/2012	28/02/2012	28	4	3
8	28/02/2012	28/03/2012	29	4.1	3

The varying time periods of deployments are due to delivery mix ups and access to the Deephams site.

The samplers were delivered in cool-boxes via courier in the morning of the day of deployment, and taken from Brunel University to the IPR treatment site at Deephams sewage treatment works in the afternoon. On each day of deployment and collection, general observations of the site and weather were made, Table 3.7.

Table 3.7 Record of temperature and general site observations taken during passive sampler deployment and retrieval

Deployment	Date deployed	Temp. (°C)	General observations	Date removed	Temp. (°C)	General observations
1	17/08/2011	21	Bright and sunny, no exhaust fumes or similar	13/09/2011	19.5	Bright and sunny, no exhaust fumes or similar
2	13/09/2011	19.5	Bright and sunny, no exhaust fumes or similar	14/10/2011	18	Bright and sunny, no exhaust fumes or similar
3	14/10/2011	18	No engine fumes	14/11/2011	10	Grey and drizzle, no exhaust fumes or similar
4	14/11/2011	10	Grey and drizzle, no exhaust fumes or similar	06/12/2011	7.5	Clear and sunny, no exhaust fumes or similar
5	06/12/2011	7.5	Clear and sunny, no exhaust fumes or similar	18/01/2012	11	No exhaust fumes or similar
6	18/01/2012	11	No exhaust fumes or similar	15/02/2012	10	No exhaust fumes or similar
7	31/01/2012	2	No exhaust fumes or similar	28/02/2012	15	Slight odour of exhaust fumes, there was lots of work taking place at the STW adjacent to the IPR plant.
8	28/02/2012	15	Slight odour of exhaust fumes, there was lots of work taking place at the STW adjacent to the IPR plant.	28/03/2012	21	Clear and sunny, no exhaust fumes or similar

Deployment 2: The passive sampler from the post microfiltration ballast tank was removed for 40 hours during its deployment by the Thames Water staff on site, it was placed in chilled microfiltered product water in a sealed unit, while they were dosing with powered activated carbon (PAC) The device was placed back into the ballast tank afterwards.

Deployment would always start from “clean to dirty”; therefore, the samplers for reverse osmosis would be positioned first, followed by the microfiltration, and finally the final effluent. A flat headed screw driver was used to open the metal tins in which the sampler cages were stored. Once the samplers were open to the air, the blank disc was also removed from its foil and left on the side the entire time the test samplers were open to the air. The cage was tied with rope and lowered into the relevant ballast tank. The control disc was then covered with foil and placed in the on-site fridge. For each treatment process where there was a POCIS deployment, there were three POCIS discs with the addition of the blank.

The passive samplers were left in the relevant ballast tanks for approximately four weeks (Table 3.4). When their deployment was over, they were collected. During collection when the samplers were exposed to the air, the corresponding blanks were also exposed and left to one side. They were transported back to Brunel in chill ice boxes, the discs removed from the cages, and membrane discs were wrapped in ethanol rinsed foil and stored at -20°C and extraction has begun.

3.6.2 POCIS Extraction

The method for extraction of the Pharm-POCIS discs was modified from both Dr Frances Orton’s thesis (Orton, 2008) and the Environment Agency’s method of extracting POCIS using the solvent mix for pharmaceutical-POCIS (HLB-oasis sorbent) supplied via personal communication.

All glassware was acid washed before use, and then solvent rinsed before each use. There was sufficient glassware for sets of each item to be designated as control, reverse osmosis (1 to 3), microfiltration (1 to 3) and final effluent (1 to 3); therefore following their initial acid wash, between sets of deployments, a solvent rinse was carried out. The solvent rinse consisted of once with methanol and twice with ethanol, and then the glassware was left to dry (for at least 2 hours, but usually overnight). This included the glass vials, pipettes, burettes and flasks.

Four POCIS discs at a time were removed from the -20°C freezer and, the plastic bags they were stored in were opened to allow them to equilibrate to room temperature. Each disc was then removed from the bag, the foil wrapping was loosened, and they were left in the fume hood overnight to allow the media fully dry.

Ethanol rinsed foil was used to make small funnels, one for each disc. The nuts were loosened on the plates holding the membrane together, using an ethanol rinsed adjustable spanner, and the spanner was rinsed with ethanol between each disc.

Taking one disc at a time, the disc was placed on a piece of the ethanol rinsed pre-creased tin foil and the membrane of the disc was cut with a scalpel blade (Figure 3.13). The sorbent from the POCIS disc was then carefully emptied onto the tin foil. This was then emptied into a glass burette, a glass ethanol rinsed Pasteur pipette filled with methanol was then used to wash any residual sorbent from the foil into the burette (Figure 3.13). The advice received from the Environment Agency was to use a minimum amount of methanol to wash the foil and get the sorbent from the sides of the burette (ideally no more than 15 ml); a volume of 10 ml was used each time.

The sorbent and methanol mixture was allowed to mix and settle; it settled quickly with methanol. A timed period of 2 minutes was allowed for each, then the stopcock was opened and the methanol was allowed to drain into a 50 ml flask. The burette was fitted with a glass frit which prevented the sorbent from being washed down with the solvent.

Once the methanol had drained through completely, 10 ml of a solvent mix (dichloromethane:isopropanol:trifluoroacetic acid 80%:20%:0.1% (8 ml (8000 µl) DCM: 2 ml (2000 µl) IPA: (0.01ml (10 µl) of TFA)) was added to the sorbent, and allowed to mix and settle, (this mixture remains in solution). After 5 minutes, the stopcock was opened and the solvent mix was allowed to drain into the 50 ml glass flask.

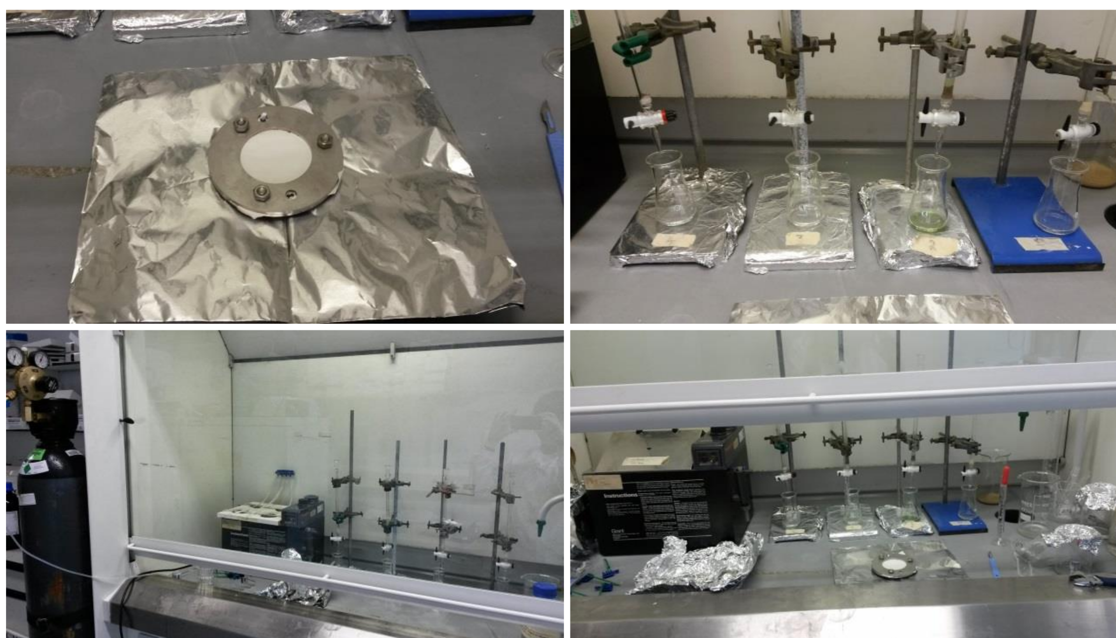

Figure 3.13 Pictures of the equipment and process used to extract from the POCIS discs. The top left is the POCIS disc on the pre-creased solvent rinsed foil just prior to cutting the membrane. The other three pictures show the extract being eluted from the sorbent in the burettes.

The four flasks were then placed in a water bath at 30°C under a nitrogen flow. The solvents were evaporated until approximately 5 ml remained. Each flask was swirled for 1 minute and

the 5 ml was transferred into an ethanol rinsed 10 ml glass vial with screw-top lid. The flask was then rinsed with 0.5 ml of ethanol to ensure all residue was collected. Then in the vials the solvent mixture continued to dry down under nitrogen using the SPE (Solid Phase Extraction) manifold.

Once the solvents had been evaporated (or when ~10 µl left likely to be the TFA), 1 ml ethanol was added, capped, wrapped with parafilm, and stored in fridge.

3.6.3 POCIS Extracts Analysis

A pooled 1ml sample of the POCIS extracts were sent to Centre for Environment Fisheries and Aquaculture Science (CEFAS) laboratories at Lowestoft. Pharmaceutical analysis was undertaken by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The method analysed the pharmaceuticals listed below with the limits of quantitation in brackets.

Ibuprofen (1 ng/l)

Dichlofenac (2 ng/l)

Paracetamol (2 ng/l)

Erythromycin (1 ng/l)

Sulfamethoxaole (2 ng/l)

Trimethoprim (1 ng/l)

Propanolol (1 ng/l)

Clofibric acid (1 ng/l)

Mefenamic acid (2 ng/l)

Dextropropoxyphene (2 ng/l)

Tamoxifen (1 ng/l)

Acetyl-Sulfamethoxazole (2 ng/l)

Clotrimazole (1 ng/l)

3.7 In vitro assays

3.7.1 Cell culture

SC5 juvenile mouse Sertoli cells (Hofmann et al., 1992) which were originally provided by Dr Ewa Raipert-de Meyts of the Copenhagen Rigshospitalet, Department of Growth and Reproduction, Denmark. The protocol was described by Kristensen et al. (2011) with slight modifications.

Media

The cell line used for the cell culture part of the project were SC5 (mouse juvenile Sertoli cells), as they produce a high quantity of prostaglandin D2. The media used to culture them was Dulbecco's modified Eagle's medium (DMEM - high glucose: Sigma D5796), with 10% heat inactivated foetal calf serum (FCS: place 50 ml aliquot in 60°C for 1 hour), 100 U/ml penicillin, 100 µg/ml streptomycin and 1mM L-glutamine. For 500 ml of DMEM the following was added; 50 ml heat inactivated FCS, 5 ml penicillin/streptomycin (Sigma P4333) and 5 ml L-glutamine (Sigma G7513), this was then referred to as full media.

Cell resurrecting

A batch of SC5 cells were removed from the liquid nitrogen store and defrosted in a water bath at 37°C. The cells are stored in dimethyl sulfoxide (DMSO), so, once defrosted, they have to be handled quickly to prevent degradation of the cells. Once defrosted, 1 ml of the cell suspension was transferred to 10 ml of full media, with some of the media being used to rinse out the tube the cell suspension was stored. The mixture of cells and media was then placed in the centrifuge for 5 minutes at 1000 rpm; while the cells were being spun, 8 ml of full media was added to a 75 cm^3 cell culture flask. The cells were then removed from the centrifuge and then as the cells were contained in a pellet at the bottom of the tube, the media now containing the DMSO was discarded, and 5 ml of fresh full media was added to the tube. The media was then pipetted up and down to re-suspend the cells; once the cells were re-suspended, the entire 5 ml cell suspension was added to flask containing 8 ml of full media, giving a final volume of 13 ml of media. The cells were then incubated at 37°C with 5% CO_2.

After 24 hours the media was changed, to ensure the removal of any residual DMSO. On the second day the cells were checked for normal growth and any signs of contamination. Once the cells have reached 60-70% confluence they were split/sub-cultured; if they were allowed to become $\geq$80% confluence they would stressed. Splitting the cells was carried out by removing the media and washing with 3 ml of Hank's Balance Salt Solution (HBSS), once

this was removed 2 ml of trypsin was added and left in incubator for approximately 2-3 minutes. In the two fresh 150 cm^3 cell culture flasks 19 and 18 ml of fresh full media were added to each. The trypsinated cells were then examined to ensure they were lifting from the surface of the flask and aggregated, then 8 ml of fresh full media was added to the flask; this acts by neutralising the trypsin. The cell suspension was then pipetted up and down to break up the aggregated cells, and 1 and 2 ml of the cell suspension were added to the clean flasks containing 19 and 18 ml of media, respectively, to make a final volume of media of 20 ml in each. The remainder of the cell suspension was discarded. The two different volumes of cell suspension were used to ensure there was sufficient cell growth, but a higher concentration can sometimes grow too rapidly and become fully confluent before there is time to split the cells. Splitting the cells is also known as sub-culturing or passaging; with this particular cell line they should not allowed to go past passage 29, as the cells start producing increased/decreased levels of prostaglandin, therefore the number of passages each batch of cells were on were recorded, and a new batch was resurrected when 29 passages were reached.

3.7.2 Prostaglandin Inhibition Assay

The Prostaglandin D_2-Methoxime (MOX) Enzyme Immunoassy (EIA) kit manufactured by Cayman Chemical Company was used. The protocol from the kit was followed but, briefly, the same full media was used as previously described in Section 3.7.1

Day1: Seeding cells in 24 well plates

The EIA plate allows 32 samples to be processed at a time. The cells should be checked and should be 70% confluent, then the cells were trypsinased as described before and 20 µl of the resulting cell suspension was taken for the haemocytometer. While the cells were being counted, the suspension was placed in the centrifuge for 5 minutes at 1000 rpm. The SC5 cells had to be at a concentration of 1x 10^5 cells/ml. Cell counts from the four corners of the haemocytometer were made, and an average number of cells calculated. The following calculation was used to determine the volume of cell suspension required:

$$\text{Cells per ml} = \frac{\text{Average number of cells in 4 grids counted}}{\text{Volume of the grid}}$$

$$\text{Total number of cells} = \text{Cells per ml} \times \text{Volume of cell suspesion}$$

$$\text{Final Volume (ml)} = \frac{\text{Total number of cells}}{\text{Target cell density}}$$

The cell suspension was used to seed two 24 well plates with 500 µl of cell suspension being placed in each well providing a subsequent 5x 10^4 cells per well concentration. The plates were then placed at 37°C with 5% CO_2 overnight to allow the cells to attach to the surface of the well plates.

Day 2: Treatment with chemicals

It was ensured, prior to starting, that clear plate plans had been created, ensuring wells for positive and negative controls, ibuprofen control, and treatment chemicals. Serial dilution of the test compounds and vehicle in full media were prepared, and known as treatment media. A negative control was prepared consisting of a 3 ml of 1% ethanol media. In addition to the negative control, a positive control was also prepared to test for cell toxicity, 3 ml of 2% triton (triton causes 100% cell death and the ethanol control would be assumed to cause 0% cell death, therefore the treatments would be expected to be somewhere in between these two controls) is used to normalise media. Ibuprofen was used as a positive control for the EIA itself. The 24 well seeded plates from the previous day were checked for cell growth, attachment and any signs of contamination; if satisfactory, the media from the wells was rapidly but gently removed, and 200 µl of full media was added to each well to prevent the cells from drying out. The 200 µl of treatment media was added to the relevant wells, consequently the test chemicals were now 200 times diluted, and each well contained 400 µl media. The plates were then returned to the incubator at 37°C with 5% CO2 for 18-24 hours.

	1	2	3	4	5	6
A	Blanks RO	Ibu 1	Ibu 5	RO 1	RO 5	EtOH
B	Blank MF	Ibu 2	Ibu 6	RO 2	RO 6	EtOH
C	EtOH	Ibu 3	Ibu 7	RO 3	RO 7	Blank Raw
D	Triton	Ibu 4	Ibu 8	RO 4	RO 8	Triton

	1	2	3	4	5	6
A	Blanks RO	MF 1	MF 5	FE 1	FE 5	Triton
B	Blank MF	MF 2	MF 6	FE 2	FE 6	EtOH
C	EtOH	MF 3	MF 7	FE 3	FE 7	Blank Raw
D	EtOH	MF 4	MF 8	FE 4	FE 8	Triton

Figure 3.14 An example of the plate plan for the seeded 24 well plates. Ibu 1-8 serially diluted ibuprofen acting as the positive EIA control; RO1-8: serially diluted extract from the POCIS sampler position post reverse osmosis; MF1-8: serially diluted extract from the POCIS sampler position post microfiltration; FE 1-6: serially diluted extract from the POCIS sampler position post pre-filtration also known as final effluent (FE); EtOH: ethanol blanks as negative controls; Blanks: the extracts taken from the blank POCIS discs at the different sampled point along the IPR plant; and Triton: the positive control for the cell toxicity assay.

Day 3: Prostaglandin D2-MOX EIA set up

The protocol provided by Cayman Chemicals was followed but, briefly, the procedure was as follows: initially the heating block and centrifuge were switched on and set at 60°C and 4°C, respectively. The MTT solution was removed from the freezer. Two sets of Eppendorf tubes were labelled for each sample and negative control. The EIA buffer from the kit was prepared by dissolving 10x concentrate EIA buffer in 90 ml ultra-pure water. The methyloximating reagent (MOX) was prepared by making up a 10 ml solution of 1:9

ethanol:water mix, and adding a small volume of it to the 0.1 g methoxylamine HCL, which was then vortexed and transferred to 0.82 g sodium acetate mixed well and added to the rest of the 10% ethanol solution. From this 10 ml solution 100 µl was added to one set of Eppendorf tubes and place in the fridge.

The prostaglandin D2 standard was then derivatised by diluting 100 µl standard with 900 µl Millipore water. Then, immediately after diluting the standard, a 1:1 solution of diluted standard and MOX were prepared and then heated in the block at 60°C for 30 minutes. This mixture was then 20 ng/ml standard, and could be stored in the fridge for one month and used in subsequent assays.

While the prostaglandin standard was on the heating block, 200 µl medium from the exposed cells was removed without disturbing them. This medium was transferred to the pre-chilled Eppendorf tubes and kept on ice. The remaining 200 µl media in the wells was left, and the plates returned to the incubator; this was in preparation for the MTT assay. The Eppendorf tubes were then pulsed on the centrifuge at 4°C, following the centrifuge, 100 µl medium was removed, leaving any debris at the bottom of the tube, and this 100 µl media was transferred to the chilled Eppendorf tubes containing the 100 µl MOX solution previously added These tubes were then heated at 60°C for 30 minutes. Once the tubes were heated for 30 minutes they were removed from the block and stored on ice until ready to use. While the samples were on the heating block the standard solution series was prepared, 200 µl of the derivatised standard (20 ng/l) was transferred to a new Eppendorf tube and diluted with 600 µl Millipore water, creating a stock concentration of 5 ng/ml. Then 8 Eppendorf tubes were labelled, with the first containing 950 µl full media, and the remaining tubes (2 to 8) containing 500 µl full media. The 5 ng/ml stock was then used for the serial dilution by adding 50 µl to the first tube, and then transferring 500 µl from the first to second, 500 µl being added from second to third and so on, mixing by pipetting up down between each dilution. By this point the treatment samples would have been ready and placed on ice. The D2-MOX AChE Tracer was then reconstituted; a whole vial of tracer was added to 30 ml of EIA buffer, which could then be stored in the fridge for one month to be used in subsequent assays. A volume of 6 ml of this tracer was used for each EIA plate, therefore 6 ml of this tracer was transferred into a separate tube and 60 µl tracer dye was added; this dye was added to the aliquot to be used on the day of the assay, not to the tracer stock. The D2-MOX EIA antiserum was also reconstituted in a similar way, the whole vial of antiserum was reconstituted in 30 ml of EIA buffer, and then could be stored in the fridge for one month. Again 6 ml of this was aliquoted out, and 6 µl of antiserum dye added to the aliquot. At this point it was time to prepare the EIA plate itself.

As with the 24 well treatment plates, a clear plate plan was created earlier, and 50 µl full media was added to the NSB wells, 50 µl EIA buffer was added to NSB and B0 wells, 50 µl of standard and samples were added to the relevant wells as per following the plan, and 50 µl of the tracer (except for the TA and blank) followed by 50 µl of antiserum (except for TA, blank and NSB). The plate was then sealed and placed in the fridge overnight.

1	2	3	4	5	6	7	8	9	10	11	12
ELISA blank	Std 1	Ibu 1	RO 1	MF 1	Raw 1	Raw 1	MF 1	RO 1	Ibu 1	Std 1	BO
NSB	Std 2	Ibu 2	RO 2	MF 2	Raw 2	Raw 2	MF 2	RO 2	Ibu 2	Std 2	BO
Etoh 1	Std 3	Ibu 3	RO 3	MF 3	Raw 3	Raw 3	MF 3	RO 3	Ibu 3	Std 3	POCIS blank Raw
Etoh 2	Std 4	Ibu 4	RO 4	MF 4	Raw 4	Raw 4	MF 4	RO 4	Ibu 4	Std 4	Etoh 4
POCIS blank RO	Std 5	Ibu 5	RO 5	MF 5	Raw 5	Raw 5	MF 5	RO 5	Ibu 5	Std 5	Etoh 5
POCIS blank MF	Std 6	Ibu 6	RO 6	MF 6	Raw 6	Raw 6	MF 6	RO 6	Ibu 6	Std 6	Etoh 6
Etoh 3	Std 7	Ibu 7	RO 7	MF 7	Raw 7	Raw 7	MF 7	RO 7	Ibu 7	Std 7	NSB
BO	Std 8	Ibu 8	RO 8	MF 8	Raw 8	Raw 8	MF 8	RO 8	Ibu 8	Std 8	ELISA blank

Figure 3.15 An example EIA 96 well plate plan. EIA blank: background absorbance caused by Ellman's Reagent; NSB: Non-Specific-Binding, non-immunological binding of the tracer to the well; B0: Maximum binding, the maximum amount of the tracer that the antibody can bind in the absence of free analyte; Std 1-8: the serially diluted prostaglandin D2 standard, Ibu 1-8 serially diluted ibuprofen acting as the positive EIA control; RO1-8: serially diluted extract from the POCIS sampler position post reverse osmosis; MF1-8: serially diluted extract from the POCIS sampler position post microfiltration; FE (which is labelled as RAW in the figure) 1-6: serially diluted extract from the POCIS sampler position post pre-filtration also known as final effluent (FE); Etoh: ethanol blanks as negative controls; and POCIS blanks: the extracts taken from the blank POCIS discs at the different sampled point along the IPR plant.

At this point the cell toxicity/MTT assay can be carried out, this is described below in section 3.7.3.

Day 4: Reading the EIA plate

Firstly, wash buffer was prepared by dissolving 5 ml concentrated wash buffer in 2 litres of purite water, and 1 ml of polysorbate was added. A vial of Ellman's Reagent was reconstituted into 50 ml Millipore water, and then the tube was covered with foil to protect the reagent from the light. The automatic plate water was used to empty the wells from the EIA plate, and it was rinsed five times with the wash buffer. With the use of a multichannel pipette, 200 µl of the reconstituted Ellman's reagent was added to each well. The plate was re-covered with the film lid and wrapped in tin foil to protect the reagent from the light. The covered plate was then placed on the plate shaker at speed 5, and left to develop for 96 minutes. Once the plate had developed, it was read on the spectrophotometer plate reader at 405 nm; the absorbances of the B0 wells should have reached a minimum of 0.3

A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5 the plate would have been washed again and fresh Ellman's reagent added, and left to develop again.

3.7.3 Assay for cytotoxicity/MTT assay

This assay was carried out during day 3 of the EIA. Initially the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was removed from the freezer, covered in foil, and left in the water bath at 37°C. The MTT solution was 5 mg/ml MTT in Phosphate Buffered Saline (PBS) or Hank's Balanced Salt Solution (HBSS), and was stored at -20°C. This MTT solution was used to make MTT medium, which was prepared into a 1 in 20 dilution of MTT in growth medium; 20 ml of this MTT medium was made each time (1 ml 5 mg/ml MTT solution into 19 ml of full media).

The cell culture/treatment media from each of the wells of both 24 well plates was gently removed, and 200 µl MTT medium was added. These were then incubated for 45 minutes at 37°C with 5% CO_2. Subsequently the MTT media solution was removed, and the cells in each well were washed with 200 µl HBSS. The HBSS was then removed, and the crystals that had formed in the bottom of the wells were then dissolved with 200 µl of dimethyl sulfoxide (DMSO). The plates were then covered with tin foil to protect them from the light and placed on a plate shaker for 20 minutes. The contents of each well were then transferred to a 96 well plate and read on the spectrophotometer plate reader at 570 nm and 620 nm.

The negative controls were normalised as 100%, and any well that fell below the 2 times standard deviation was considered to be toxic.

3.8 Additional *in vivo* exposures

3.8.1 NSAIDs exposures

Additional zebrafish embryos exposures were conducted using three of the most potent NSAIDs analysed on the prostaglandin inhibition EIA. The three NSAIDs tested were diclofenac sodium (Sigma-Aldrich, 99.9% Pharmaceutical secondary standard traceable to USP, PhEur and BP, certified reference standard), mefenamic acid (Sigma-Aldrich, $\geq$98%) and naproxen (Sigma-Aldrich, 99.9% Pharmaceutical secondary standard traceable to USP, PhEur and BP, certified reference standard).

Stocks and exposure concentrations

The chemical stocks were made in ethanol and relevant dilutions are described in Table 3.8.

Table 3.8 The concentration of the stocks for each of the NSAIDs

Stocks in ethanol	NSAIDs		
	Diclofenac sodium	Mefenamic acid	Naproxen
Master stock (µg/l)	30 000	30 000	50 000
Working stock 1 (µg/)	3000	300	1250
Working stock 2 (µg/)	300	30	500
Working stock 3 (µg/)	30	3	50
Working stock 4 (µg/)	3	0.3	5
Working stock 5 (µg/)	0.3	N/A	0.5

The master stock of each of the NSAIDs was used to make up the first working stock, which was then serially diluted resulting in 5 or 4 (in the case of mefenamic acid) working stocks. Each of these working stocks were used to make the final test concentration, which was done by pipetting 1 ml of each into a 100 ml Duran bottle. The 1 ml of working stock was then left in the fumehood to evaporate. Following evaporation of the ethanol, 100 ml of header tank water was weighed into the bottle, the bottle was then shaken vigorously and stirred with a magnetic stirrer.

Table 3.9 The concentration of the final exposures for each of the NSAIDs

Final exposure concentrations (ng/l)	NSAIDs		
	Diclofenac sodium	Mefenamic acid	Naproxen
Made from Working stock 1	30 000	3000	12500
Made from Working stock 2	3000	300	5000
Made from Working stock 3	300	30	500
Made from Working stock 4	30	3	50
Made from Working stock 5	3	N/A	5

A solvent control and header tank water were also used; the solvent control consisting of 1 ml of ethanol and being allowed to evaporate.

Zebrafish embryo exposure (72 hour)

The same zebrafish breeding regime used in previous exposures was followed, with the breeding adults being paired the day before the exposure was to start. Twenty four well plates were used, and 2 ml of the exposure media was placed into each well the day before the start of the exposure, and the plates were left in the incubator overnight. In the morning of start of exposure, the exposure media from the previous day was removed, and fresh media was added to each well. The plates were returned to the incubator to allow the correct temperature to be reached. There was a plate for each concentration, control (header tank water) and solvent control, and on each plate there were four control wells with header tank water. Basic physical chemical properties of the water were taken at the start of the study. These included dissolved oxygen, temperature (HACH probe), pH, general hardness, carbonate, nitrate and nitrite (API 5 in 1 test strips) and ammonia (API ammonia test strips). The temperature within the incubator was recorded with a thermometer to check on the spot and also continuous readings were taken using the two Tiny Tag data loggers.

	1	2	3	4	5	6	
A	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	A
B	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	B
C	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	Chemical exposure	C
D	Chemical exposure	Chemical exposure	Control	Control	Control	Control	D
	1	2	3	4	5	6	

Figure 3.16 Representation of the plate lay out. There was a plate with this lay out for each concentration of the NSAID and solvent control, and there was a plate where all 24 wells were control.

The embryos were sorted and one embryo per well was added. The plates were returned to the incubator, with daily observations being carried out on a daily basis until 72 hours post fertilisation. At 24 hours post fertilisation, the number of movements within a 20 second period were recorded for every surviving embryo. At 48 hours post fertilisation the number of heart beats per 30 seconds were recorded for every surviving embryo. At every observation, signs of developmental delay and malformations were made, photographs taken, and the

number of hatchings and number of mortalities recorded. At the 72 hour point, final observations were made and the embryos were preserved in RNAlater® stabilising solution, the normal looking ones being pooled together, and any abnormal looking embryos were separately stored. Each exposure for the individual NSAIDs was repeated twice.

3.8.2 Tyrosinase inhibitor exposures

Additional zebrafish embryos exposures were conducted using three tyrosinase inhibitors. The three tyrosinase inhibitors tested were 4-n-butylresorinol (supplied by TCI, >98%), kojic acid (supplied by Sigma Aldrich, ≥98.5%) and niacinamide (supplied by Sigma Aldrich, 99.7%).

Stocks and exposure concentrations

Table 3.10 The concentration of the stocks for each of the tyrosinase inhibitors

Stocks in double distilled water	Tyrosinase inhibitors		
	4-n-Butylresorinol	Kojic acid	Niacinamide
Master stock (mg/l)	100	500	10000
Working stock 1 (mg/)	N/A	50	N/A

Based on the desired final concentrations, and the fact that none of the tyrosinase inhibitors required to be dissolved in solvent, a serial dilution was not needed. The volume of the stock was varied in relation to the final concentration, with the final volume being made with water from the header tank, and made up to 500 ml in a larger Duran bottle. Again, these were then vigorously shaken and placed on the magnetic stirrer. There was also no requirement for a solvent control, so one control was used using solely header tank water. As there were limited data on the toxic concentrations, and known environmentally relevant concentrations, only three concentrations of the three chemicals were chosen.

Table 3.11 The concentration of the final exposures for each of the tyrosinase inhibitors

Final concentration of tyrosinase inhibitors		
4-n-Butylresorinol (µg/l)	Kojic acid (µg/l)	Niacinamide (mg/l)
10	5	10
100	50	100
1000	500	1000

Zebrafish embryo exposure (72 hour)

The same zebrafish breeding regime used in previous exposures was followed, with the breeding adults being paired the day before the exposure was to start. Crystallising dishes were used, with a capacity of 300 ml; 200 ml of the exposure media was placed into each dish on the morning exposure was due to start, and left in the incubator to acclimatise. There were two dishes for each concentration and control (header tank water). Basic physical chemical properties of the water were taken at the start of the study; these included dissolved oxygen, temperature (HACH probe), pH, general hardness, carbonate, nitrate and nitrite (API 5 in 1 test strips) and ammonia (API ammonia test strips). The temperature within the incubator was recorded with a thermometer to check on the spot, and also continuous readings were taken using the two Tiny Tag data loggers.

The embryos were sorted, and 50 embryos were added per dish. The dishes were returned to the incubator and observations were carried out on a daily basis until 72 hours post fertilisation. At every observation, signs of developmental delay and malformations were made, photographs taken, number of hatchings, and number of mortalities recorded. At the 72 hour point, final observations were made, and then preserved in RNAlater® stabilising solution; the normal looking ones being pooled together, and any abnormal looking embryos were separately stored. Each exposure for the individual NSAIDs was repeated twice.

CHAPTER 4: Investigating the Chemicals Remaining in the Product Waters Following Treatment

4.1 Introduction

During the running of the pilot IPR plant at Deephams waste water treatment plant, Thames Water had been taking twice weekly samples of the water from the various processes and sending them for chemical analysis. There are data for over 400 chemicals, which had been categorised by Thames Water into classes such as Endocrin Disrupting Chemicals (EDCs) and Pharmaceuticals, Pesticides, Organics, Inorganics, Nutrients and Microbiology.

4.1.1 Daily and seasonal fluctuations

Thames Water stated that the composition of the effluent from the Deepham's sewage treatment works did not show any alteration due to day of the week or season. However, it has been stated that there is an expected alteration in the composition during the day due to peaks in water usage, Table 4.1 shows the range of concentrations during the a 24 hour or 15 hour period for ammonium, Total Organic Carbon (TOC), Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD).

Table 4.1 Diurnal data supplied by Thames Water

Measurement	December 2009		June 2010	
	Minimum (mg/l)	Maximum (mg/l)	Minimum (mg/l)	Maximum (mg/l)
Ammonium as NH_4[a]	0.05	0.6	0.05	0.33
TOC[b]	8.8	12.7	0.4	13.1
BOD[b]	1.8	4.1	1	4.9
COD[b]	27.9	41	25.2	33.8

[a]From 1 am to midnight

[b]From 9 am until midnight

TOC: Total Organic Carbon

BOD: Biochemical Oxygen Demand

COD: Chemical Oxygen Demand

4.1.2 Categorising the contaminants

For this project the contaminants were further categorised and work examining the reduction of the contaminants based on their category was carried out (data not shown), to establish if there was a specific category of contaminant that was persisting within the IPR plant treatment process. I used very specific categories, for example pharmaceuticals were further categorised into their mode of action, for example Pharmaceutical-Beta blocker. Once these categories were established, literature searching was undertaken to determine specific

toxicological endpoints targeted by each contaminant that had been detected above the limit of detection (LoD) from at least one point along the treatment process. This was accomplished by taking a contaminant in turn and performing a literature search to determine if there are specific target organs and/or effects such as carcinogenicity, genotoxicity, reproductive and/or developmental effects and if it is endocrine disrupting. In addition to these potential human health impacts, certain physicochemical properties were noted as well as fate and behaviour and limited ecotoxicological data. Once these had been noted, a category was assigned to each endpoint. This was then used to determine if any product water from any point along the treatment process would have had the potential to exhibit a specific toxicological effect, and subsequently, a hypothesis could be formulated as to the likelihood of an effect that might be observed in the development of zebrafish and/or gene expression results.

Whilst completing the literature searching and the categorisation it became evident that many of the contaminants often had more than one potential endpoint.

4.1.3 Toxicological endpoints along the treatment process

Figure 4.1 below illustrates the frequency of pesticides being detected in the different product waters with a specific target organ and/or toxicological endpoint. It is important to note that one contaminant can have multiple health effects, and these data do not take into consideration potencies or the concentration at which they were detected, just that they are present. The figure shows, for example, that in final effluent there have been 34 individual pesticides detected above the limit of detection that caused an effect on the gastrointestinal tract and/or liver of experimental mammals. However, in the final product there were only 3 pesticides detected above the limit of detection that exhibit a similar effect. The figure shows that the number of pesticides with specific health outcomes is being reduced along the treatment process.

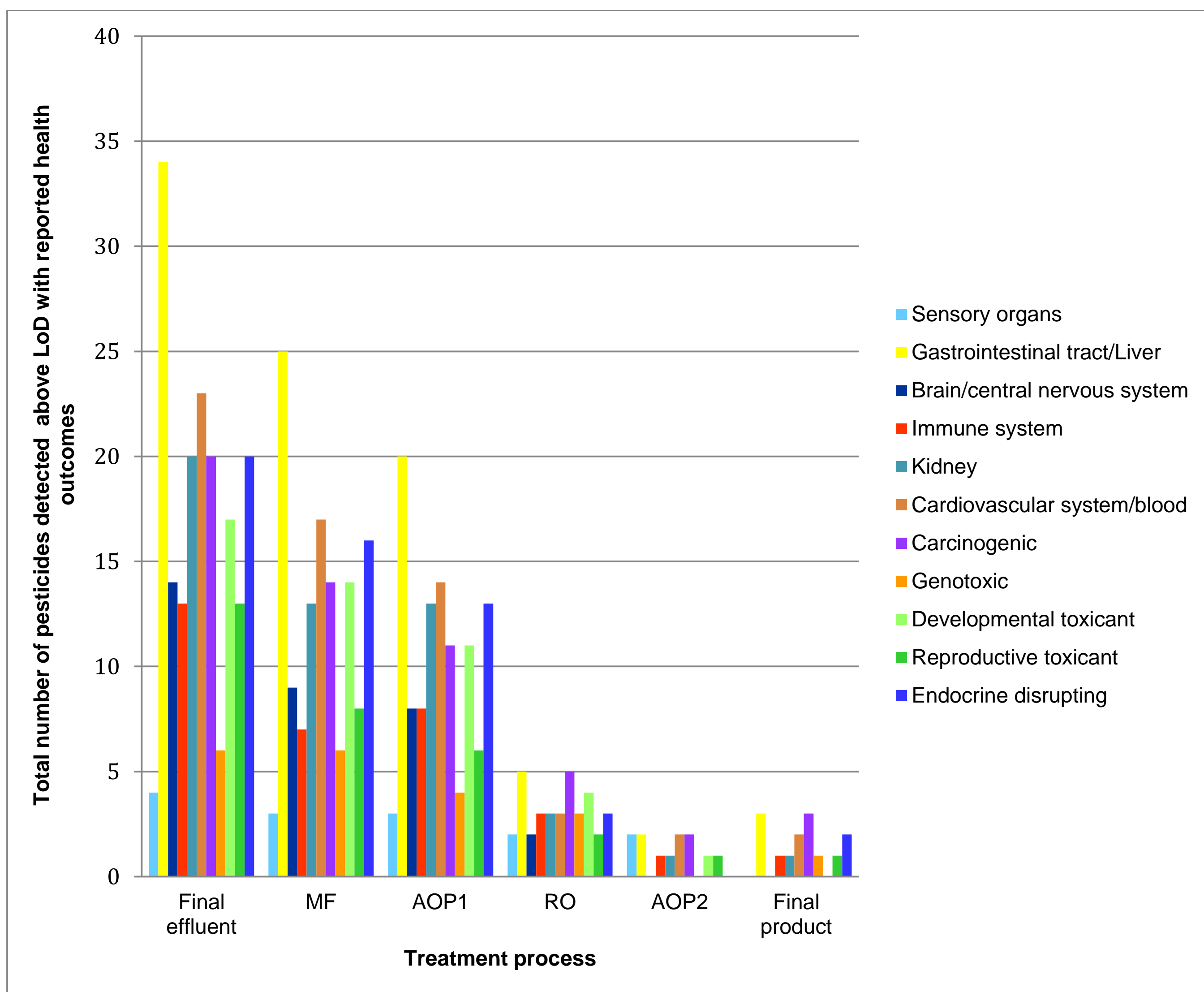

Figure 4.1 Total number of pesticides detected at concentrations above limits of detection at the various points along the treatment process, with reported health outcomes

4.1.4 Chemical analysis data on the aquarium water

Analysis was also conducted on the aquarium and tap water used as controls in the zebrafish embryo exposures. Samples were taken on three separate occasions in August 2013. The results of this sampling are displayed in the Appendix. The chemistry was organised by Thames Water Ltd.

4.2 Passive samplers

Passive samplers vary in design, but simply they are devices that enable continuous monitoring of contaminants, both atmospheric and aqueous. In the aqueous environment they remove the difficulties associated with sampling living organisms (Zhang et al., 2008) and provide a more representative sample of the contaminants that organisms are exposed

to. Contaminants are absorbed onto a membrane/material, allowing them to be extracted, which then enables them to be identified and sometimes (dependent on the membrane and target contaminants) a relative concentration of the contaminants can be determined or the extract can be used in exposure experiments and *in vitro* bioassays.

There are two standard passive samplers used in aquatic systems and these are Semi-Permeable Membrane Device (SPMD) and the Polar Organic Integrative Sampler (POCIS). There is also a third, type, but less widely used, the Diffusive Gradients in Thin Films (DGT). SPMD and POCIS are commonly used together due to them targeting different contaminants and combining the two increases the variety of contaminants sampled.

4.2.1 SPMD

SPMDs are designed to function in a similar way to the processes involved bioconcentration in an organism, but with the addition of the ability of enabling semi-quantitative to quantitative estimates of concentrations of hydrophobic organic chemicals (Huckins et al., 2006). SPMD is “an envelope of lay-flat low density polyethylene containing triolein, a major component of fish lipid” (Environment Agency, n.d.). SPMD will absorb hydrophobic compounds present in the water; these have log Kow of >3 but are not bound to sediment, such as PAHs, PBCs, organochlorine pesticides and synthetic pyrethroids. Only contaminants that are dissolved in the water and are bioavailable will be absorbed by the device (Environment Agency, n.d.). It is reported that a standard SPMD will remove the contaminants present in the equivalent of approximately 1 to 4 litres of water per day (Environment Agency, n.d.).

4.2.2 POCIS

POCIS consist of a solid receiving hydrophobic phase (sorbent) with two hydrophilic microporous polyethersulfone (PES) membranes either side (Zhang et al., 2008). POCIS are reported to absorb a complimentary array of hydrophilic organic chemicals, as SPMD does not absorb compounds with a log Kow <3. POCIS absorbs compounds such as pharmaceuticals, steroid hormones and veterinary medicines (Environment Agency, n.d.). As with the SPMD, the POCIS will only absorb compounds that are dissolved and bioavailable, unbound to the sediment (Environment Agency, n.d.).

The absorption of hydrophobic compounds to the sorbent is prevented by the membrane, and only the hydrophilic compounds that are able to pass through the membrane consequently absorb and accumulate on the sorbent (Environment Agency, n.d.).

4.2.3 DGT

The DGT samples ionic species from the water, and as with the SPMD and POCIS, only dissolved and bioavailable compounds will be absorbed (Environment Agency, n.d.). DGT is used to sample toxic metals such as nickel, copper, chromium, cadmium and zinc and phosphate (Environment Agency, n.d.).

4.2.4 Deployments

At the Deephams IPR plant, pharm-POCIS devices were deployed. The extracts could then be used for subsequent in vitro assays.

CHAPTER 5: Identifying Apical Endpoints in Developing Zebrafish Embryos Exposed to the Different Product Waters from the IPR Treatment Process

5.1 Hypothesis

As previously stated, due to the complex chemical composition of the different product waters from the Indirect Potable Reuse (IPR) plant, and therefore (by interference) the many possible biological activities and potential effects, this project was non-hypothesis led at the start. Therefore, the main research question to address initially was whether any biological effects could be observed following exposure to the various product waters from the IPR plant at different treatment stages, and secondly, whether the treatment process itself could remove or enhance these effects. Specifically for this stage of the research I was interested in finding out if any obvious developmental malformations or other apical endpoints occurred following exposure to the different product waters from the IPR plant.

5.2 Aims and Objectives

5.2.1 Aim

To determine if product waters from the different treatment stages of the IPR plant caused observable effects on developmental endpoints.

5.2.2 Objective

Expose zebrafish embryos to each of the product waters from the IPR plant, including final effluent (FE), microfiltration (MF), advanced oxidation following MF but in the absence of reverse osmosis (AOP1), reverse osmosis (RO), AOP following MF and RO (AOP2) and final product (FP). Observe the embryos during their development at defined developmental time-points, recording survival, hatching, heart rates and abnormalities. Compare these measured endpoints to zebrafish embryos reared in purified aquarium water (referred to as Control) and tap water.

5.3 Introduction

5.3.1 Test Organism

Embryos from the zebrafish, *Danio rerio* Tübingen strain, were used as the test organism for all the studies described in this chapter. A detailed description of the zebrafish as a model organism and their use in research is detailed in the General Introduction, section 2.7.1.

5.3.2 Fish Embryo Toxicity (FET) Test

As part of environmental risk assessments, there is a regulatory requirement to test chemicals, pesticides, biocides and pharmaceuticals in the 96 hour acute fish toxicity test,

and initially the fish embryo test was seen as a potential replacement for this test (Scholz et al., 2008). In the 96 hour acute fish test (OECD 203), sub-lethal effects are not considered, and only the LC50 (Lethal Concentration for 50% of the individuals in the test group) is reported. Therefore this test is likely to underestimate the potential adverse long-term environmental effects, and short-term mortality is only specifically useful in the case of accidental spills (Nagel, 2002). Additionally, the acute fish test uses large numbers of adult fish and puts them under a significant degree of pain and suffering as well as being time consuming and costly. Consequently, there has been increasing pressure to find relevant alternative(s). Also, it is difficult to derive an LC50 for a complex and largely undefined mixture of chemicals.

Lange et al., (1995) compared the suitability of using zebrafish embryos in toxicity testing as a replacement for the acute fish test using adults. They also compared cytotoxicity assay in the RTG-2 cells (Rainbow Trout Gonad), where the cell viability was tested using Neutral Red uptake, and the MTT assay. They found that for the majority of the 10 compounds tested, the zebrafish embryo test was more sensitive than the acute fish test using adult zebrafish (Lange et al., 1995). In that study it was also noted that the embryo test was more sensitive for all 10 compounds tested compared to the RTG-2 cell cytotoxicity assay (Lange et al., 1995). In Germany, the fish embryo test has been adopted as a routine test to determine the toxicity of wastewater effluents, with results that are comparable to those of the acute toxicity fish tests using adult fish (Scholz et al., 2008).

The Organization for Economic Co-Operation and Development (OECD) has issued a number of guidance documents for the fish embryo test (FET). The first draft of the OECD guideline of the FET was submitted to the OECD in 2006 and was approved in 2013 following review (OECD, 2006, 2013). The OECD draft FET test guidelines specify the fish species to be the zebrafish (*D. rerio*), but include the caveat that the method could be adapted for fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and other relevant species of interest (OECD, 2006). However, in the finalised revised version of the guidelines, only zebrafish are specified (OECD, 2013).

The zebrafish embryotoxicity test (ZET) is widely used, but there are still reported to be inter-laboratory differences in its responses, and there is a lack of standardisation in a number of key areas. Variations include factors such as the specific developmental endpoint(s) that are considered and the developmental stages at which endpoints are measured, whether cytotoxicity is considered or not, and differences in approaches to determine exposure to the test compound (i.e. within the embryo or in the water), if carried out at all (Beekhuijzen et al., 2015). Key points in the experimental design still vary greatly in the literature; for

example variations exist as to when the start of the exposure occurs, from ≤2, 6-8 or 24 hours post fertilisation (hpf) (Beekhuijzen et al., 2015; Hermsen et al., 2011; Selderslaghs et al., 2012). Beekhuijzen et al. (2015) discussed the use of a standardised starting point, and suggested that this should be comparable with that of mammalian developmental toxicity studies in rats and rabbits. In these mammalian developmental toxicity studies, maternal exposure is started following the completion of implantation of the embryo also referred to as the gastrulation period (OECD, 2001). In zebrafish embryos this would correspond to 5¼ hpf (Kimmel et al., 1995). Beekhuijzen et al. (2015) argue that this standardised approach will enable the results from both zebrafish embryo studies and mammalian developmental studies to be compared and more effectively used in predictive models.

Another variation in experimental design is the temperature at which the embryos are reared. The updated OECD guidelines for the FET test, OECD 236, state a temperature of 26°C (OECD, 2013). However, 28.5°C has also been reported to be the best temperature to rear zebrafish embryos in order for accurate developmental staging (Kimmel et al., 1995; Westerfield, 2007). It is also stated that zebrafish embryos can tolerate a temperature range of 24 to 33°C (Westerfield, 2007). Beekhuijzen et al. (2015) compared some results of toxicity studies at both 26°C and 28°C, and concluded that at 28°C there was increased mortality whereas at 26°C there were an increased number of malformations. At the temperature of 26°C the rate of development is reduced, and therefore the reduced number of malformations at 28°C might be due to a greater developmental rate which effectively means that the embryos spend less of their time being exposed to the chemical during specific windows of development. Embryos raised at 26°C develop more slowly, which increases the duration of exposure at each developmental stage, thereby potentially increasing their sensitivity to teratogenic effects (Beekhuijzen et al., 2015). It was also highlighted that at a higher temperature evaporation of the exposure media would increase, potentially increasing the concentration of non-volatile test compounds in the media (Beekhuijzen et al., 2015). Beekhuijzen et al. (2015) concluded that using a temperature of 26°C would increase the predictability of the zebrafish embryo assay, but that more research was required to determine an optimum temperature. Certainly, being aware of these experimental differences, combined with the knowledge of how they could potentially affect the experimental outcome, leads to a lack of confidence in the reliability of data when comparing results derived from exposures adopting slightly different temperatures. For my exposures, a temperature range between 27 and 28°C was used, based on information reported in a number of credible sources (Kimmel et al., 1995; Westerfield, 2007).

There is also debate over whether it is better to remove the chorion (egg shell) when exposing zebrafish embryos. The chorion covers the embryo until hatching between 48 and

72 hpf (Kimmel et al., 1995). The chorion is a permeable shell consisting of pore canals which are between 500-700 nm in diameter (Gustafson et al., 2012). It is considered that for certain compounds, the chorion might act as a barrier and prevent or reduce the embryo itself from being exposed to the test compound, especially larger compounds which would be unable to pass through the pore canals. Therefore, some researchers remove the chorion whereas others do not, as it is time consuming to do so, consequently reducing the advantage of the assay being high through-put. More importantly, some researchers have found that dechorionated embryos have increased mortality and abnormality rates compared to chorionated ones (Beekhuijzen et al., 2015; Selderslaghs et al., 2012). Subsequently, the results obtained from the differing procedures are not comparable. During an inter-laboratory study for the harmonisation of the zebrafish developmental assay Gustafson et al. (2012), determined that the chorion did not prevent the uptake of any of the 20 compounds tested. Additionally, silver nanoparticles have been reported to pass through the pore canals of the chorion (Gustafson et al., 2012). However, Selderslaghs et al. (2012) reported on two studies whereby it was confirmed that the chorion of the zebrafish embryo acted as a barrier to a cationic polymer (Luviquat HM 522) and thalidomide, whereby teratogenic effects were only observed following removal of the chorions. However, the second treatment process used at the IPR plant was microfiltration and microfiltration is reported to remove particles between the size of 0.05 and 5 µm (Gray, 2010). Consequently, with post treatment with microfiltration and beyond it, chemical contaminants still present in the treatment water would be of the size range to pass through the pores of the chorion.

Different researchers report using different test chambers. For my research, 24 well plates and 300 ml glass crystallising dishes were both used, depending on the specific experimental outcome being measured. From the literature, 96 and 24 well plates are widely used, and the OECD 236 guidelines state that 24 well plates should be used (OECD, 2013). It has been reported that within the well of a 96 well plate there is insufficient room for the embryo to move and that skeletal malformations in the form of tail kinks have been observed at an increased frequency compared the frequency in embryos raised in 24 well plates (Selderslaghs et al., 2009). However, the authors of that research also observed a greater number of skeletal malformations at the 144 hpf time-point compared with embryos at 55 and 72 hpf, but still with a greater number in the smaller wells of the 96 well plate (Selderslaghs et al., 2009). Also, each 96 well has a reduced volume compared to wells of a 24 well plate (360 µl and 3000 µl, respectively), potentially causing a reduced dissolved oxygen concentration in the smaller well over the length of the exposure and increased evaporation over time. As the majority of testing for this project consisted of using treated sewage effluent, there was the need to consider the possibility for depletion of dissolved

oxygen, especially with the product water from final effluent and microfiltration, and therefore the 24 well plates were favoured. The well plates are of particular benefit when observing the zebrafish embryos for malformations or delays in development. One embryo was added per well, enabling individuals to be monitored throughout the entire period of the exposure. Also, should death occur, the decomposing embryo in a well remains isolated from the other embryos. Dishes were used when larger numbers of individuals were required to produce a pooled sample for the genomic work. Due to the large number of dishes required, they were placed into heated water baths as opposed to the incubator.

Further examination of the variations in experimental design adopted by different research groups showed the length of exposure time to be variable also. The OECD 236 now specifies a period of 96 hpf (OECD, 2013). The draft version of OECD FET test released in 2006 stated an exposure time of 48 hours (OECD, 2006). In Germany, zebrafish embryos are exposed for 48 hours in their standardised whole effluent testing, which was established in 2002, and made mandatory in 2005 (Braunbeck & Lammer, 2005). When Schulte & Nagel (1994) developed a test design for the zebrafish embryo assay, they stated a exposure time of 48 hours, with the assumption that pain and suffering could be avoided up to that time point. According to The Animals (Scientific Procedures) Act 1986, fish in the embryonic form are classified as a protected animal from the stage at which it becomes capable of independent feeding. For zebrafish this is after 96 hpf, and can be determined by the complete resorption of the yolk sac.

The method of exposure varies among researchers; flow-through is always the preferred method for the majority of aquatic toxicity studies, allowing a more stable exposure to the target concentration and keeping the dissolved oxygen level at a steady state. However, it is not possible to use the standard flow-through methods used for adult fish in large tanks and flowmeters, due to the small size of the embryos and the need to examine development under a microscope on a daily basis. Consequently, usually a static or semi-static/renewal system is applied. The OECD FET test guideline (OECD 236) recommends that the test media should be refreshed on a daily basis for compounds that are susceptible to degradation over a 96 hour period (OECD, 2013). The static approach is also favourable in terms of cost and time, and was therefore used for all the described experiments reported in this chapter.

Variations in the FET test also occur in choice of dose range and use of solvents. For the exposures described in this chapter a dose range was not required, as it was decided that the embryos should be exposed directly to the different product waters. As no additional chemicals were directly added to the IPR plant treatment waters, no solvent was required.

The use of solvents in the FET assay will be discussed later, when direct chemical exposures were carried out.

Test Validity

The OECD 236 stipulates the following criteria to classify a FET as valid (OECD, 2013):

- Overall fertilisation of eggs to be $\geq$70%.
- Water temperature to be maintained at 26±1°C.
- The overall survival of embryos in the control group to be $\geq$90% for the length of the 96 hour exposure.
- Use of a positive control (e.g. 3,4-dichloroaniline) and for the embryo survival rate within this group to be at a minimum of 30% at the end of the 96 hour exposure.
- The hatching rate in the negative control should be $\geq$80% at the end of the 96 hour exposure.
- Dissolved oxygen in the negative control and highest test concentration should $\geq$80% saturation at the end of the 96 exposure.

5.3.3 Exposure during development/Sensitive Window

The embryonic and foetal stages are particularly sensitive to chemical exposure because the stages of embryogenesis are easy to disrupt. Disruption to these early developmental stages can cause death, malformation, growth retardation and functional disorders (Timbrell, 2001). Disruptions to development can cause organisational and functional effects to the organism which will persist throughout life. Two clear mammalian examples of this are illustrated by the consequences of developmental exposure to two pharmaceuticals: diethylstilbestrol and thalidomide. WHO/UNEP (2013) reviewed the case of diethylstilbestrol (DES). DES is a potent synthetic oestrogen that was widely used between the 1940s and 1970s in Europe and in the US as a treatment for pregnant women to prevent/reduce the risk of miscarriages. Initially it was only prescribed to women deemed to be in a high risk group, but later was more widely distributed, and it use was claimed to improve the health of babies. Consequently, the numbers exposed were vast (WHO/UNEP, 2013). DES was found to be ineffective at preventing miscarriages and significant health effects were observed in the children of the exposed mothers, so its use was discontinued. It was associated with a rare type of vaginal cancer in a low percentage (<0.1%) of adolescent daughters who were exposed to it during *in utero* development. These adverse effects were only observed in the offspring, and not in the mothers themselves, and also the effects were not observed until the children had entered adolescence. This delay in the manifestation and discovery of the adverse health effects meant that *in utero* exposure to DES continued unabated for many years, and the numbers of exposed children increased dramatically as a consequence.

Following this discovery of a low level of cancer, DES exposure to daughters *in utero* was associated to more frequent (approximately 90-95%) benign reproductive problems including reproductive tract malformations and dysfunction, miscarriage, pre-term delivery and low birth weight, ectopic pregnancies and premature labour and births. When DES exposure occurred *in utero* it was found to change the normal genetic programming that acts on differentiation of the reproductive tract (WHO/UNEP, 2013). Effects were not only observed in the daughters but also in the reproductive system of sons, who displayed increased risks of developing cancer, namely testicular germ cell cancer (WHO/UNEP, 2013).

Another example is thalidomide, which was used extensively in the late 1950s as a treatment in pregnant women for morning sickness. It was found to cause severe birth defects in the offspring in the form of reduced limb size and malformed limbs. Contrary to DES, however, the adverse effects were not observed later in the child's life, for limb malformations were observed from birth, but again without causing any maternal toxicity. Along with the limb malformations, thalidomide was also linked to congenital heart disease, malformation of the inner and outer ear, and ocular abnormalities (J. H. Kim & Scialli, 2011).

5.3.4 Endpoints

As previously mentioned, the precise choice of endpoints selected during zebrafish embryo assays will be dependent on the main research aim and hypothesis.

Schulte & Nagel (1994) separated the toxicological endpoints from a 48 hour exposure for the zebrafish embryo into two distinct groups, lethal and specific mode of action, which are illustrated in Table 5.1.

Table 5.1 Classification of identified endpoints in the 48 hour zebrafish embryo toxicity test (Schulte & Nagel, 1994).

Lethal endpoints	Endpoints related to specific mode of action
Coagulation of the embryo	Reduced number of somites
Gastrulation not initiated	Absence of circulation
Non completion of gastrulation	Absence of eyes
Absence of somites	Absence of spontaneous movement after 24 hours
No extension of the tail	Significantly reduced heartbeat
Absence of heartbeat	Absence of otolith
Absence of spontaneous movements after 48 hours	Absence of pigmentation

Survival

The simplest determination of lethality in a zebrafish embryo at any stage of development is coagulation. However, Table 5.1 details that at the 48 hpf there are number of other observations which, if made, also indicate death of the embryo. Lethality is a simple and straightforward endpoint to assess, and can indicate general toxicity as well as adverse developmental effects.

Hatching

Zebrafish embryos reared at 28.5°C hatch from their chorions between 48 and 72 hpf, but this depends on the thickness of the chorion and the muscular activity of the embryo itself (Kimmel et al., 1995). Taking this into consideration, it is feasible to assume that if a compound reduces the movement of the embryo, then hatching will be delayed and vice versa. Release from the chorion can also occur from handling of the embryos, therefore care must be taken when handling the embryos as so not to skew hatching data.

Heart Rate

Due to the transparent nature of the eggs, the heartbeat of a zebrafish embryo should be visible by 48 hours and therefore the heart rate can be measured. Heart rate was reported to be an important sub-lethal endpoint (Nagel, 2002).

Abnormalities

Abnormalities (determinants of developmental toxicity) can be classified using some of the criteria in Table 5.1 and in more detail by including the information displayed in Table 5.2.

This was the case in a study conducted to determine if a chemical's physical properties, in this instance BCF or Log P, can be used to predict its toxicity and developmental effects. This was done by comparing LC50 data of 133 chemicals against the relevant physical properties. The researchers found that BCF and LogP were not predictive of a chemical's biological effect; however, developmental effects could be predicted using the abnormalities themselves. For example pericardial oedema and yolk sac oedema are more reliable predictive endpoints of whether a compound exhibits developmental toxicity or not (Ducharme et al., 2013).

Whilst developing a zebrafish embryo teratogenicity assay, Brannen and colleagues would classify embryos as non-viable if any of the following endpoints were noted at the 5 day post fertilisation (120 hpf) stage; no heartbeat, all or part of the body degraded, and/or growth retardation/malformation so severe that short-term survival would be highly unlikely (Brannen et al., 2010). They then scored the viable larva at the 5 day post fertilisation stage based on body length, motility, cardiovascular function, pigmentation and morphology of most anatomical structures (Brannen et al., 2010). These were then scored 1 to 5 based on severity, in order to generate a quantifiable approach to assessing the abnormalities. In a related study, the following morphological endpoints of 5 day old zebrafish larva were assessed: upper jaw, lower jaw, anterior lower jaw, pharyngeal arch, forebrain, midbrain, hindbrain, eyes, otic capsules, heart, swim bladder, yolk, intestine, notochord, somites, tail and fins. These were then scored 0.5 to 5, with 5 being normal in appearance and 0.5 being grossly malformed. From these scores they determined a NOAEL and teratogenic index (LC25/NOAEL), which was used in a predictive model (Panzica-Kelly et al., 2010). In addition, further endpoints (including body length, swim bladder inflation, pigmentation, liver and stomach morphology) that were particular to specific developmental stages, or as indicators of unusual types of toxicity, were included. These endpoints were not used to determine the NOAEL, but were included to determine whether any abnormalities observed may be due to a delay in development or were caused by direct effects upon the morphology, thereby subsequently impacting endpoints used to determine the NOAEL (Panzica-Kelly et al., 2010). The scoring system firstly classified the severity of the abnormality, after which it then classified the abnormality according to whether it was due to developmental delay or an irreversible severe effect (Panzica-Kelly et al., 2010).

Table 5.2 Endpoints that are observed and the various significant time-points during zebrafish development that, if present or absent, can indicate a disruption to normal development and is therefore categorised as an abnormality. Some of these endpoints have been determined as lethal, sub-lethal or teratogenic. " * " indicates that the endpoint will be observable at that specific time-point (modified from (Braunbeck & Lammer, 2005; Nagel, 2002; Schulte & Nagel, 1994).

	8 hours	24 hours	48 hours	120 hours
Lethal endpoints				
Coagulation	*	*	*	
Tail not detached		*	*	
No somite formation		*	*	
No heart-beat			*	
Sub lethal/developmental endpoints				
Completion of gastrula	*			
Formation of somites		*		
Development of eyes		*	*	
Spontaneous movement		*	*	
Heart-beat/ blood circulation			*	
Heart-beat frequency			*	
Pigmentation			*	
Formation of oedema			*	
Endpoints of teratogenicity				
Malformation of head		*	*	
Malformation of sacculi/otoliths		*	*	
Malformation of tail		*	*	
Malformation of heart		*	*	
Modified chorda structure		*	*	
Scoliosis		*	*	
Rachitis		*	*	
Yolk deformation		*	*	
General growth retardation		*	*	
Length of tail				*

Abnormalities occurring during development of an organism are often caused by teratogens. Teratogens are defined as "any agent that physically or chemically alters developmental processes and produces structural deformities in an organism" (Conley & Richards, 2010). In general, teratogens do not cause toxicity to the mother, but specifically target a certain developmental stage of the embryo, causing a deformity (Timbrell, 2001). These deformities are commonly referred to as birth defects. In the past only gross anatomical abnormalities were classified as being a teratogenic effect. However, more recently teratogenic effects

have been understood to include all morphological abnormalities, including subtle effects observed in behaviour, biochemistry and learning (Conley & Richards, 2010). As teratogens impact different developmental processes, the timing of exposure is crucially important to the overall developmental effect (Timbrell, 2001). Teratogens differ from mutagens because teratogenic effects are not heritable, as they disrupt the somatic cells rather than the germ cells. Mutagens cause alteration in the germ cells, causing genetic disruptions that are passed on to future generations. Additionally, mutagens can cause mutagenicity at any stage of life of an organism, conversely teratogens can only exert an effect during the developmental stage (Conley & Richards, 2010).

The severity of an abnormality caused by exposure to a teratogen is directly related to the specific point during development at which the organism is exposed, the duration of exposure and dosage (Conley & Richards, 2010). Teratogenic effects can lead to malformations, functional disorders and growth retardation. If these effects are severe, the endpoint that is measured is death, and consequently teratogenic effects can go unreported (Conley & Richards, 2010).

In the environment, teratogens are divided into three categories: mechanical disruptors, environmental factors, and chemical contaminants. Mechanical disruptors include abnormalities caused by parasites forming cysts on a developing limb, or predators removing a limb that then regenerates with a deformity due to external factors such as abrasion from sand and gravel. Environmental factors include variables such as ultra violet (UV) radiation, oxygen levels, temperature and pH. UV radiation can directly disrupt cells of a developing organism, but can also transform certain chemical contaminants via photolysis into potent teratogens (Conley & Richards, 2010). A number of chemicals have been identified as being teratogens, some of these are classed as xenobiotics (substances which have no essential biological function) and others are classed as essential substances, such as some metals and nutrients, but at an inappropriate dose (too little or too much) can cause a teratogenic effect (Conley & Richards, 2010).

5.4 Material and methods

Three sets of developmental exposures were carried out, all using slightly different experimental designs depending on their ultimate purpose. The three sets of exposures were intended for developmental observations, an extended exposure and exposures specifically for gene expression analysis. The methodologies for each type of exposure are detailed in Chapter 3; any deviations from that are detailed below.

5.4.1 Developmental observation

Exposures specifically intended for developmental observations were carried out during December 2011 and February 2012, and were independently repeated four times. The second exposure experiment was, however, stopped after 24 hours due to the high mortality rate in all treatments and control.

For the first developmental exposure it was not possible to collect a composite sample post final product, due to unplanned maintenance work at the IPR plant. However 24 hour composite samples were prepared from the other five treatment processes, and a spot sample was taken of the final product water. In this exposure, one 24-well plate was used per treatment, with 4 wells on each plate dedicated to the control (n=20 total for each treatment) Tap water was not included as a treatment in this exposure.

In the second observation there were operational issues at the IPR plant and again it was not possible to collect composite samples from all the treatment processes, and therefore spot samples were collected instead.

In the third observation, composite samples were collected from all treatment processes except for AOP2, where a spot sample was taken because an insufficient number of auto-samplers were present on-site at the time of sample collection. The exposure/plate configuration as detailed in Chapter 3, Section 3.4.2 was used.

In the fourth observation, 24-hour composite samples were collected for all 6 treatment processes. The 8 hour post fertilisation (hpf) time point was missed out due to a fault in the microscope causing the 4 hpf observation point to take several hours. For both the third and fourth observation studies it was not possible to travel to the laboratory to carry out the observation at the 36 hour time-point.

5.4.2 Extended embryo exposures

These exposures were repeated four times, but in the third and fourth exposures the tap water and final product water were omitted because a reduced number of zebrafish embryos than expected were obtained.

5.4.3 Gene expression exposures

These exposures were repeated three times, although in the second experiment final product and AOP2 treatment water samples of the IPR plant were unavailable due to a fault at the site. However, there was limited time left for the plant before decommissioning, so it was decided to carry out the exposure in case the fault remained until the end of the trial.

5.5 Results

5.5.1 Survival

None of the treatments significantly affected survival of the embryos over the 0 to 48 hour time period compared to the aquarium water control. The OECD 236 guidelines state there is a requirement for ≥90% survival in the controls for the test to be a valid OECD test, whereas survival in the control group typically ranged from 66.67 to 70.83% with an average survival of 68.75% ±2.08 (mean ± SD). However, a number of variations were tried including use of an embryo media, but the survival rate within the controls and treatments remained variable. This could have been due to temporal variations in the quality of embryos, and/or small variations to water quality from one experiment to the next.

The survival rate was analysed individually for the three types of exposures (48 hour developmental observations, 48 hour gene expression and the extended exposure) as they were carried out slightly differently and for different endpoints, but they were also combined. Figure 5.1 displays the average percent survival from all exposures over 48 hours, as all zebrafish embryos, regardless of exposure purpose, were observed for a 48 hour time period, with observations being made at regular time-points (4, 8, 12, 16, 24, 36 and 48 hours post fertilisation).

The control group survival rate in the first observation after 48 hours was only 67% with no other visible signs of abnormal development in any of the embryos. This was also true for the third and fourth exposures. In all three exposures, between 36 and 48 hpf very few (if any) additional embryo deaths were observed in any of the treatments. The majority of deaths occurred between 4 and 12 hpf, irrespective of treatment.

Figure 5.2 displays the percent survival data at 48 hpf from three of the four developmental observations (see Section 5.4.1). Experiment 2 was omitted due to operational issues at the plant. These data at the 48 hour time point were used in statistical analysis to allow comparison with survival in the extended and gene expression exposures (Figure 5.3 and Figure 5.4). Both Figure 5.1 and Figure 5.2 suggest that there were no obvious differences in the survival rates in relation to the treatment water the embryos were exposed to. Univariate analysis of variance using SPSS confirmed that there was no significant difference in the survival of the embryos at 48 hours based on the treatment water they were exposed to (P. value >0.05). Additionally, there was good consistency in the survival measured in all three exposures (1, 3 and 4) (P. value >0.05).

Figure 5.3 displays the mean percent survival of the embryos exposed to the different IPR product waters at the 48 hour time-point in the extended exposure studies. In each case

there were three replicates, and the exposures were repeated four times. In the figure it can be observed that there was a large difference in survival between the different exposures. The second exposure had the largest survival rate, with the third exposure having the lowest level of survival out of the four exposures. However, as with the initial developmental exposures, the different treatment waters produced quite consistent survival rates across the different treatments within each exposure. This was tested using univariate analysis of variance in SPSS and it was found that there was no significant difference in the survival of the embryos at 48 hours based on the treatment water to which they were exposed (P. value >0.05). However, as is evident from Figure 5.3, there was a significant difference between the number of surviving embryos between the different exposures (P. value <0.05).

Figure 5.4 displays the mean per cent survival in the groups of embryos sampled at 48 hours in the gene expression exposures. There were two replicate dishes for each treatment, and each exposure was repeated independently on three separate occasions. The survival rate was lower overall in this type of exposure compared to the other two types of exposures (Figure 5.2 and Figure 5.3). Univariate analysis of variance revealed a significant effect on the survival of the embryos (P. value 0.041). Using the LSD post-hoc test, these differences were present between the following treatments; control and tap (P. value 0.035), control and AOP1 (P. value 0.033), tap and final effluent (P. value 0.022), final product and final effluent (P. value 0.017), AOP2 and final effluent (P. value 0.041), reverse osmosis and final effluent (P. value 0.005) and AOP1 and final effluent (P. value 0.002). This would indicate that the survival rate in the final effluent was significantly reduced relative to most of the other treatment waters from the IPR plant. However there was no significant difference between per cent survival in the final effluent and the control (P. value >0.05). Additionally, the different exposures (1, 2 and 3) did not significantly impact the survival (P. value >0.05).

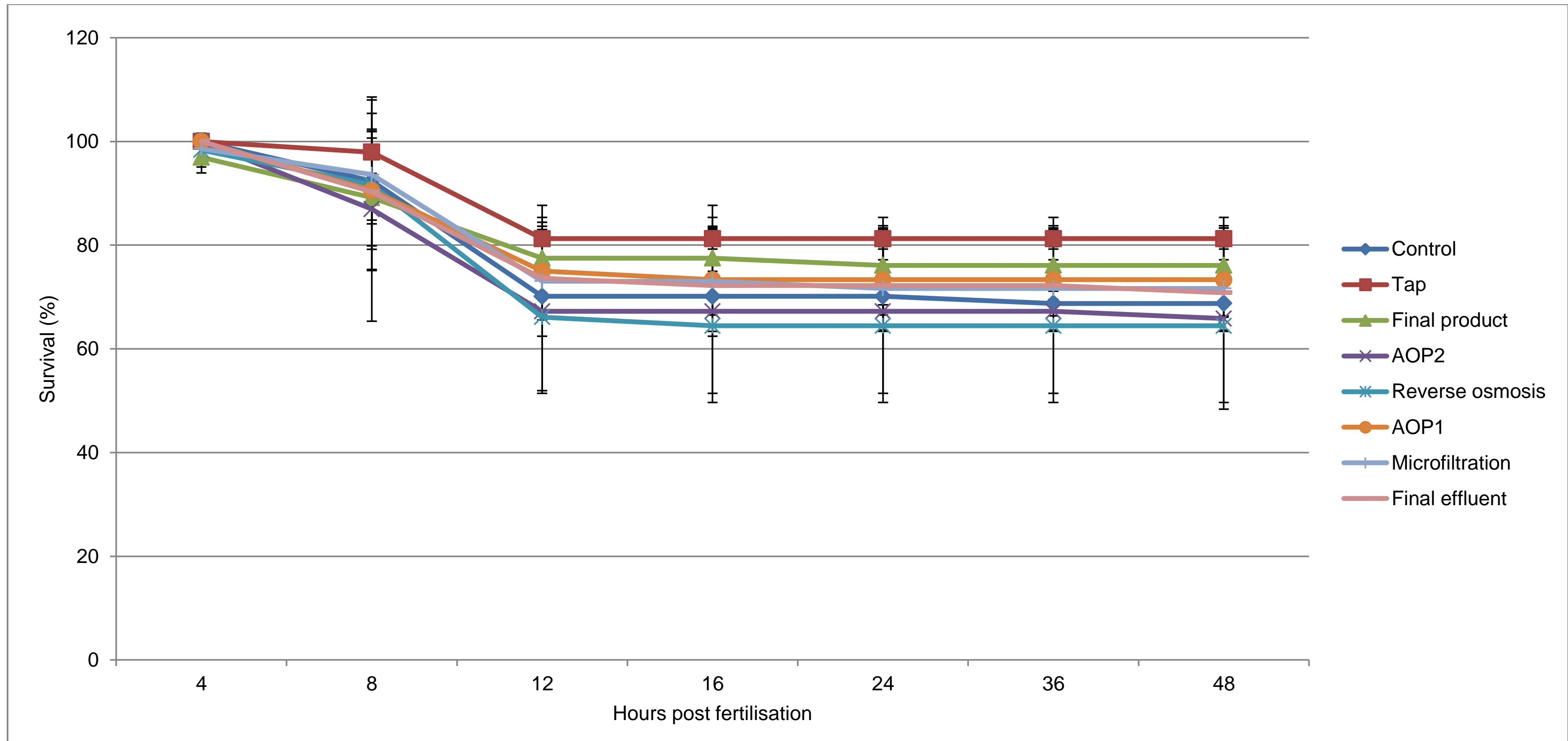

Figure 5.1 Average percent survival from all 48 hour developmental observation exposures, showing the 95% confidence intervals.

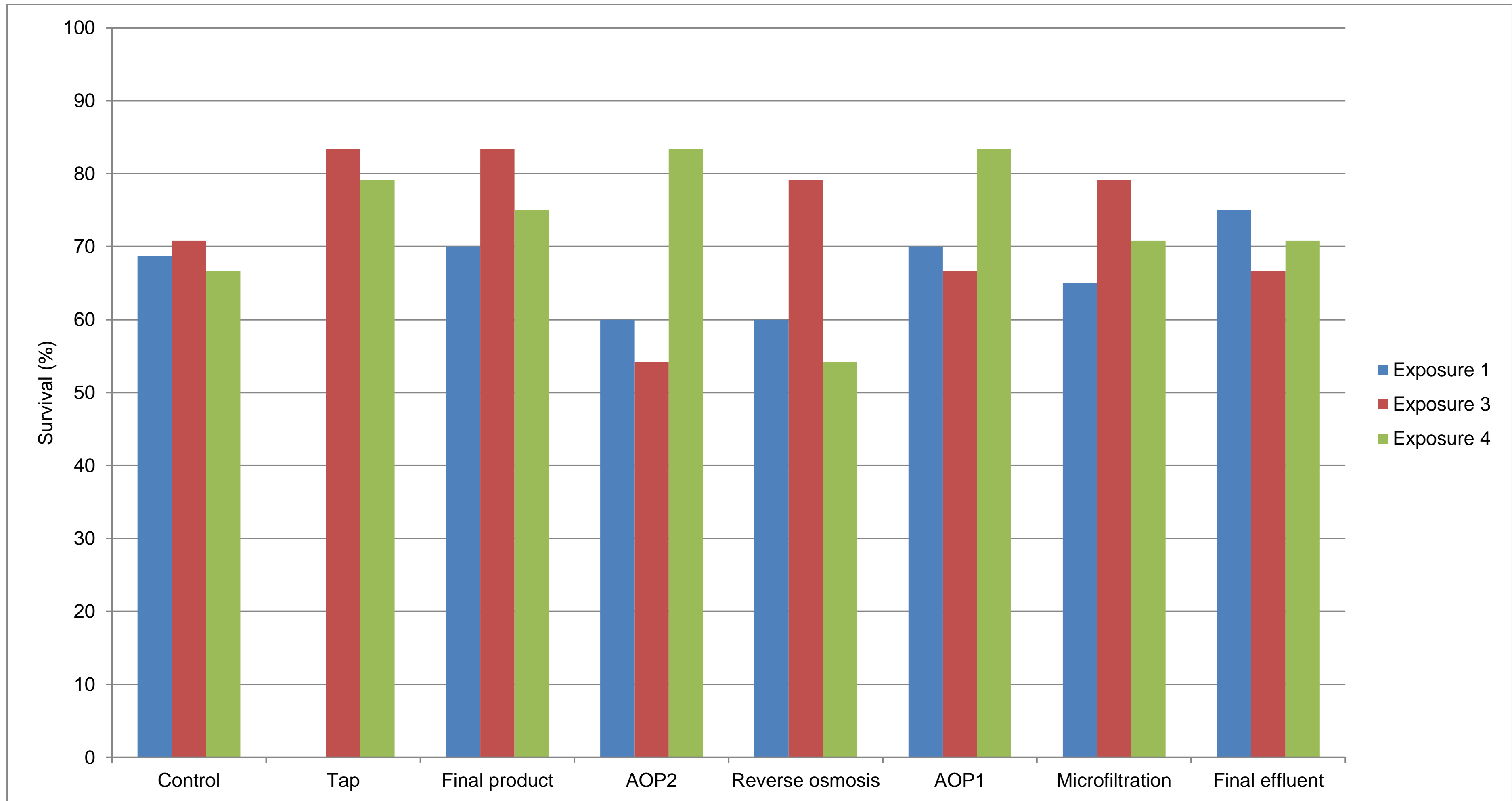

Figure 5.2 Survival (%) observed in the first 48 hours of the developmental exposures. The exposures were repeated four times (Experiment 2 is not included due to high mortality) and in Exposure 1 no tap water treatment was tested.

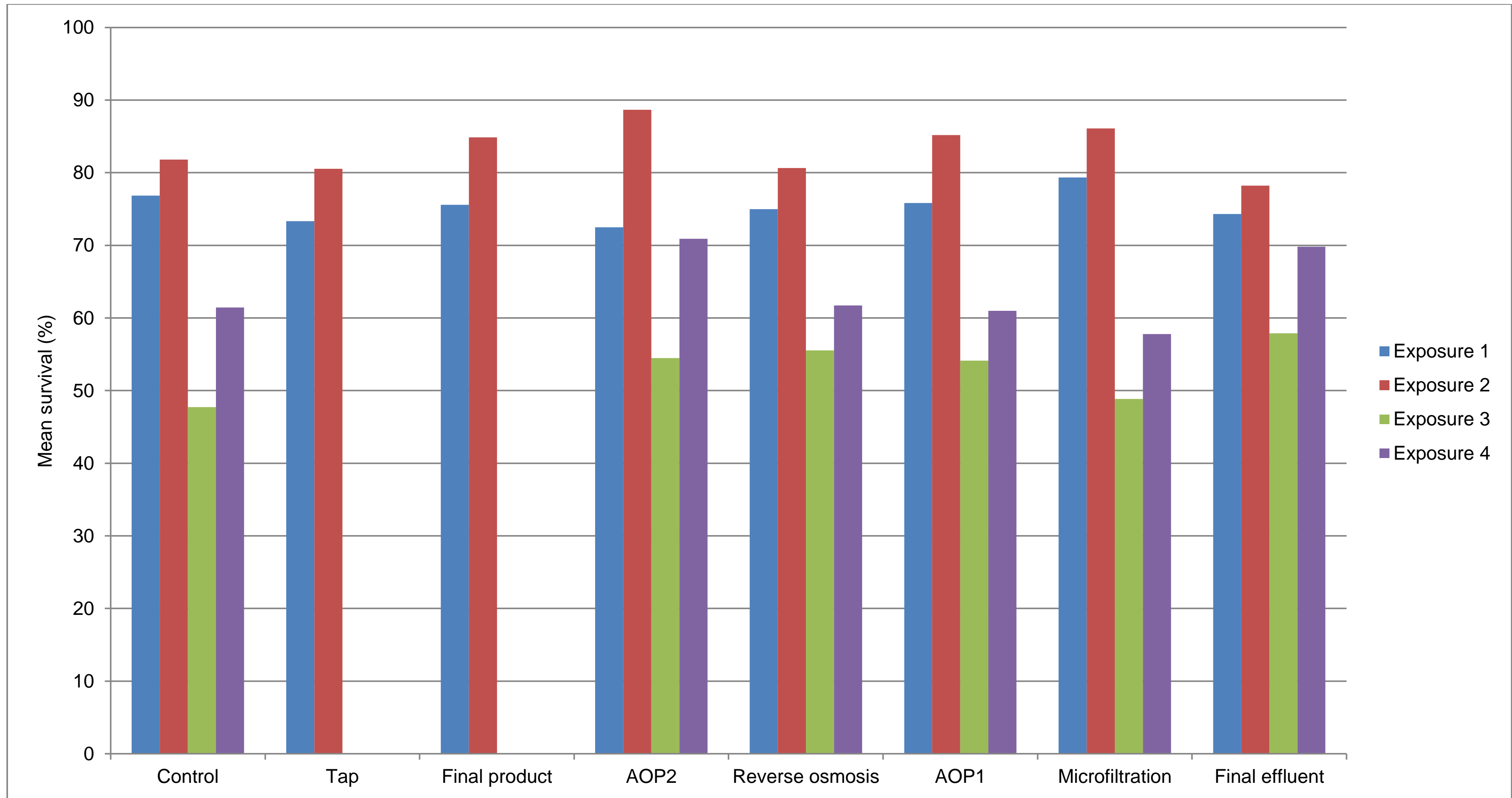

Figure 5.3 Survival (%) observed in the first 48 hours of the extended exposures. The mean was calculated from the three replicates for each treatment, and the exposures were repeated four times. However in the third and fourth exposures, no tap or final product treatments were used.

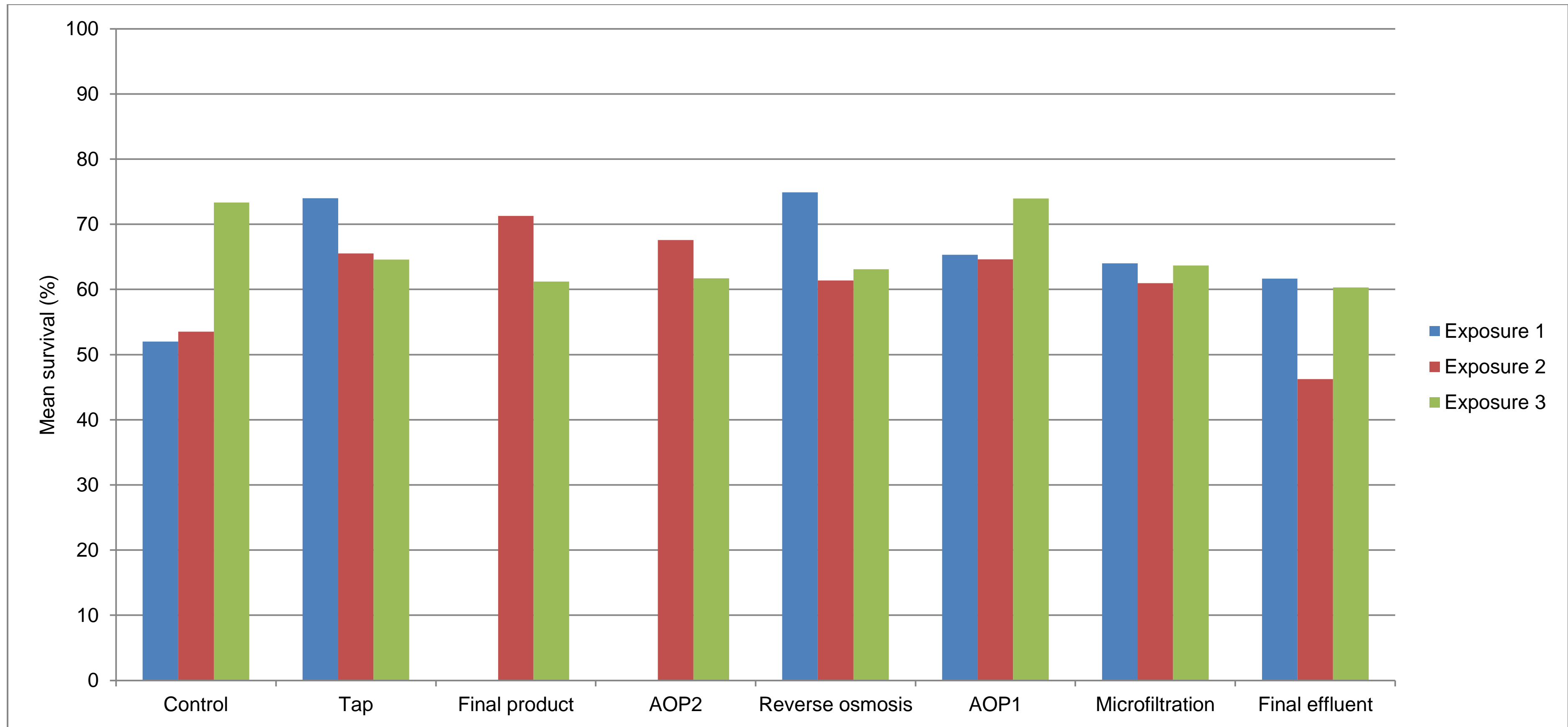

Figure 5.4 Survival (%) observed in 48 hour group from the gene expression exposures. The mean was calculated from the two replicates for each treatment, and the exposures were repeated three times. However, in the first exposure, final product and AOP2 treatments were not available from the IPR plant, so these groups had to be omitted.

5.5.2 Hatching

The numbers of hatched embryos were recorded at 48hpf in the developmental exposures and the extended exposures. It was not possible to record the number of hatched embryos at 48hpf in the gene expression exposures as sampling was extremely time dependent, and priority was given to recording the numbers and types, if any, of malformed embryos.

Figure 5.5 shows the mean number of hatched embryos at 48 hours in Experiments 1, 3 and 4 of the initial developmental exposures. The 95% confidence intervals indicate a large variation in the numbers of embryos hatching in all treatments and control waters when comparing all three exposures. In Figure 5.6, hatching results for the first exposure were removed and data from just the last two exposures were examined. The decision to omit Experiment 1 from the analysis was taken on the basis that this was the first embryo exposure that I conducted in my research, and the hatching data seemed largely at odds with the subsequent experiments. The confidence intervals for these data are now much smaller and the results seem a lot more consistent; embryos reared in the control water do seem to have a reduced hatching rate compared to the other exposures, and reverse osmosis, final product and microfiltration all had similar numbers of hatched embryos that were increased in number compared to the control.

An initial chi-square test was conducted on frequency of hatched embryos reared in the different waters and it was determined that there was an association between treatment and the number of hatched embryos at 48 hours in all three experiments (observation 1, $X^2 = 21.31$, df = 6, P value = 0.0016 (<0.05); observation 3 $X^2 = 14.08$, df = 7, P value = 0.0497 (<0.05); and observation 4 $X^2 = 28.52$, df = 7, P value = 0.0002 (<0.05)).

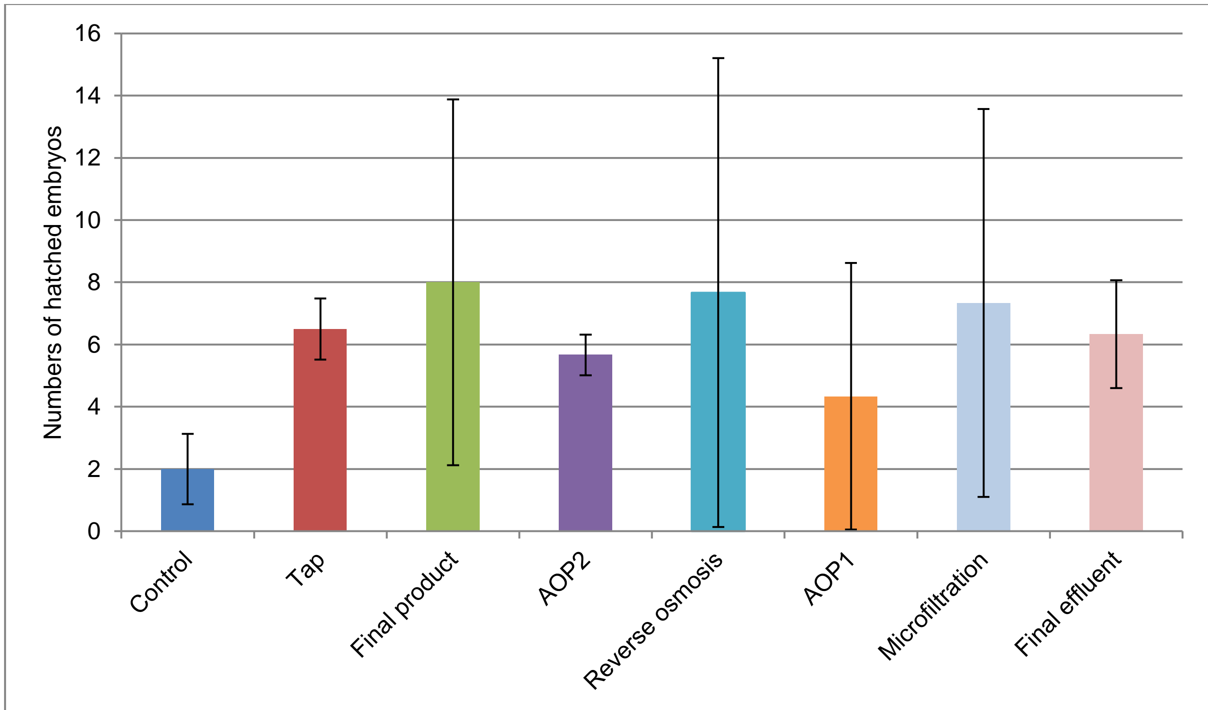

Figure 5.5 Average numbers of hatched embryos at 48 hours in developmental Exposures 1, 3 and 4 with 95% confidence intervals.

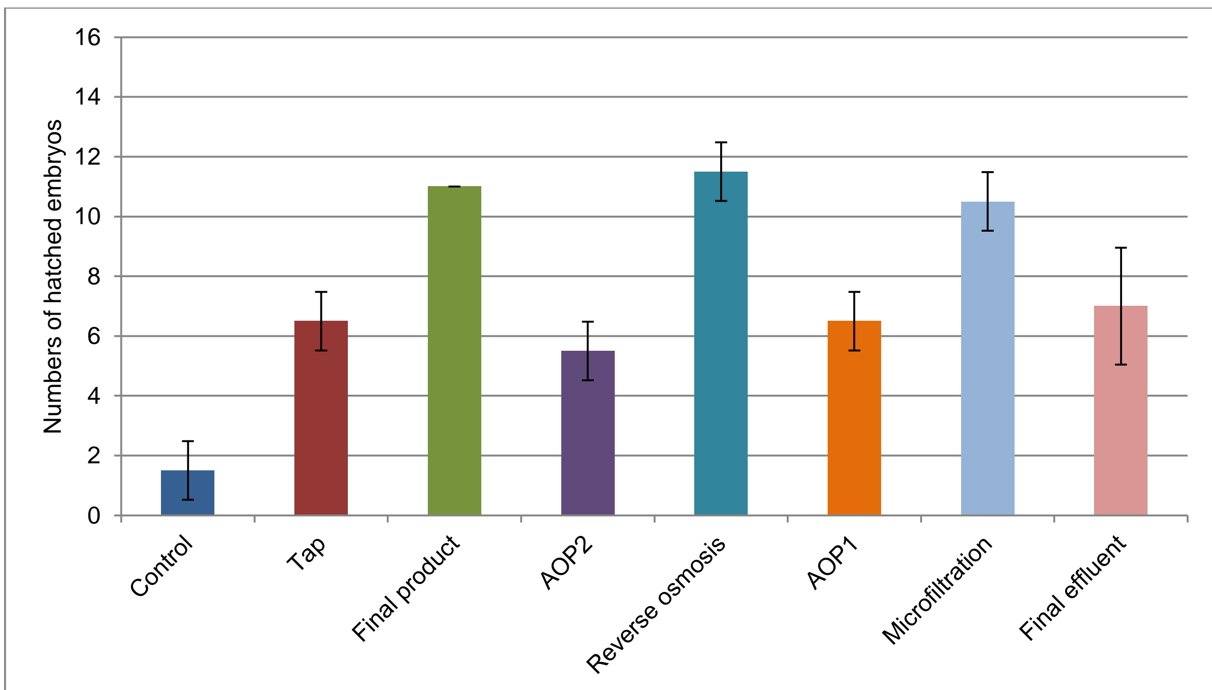

Figure 5.6 Average number of hatched embryos at 48 hours in developmental Exposures 3 and 4 with 95% confidence intervals

Further statistical analysis on the hatching data determined that the frequency of hatched embryos in the first exposure was very different from the frequencies recorded in the other two. This result reaffirmed the decision to remove the data from exposure 1 from the

analysis. Univariate analysis of variance of data from Exposures 3 and 4 showed a significant effect of treatment on the number of hatched embryos (P. value <0.05). However, there was no significant difference in hatching rate between Exposure 3 and 4 (P. value 0.640). A post-hoc test, LSD was conducted on the data, and it indicated that the significant differences in numbers of hatched embryos were present between control and all the other treatments, including tap water (P. value <0.05). Other significant differences arose between the following, all with P. values <0.05; tap and final product (P value = 0.022), tap and reverse osmosis (P value = 0.001), tap and microfiltration (P value = 0.022), final product and AOP2 (P value = 0.028), final product and AOP1 (P value = 0.047), AOP2 and reverse osmosis (P value = 0.01), AOP2 and microfiltration(P value = 0.028), reverse osmosis and AOP1 (P value = 0.002), reverse osmosis and final effluent (P value = 0.008) and AOP1 and microfiltration (P value = 0.045). There was not a clear increase or decrease in the frequency of hatching as treatment process is followed from either end.

5.5.3 Heart rates

Heart rates of individual embryos were recorded in the developmental exposures 1, 3 and 4 at the 48hpf time point (Figure 5.7). The data were not normally distributed, and therefore a non-parametric statistical test was conducted. A Kruskal-Wallis test found no significant differences in the heart rates recorded in Exposure 3. In contrast, statistically significant differences in heart rates of embryos exposed to the different product waters from the IPR plant were found in Exposures 1 and 4, P values = 0.0250 and 0.0481, respectively. A post-hoc test, Mann-Whitney U was carried out to determine where these differences occurred. In Exposure 1, the following heart rates recorded in the treatments were determined to be statistically significantly different from one another; final product and control (P value = 0.0047), AOP1 and control (P value = 0.0035) and final effluent and control (P value = 0.0455). In Exposure 4 the differences occurred among the following treatments; microfiltration and tap (P value = 0.0165), microfiltration and final product (P value = 0.0185), microfiltration and reverse osmosis (P value = 0.0263), final effluent and tap (P value = 0.0193), final effluent and final product (P value = 0.0292), and final effluent and reverse osmosis (P value = 0.0286).

It was not possible to record heart rates for the other experiments.

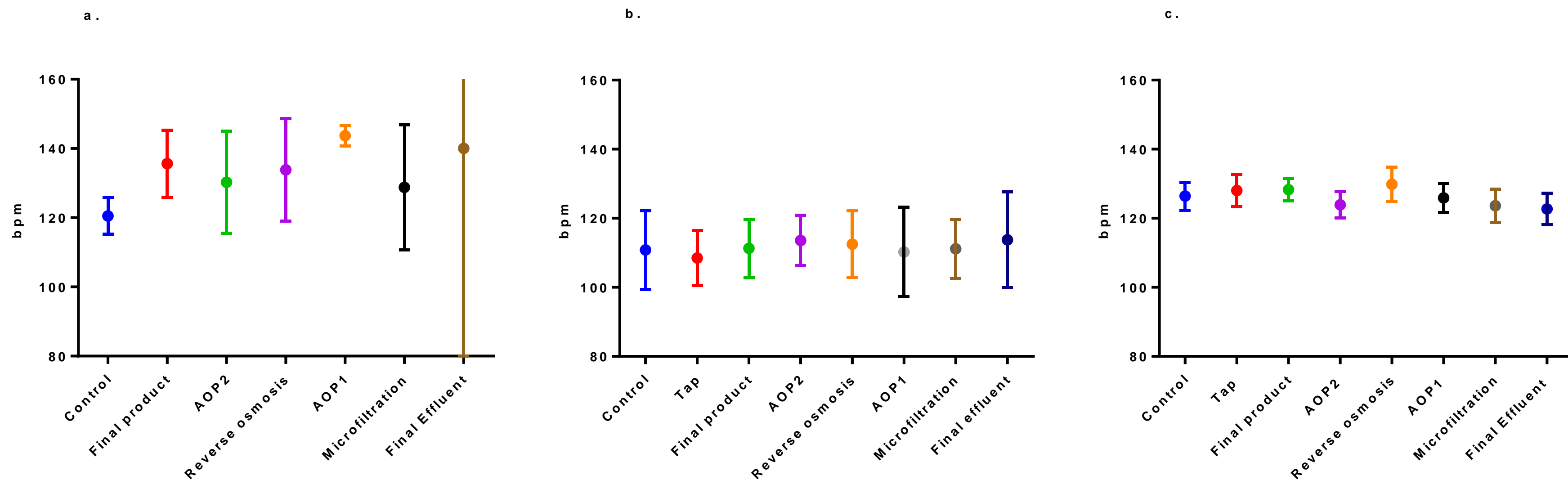

Figure 5.7 The three graphs show the heart rates (beats per minute (bpm)) measured at 48 hour from the three developmental observation exposures. The points represent the mean bpm and the 95% confidence intervals. Graph a shows the heart rates from the Exposure 1, graph b heart rates from Exposure 3 and graph c heart rates from Exposure 4.

5.5.4 Abnormalities

In all exposures carried out there were a variety of abnormal looking embryos. Abnormal embryos were individuals that were determined to be alive but showed clear malformation in structure, movement and/or appearance. These results were recorded at the 48 hour time-point. However, there were other embryos that had malformations at earlier time-points which then went on to die; these were counted as dead. It was observed that some abnormal embryos had more than one abnormality, for example a curved spine and enlarged heart. However, the frequencies of abnormalities in all incidences were too low to apply any meaningful statistical analysis, but the types of abnormality in the different treatment groups were of interest.

Figure 5.8 shows the total number of abnormalities observed at 48 hours combined from all three exposure experiments with the corresponding survival. All embryos were observed in every treatment. There was a low incidence of abnormalities in all treatments, including the control. A total of eight abnormal embryos were identified in the controls; however, it is important to note that seven of these occurred in the fourth exposure of the extended exposure and in the same replicate (dish). Survival for this group was also 60% in controls compared to >70% and >80% in the two prior exposures.

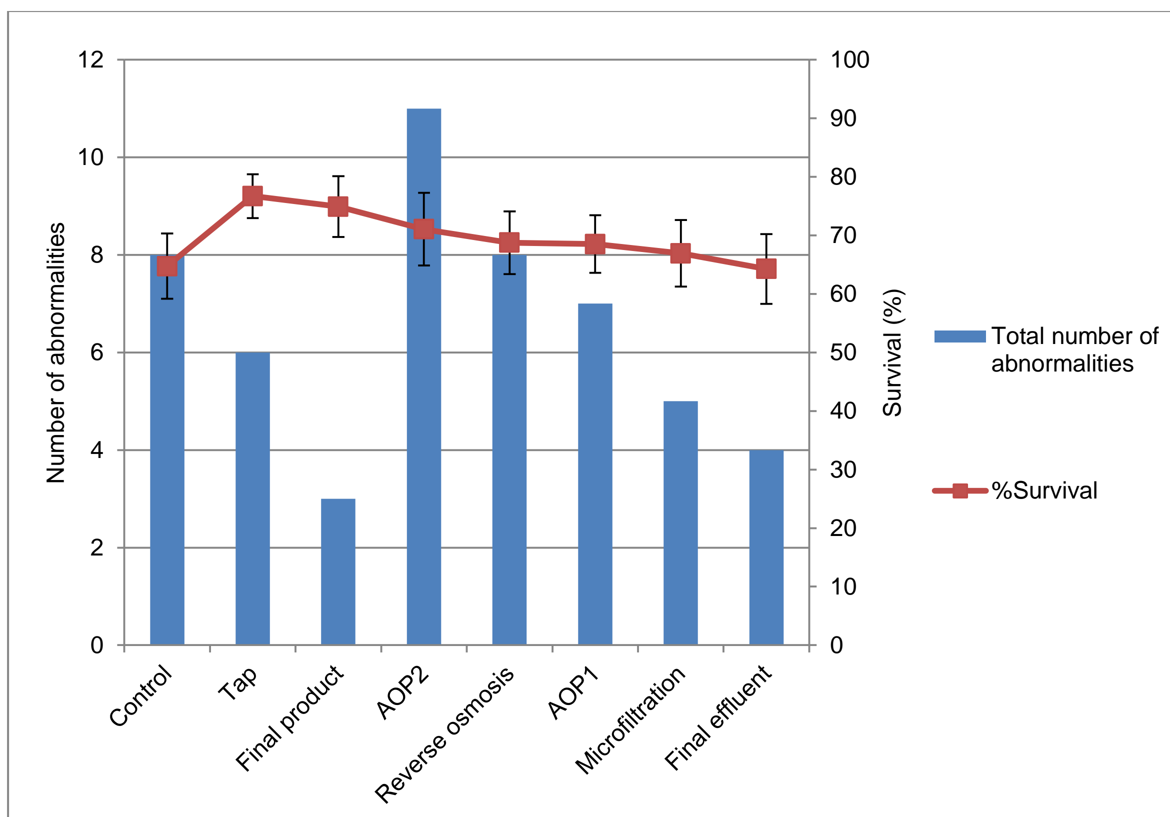

Figure 5.8 Total numbers of abnormal embryos observed and the corresponding mean survival (%) for each treatment group at 48hpf in all exposure experiments. The survival data include the 95% confidence intervals.

Table 5.2 lists the endpoints that can be observed at each specific time-point of development. For this analysis, the frequency of abnormal embryos was analysed from data collected at 48 hpf only. However, for the developmental exposures, abnormality data were collected at all time-points (4, 8, 12, 16, 24, 36 and 48 hpf). These observations were only made on a daily basis for the extended exposure studies. For the gene expression exposures, abnormalities were recorded at each sampling time point, but only selected embryos were recorded. As embryos were divided into discrete treatments and time-points for sampling, the same population was not followed/observed between 8 hours and 48 hours. Consequently, the 48 hour time-point was examined to allow comparison across the exposures. Some abnormal appearing embryos from the developmental observation exposures that were recorded as abnormal before 48hpf had died by the time the 48 hour point had been reached.

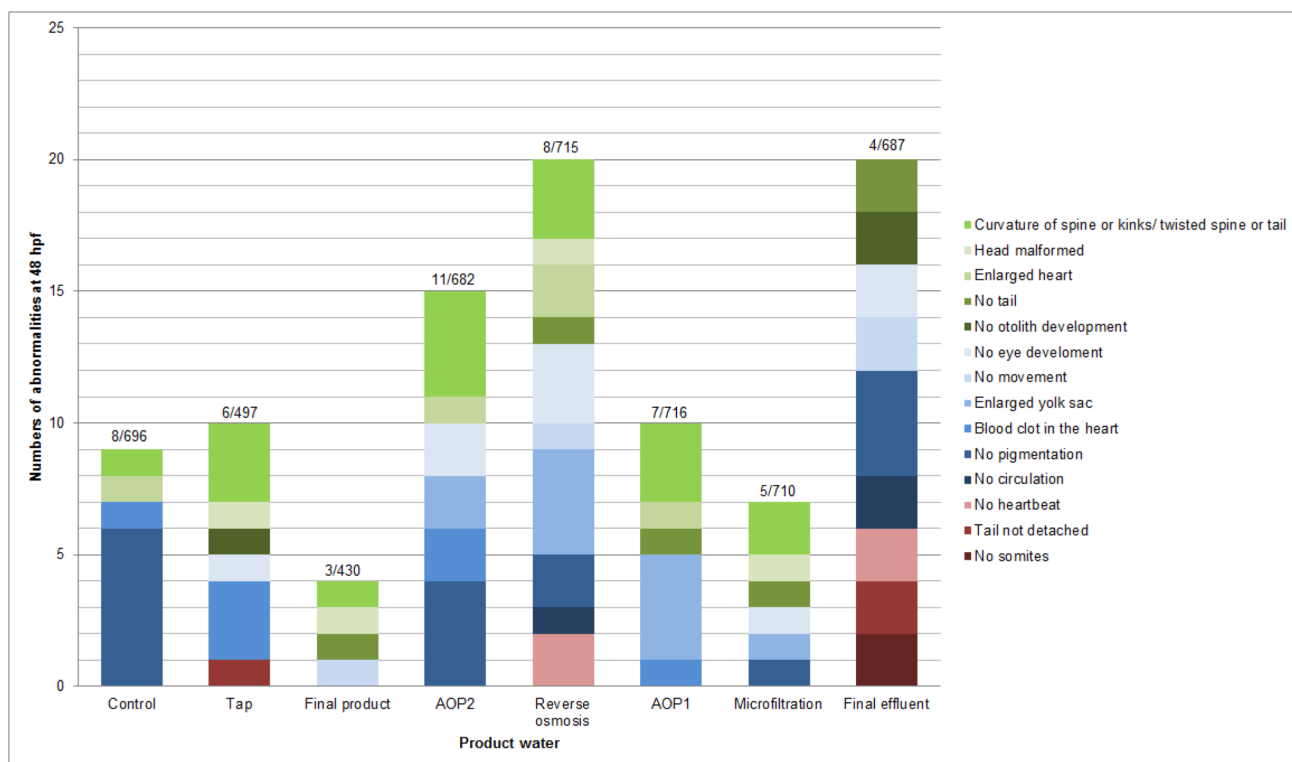

Figure 5.9 shows both the type and frequency of the various abnormalities examined at 48hpf. Abnormalities occurred in the control embryos as well as in the other treatments; however, in the control, the 8 recorded occurrences of abnormalities were categorised into just four types abnormality. The abnormalities observed in the embryos exposed to Final Product also were categorised into four types of abnormality, followed by AOP1 having five types, tap, AOP2 and microfiltration with six, final effluent with nine and reverse osmosis with the greatest number of categories of abnormality, with ten.

In the control group, abnormalities included enlarged heart (cardiomegaly), blood clot in the heart, absence of pigmentation and curvature of the spine. According to Table 5.2, absence of pigmentation and formation of oedema are both sub-lethal endpoints; however, modified chorda and malformed heart are teratogenic endpoints. Conversely, it is unclear if the blood clot in the heart would be classified as a malformed heart. Some researchers classify pericardial oedema and yolk sac oedema as teratogenic effects (Hermsen et al., 2011), whereas others classify these as sub-lethal effects (Braunbeck & Lammer, 2005; Fraysse et al., 2006).

In the Final Product water, abnormalities included absence of a tail, malformed head, curvature of the spine, and no movement. The first three types of abnormality are all classed as being teratogenic endpoints, with the last being sub-lethal.

The types of abnormalities observed in AOP1 included absence of a tail, enlarged yolk sac, enlarged heart, blood clot in the heart and curvature of the spine; all of these are classified as being teratogenic endpoints.

The abnormalities recorded in the embryos reared in tap water included; no eye development, no otolith development, tail not detached, blood clot in the heart, head malformed, and curvature of the spine. These abnormalities are all classed as teratogenic apart from the eye development at 48 hours, which is sub-lethal.

In the AOP2, the types of abnormality included absence of eye development, enlarged yolk sac, enlarged heart, blood clot in the heart, no pigmentation and curvature of the spine. Absence of eyes and pigmentation at 48 hours are classified as sub-lethal; however, the other four abnormalities are classified as teratogenic.

The types of abnormalities observed in the embryos reared in water from the microfiltration treatment included absence of eye development, absence of a tail, enlarged yolk sac, head malformed, no pigmentation and curvature of the spine. No eyes and no pigmentation at 48 hours are deemed as sub-lethal, whereas the other four abnormalities are classified as teratogenic.

In the groups of embryos which were reared in final effluent there were a total of nine types of abnormalities and these included: (i) absence of somites, (ii) absence of eye development, (iii) absence of otolith development, (vi) tail not detached, (v) absence of a tail, (vi) no movement, (vii) no heartbeat, (viii) absence of pigmentation, and (ix) no circulation. At the 48 hour time-point, no eyes, movement, circulation or pigmentation are classed as sub-lethal, whereas no somites and no heartbeat at 48 hours are considered a lethal endpoint and the remaining are classified as being teratogenic endpoints.

The abnormalities observed in the embryos reared in RO water included absence of eye development, absence of a tail, no movement, enlarged yolk sac, enlarged heart, head malformed, no heartbeat, no pigmentation, curvature of the spine and no circulation. Lack of eyes, movement and pigmentation at 48 hours are considered to be sub-lethal endpoints. No heartbeat at 48 hours is classified as lethal and the other five are all teratogenic.

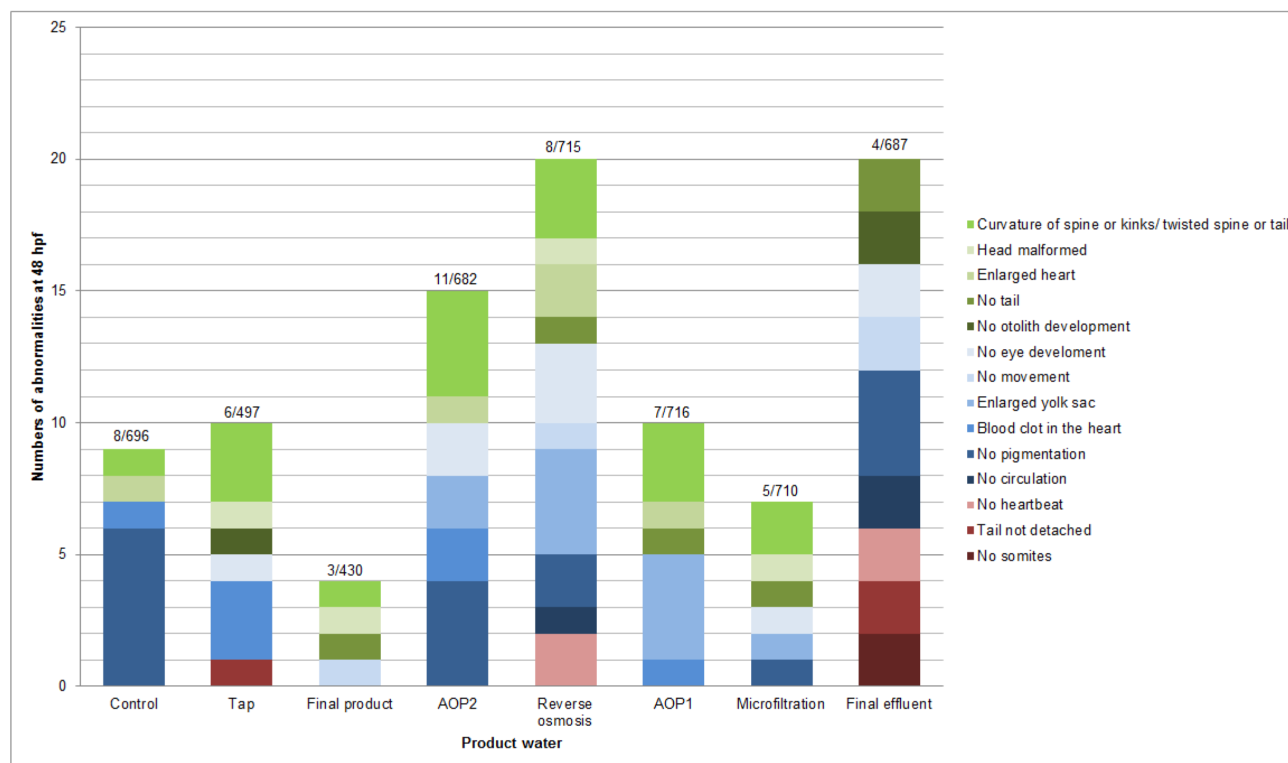

Figure 5.9 Total number of the different types of abnormalities observed at the 48 hour time-point in all three exposure types (developmental observations, extended and gene expression exposures). Numbers above the bars indicate the number of individuals with at least one abnormality recorded/out of the number of surviving embryos at the 48 hpf time point (in the extended exposures, exposures to tap water and final product were not repeated in the two final repaets, hence the lower total number of surviving embryos). The value of the bar indicate the number of abnormalities recorded in embryos exposed to each of the product waters, it was observed that some abnormal embryos had more than one classifiable abnormality, thus the difference between number of abnormal individuals and abnomalities. The proportion of the bar, idientified the different colours, indicate a specific type of abnomality. The red colours indicate abnormalities which are classied as lethal, blues indicate sublethal abnormalities and the greens indicate abnormalitites which are classified as being teratogenic (the classification are from information gained from Braunbeck & Lammer, 2005; Nagel, 2002; Schulte & Nagel, 1994)

5.6 Discussion

5.6.1 Survival

Death of an embryo is an important developmental toxicological endpoint, and can be considered an indicator of general toxicity (Brannen et al., 2010). Survival was examined over the 48 hour exposure period (exposure from 0 hpf to 48 hpf) in all exposure studies (irrespective of their ultimate purpose). For the first two study types (48 hour developmental observation and extended exposures), there was no significant difference between the survival of the embryos reared in product water from any of the treatments at the IPR plant compared to the control and tap waters. The survival of the embryos in control water (water from the header tanks at the aquarium facility at Brunel University) was below what would be considered valid in an OECD FET test; however it did not vary significantly from the test waters. A number of strategies were used to improve the survival of the controls, including the use of zebrafish embryo media and rinsing the embryos in distilled water over a fine mesh filter, but these methods did not improve the survival. It is therefore possible that the embryo survival rate achieved here was largely determined by biological (genetic) factors, including the quality of the gametes produced by the zebrafish.

Indeed, it was observed that if overall survival was decreased in one experiment, it was also decreased in all treatments, including the controls, suggesting initial embryo quality and/or experimental conditions together were driving overall survival rather than the treatments. Efforts were made to ensure that all variables were consistent across treatments, with the exception of the physicochemical properties of the product waters themselves. Temperature was kept constant in all studies by conducting exposures in an incubator or water bath. Due to differences in the level of treatment (and biological loading), it is possible that oxygen levels in the Final Effluent and microfiltration (MF) may have been lower than that of the controls or treatment water further along the treatment process (AOP1, AOP2 and FP). The same would also be true for nitrates and nitrites, and, therefore, if slightly raised levels of nitrate and nitrites (and reduce oxygen) in the less treated product waters were affecting the survival, the effects would only be observed in those earlier treatment stages. Both the product waters from the final effluent and microfiltration stage of the treatment were observed to have increased levels of nitrates and nitrites. However, from the possible explanations for the overall decreased survival on certain study events, it is likely that the quality of the embryos remains an important factor. Embryos were collected and sorted in a consistent way prior to each exposure by the same researcher. They were sorted and placed into clean aquarium/control water, with debris removed before being placed into the exposure mediums. The adults were rested for several days between breeding events.

Mixed breeding groups were used along with several different groups, to optimise the quantity of embryos, so that any groups producing less than optimum embryos could be missed.

In the gene expression exposures significant differences in survival at 48 hpf were observed between the different treatment groups, between embryos reared in final effluent (the source water into the IPR plant) and the produced waters from the IPR process. Despite this, there was no significant difference between survival of the embryos in final effluent compared to the controls. It is unclear why for this study design the survival would be similar for the control and final effluent, but different in the other treatments. Additionally, there was no significant difference in survival between treatments in independent experiments carried out on different days. Two explanations can be offered for this when considering differences that were recorded in the different experimental events/repeats in the two other study designs. Firstly, the gene expression exposures were all conducted within the same month (March 2012), meaning the conditions at the IPR plant would have been similar as there would have little change in the weather, specifically temperature and rainfall. These two factors would affect the plants efficiency and dilution. Also, the adult breeding groups were the same and there would be little difference in the age of the fish, and the conditions in the laboratory changed little over this short time frame. Conversely, the other two study designs were started in late 2011, and repeats were conducted into early April 2012, Thus providing larger scope for conditions at the IPR plant and laboratory to alter, albeit slightly. Secondly, in the first study design, the embryos were exposed in individual wells of a 24 well plate, meaning that if one embryo died, its decomposition would not impact on the other embryos in its exposure group. In the second study design the embryos were exposed to the product waters in groups in crystallising dishes, as they were in the gene expression exposure, but they checked every 24 hours and any dead embryos were removed, thereby decreasing effects of decomposition on the other embryos. In the gene expression exposure, this was not possible, and the 48 hour group were left for the entirety of the 48 hours, with any dead embryos remaining with the living ones. The initial developmental observations saw that the majority of deaths occurred within this first 12-16 hours; therefore this could have impacted survival on this group. It might also offer an explanation as to why the survival was, as a whole, less in the gene expression exposure compared to the other study designs. This highlights positive aspects of using a well plate for short-term embryo exposures or designing experiments small scale enough to enable regular observations and removal of the dead.

5.6.2 Hatching

Hatching was recorded in the first study design (48 hour developmental observations), and this study was successfully repeated three times. There were obvious differences in the hatching rate between first repeat and the last two repeats. It is considered that this could be due to the slightly different experimental design used in the first exposure compared to the last two. The first exposure was conducted at the very preliminary stages of the research project, without any previous experience, and observations, including numbers of hatched, were recorded earlier on day two of the exposure (the 48 hour point). With insight from the first exposure, a number of modifications to the experimental design were made, including staggering the start time of treatments across the plates, and ensuring that hatching was recorded as the first observation across all the well plates. As there was a large number of embryos to record (including heart rates and individual photographs taken), the observations took several hours to complete. Zebrafish begin to hatch around this time, and can emerge from the chorion within hours, which is why it was important to record hatching as the first observation for all test samples in a staged manner.

Based on the differences between the repeats of this study and potential explanations for these differences, the hatching recorded in the first exposure study was removed from further analysis. Examining the hatching data from the last two repeats of this study, significant differences in the number of hatched embryos were observed between treatments, with fewer embryos being observed to have been hatched in the control compared to the other treatments. The rate of hatching may increase if properties of the water weaken the chorion, or if the embryo movement increases. The product water from the IPR plant did undergo chloramination and tap water is chlorinated; however, the control water was also dechlorinated. There was an alteration in water hardness and pH along the treatment process.

In a study testing 133 chemicals, altered hatching rate as an endpoint was recorded to be correlated with another twenty endpoints. These other endpoints included abnormalities such as curvature, yolk sac size and yolk sac oedema (Ducharme et al., 2013). This correlation of the hatching rate and morphological endpoints suggests that hatching rate could be used as an indicator of developmental toxicity in zebrafish embryo assays (Ducharme et al., 2013).

5.6.3 Heart rate

Heart rates were recorded in the first study design, 48 hour developmental observations at the 48 hour time point, and this study was successfully repeated three times. The heart rates

were not found to significantly differ among the treatments and control in the middle of the three experimental repeats. However, heart rates were shown to be significantly different in the first and last repeats. In the first repeat, the difference arose only when comparing the heart rates in embryos reared in AOP1 product water and control and final effluent product water and control. The same differences were not repeated in the last replicate, with differences occurring with microfiltration and three other treatments - tap, final product and reverse osmosis product waters. Additionally, heart rates were significantly different when comparing final effluent and the same three treatments as were different to microfiltration. Taken in isolation this indicates that the heart rates in the embryos exposed to product water that had undergone the least amount of treatment were significantly different from three much more advanced treatments. However, this result was not repeatable in the subsequent experimental repeats. The confidence intervals became smaller in each progressive test, and are smallest in the final repeat. Due to the difficulty involved in recording the heart rates it is possible that I was able to record this endpoint more accurately with practice, and that the data became more reliable at each attempt.

A group of researchers determining endpoints in zebrafish assay to predict teratogenicity, omitted heart rate along with larval length, head-trunk angle, otic vesicle length (the estimated distance between the eye and the otic vesicle), somite number, swim bladder development, stomach morphology and liver morphology from the final analysis (Brannen et al., 2010). This decision was made on the basis that (i) they had a variable background, and could only be proven to be affected when other more predictive or reliable endpoints were also affected, (ii) they were not easy to accurately assess, and (iii) were observed not to change greatly despite the exposure group (Brannen et al., 2010). However, heart rates have been reported to be altered by certain chemicals. Nagel (2002) reported that the drugs verapamil and propranolol both reduce the heart rate in exposed zebrafish embryos, and that theophylline and isoprenalin were both reported to increase the heartbeat. Results of exposure to these four drugs were as expected, and demonstrated that the zebrafish embryo model reacted in much the same way as humans do to these drugs (Nagel, 2002). It was therefore suggested by Nagel (2002) that the zebrafish embryo assay could be used as a model to determine suitable compounds that have an effect on cardiovascular system of other vertebrates.

I found heart rate to be a very variable endpoint, and also one that was very time consuming to measure. Measuring heart rate could still prove important for hypothesis driven research (i.e. when the chemical or chemicals are hypothesised to specifically alter heart rate), but should not be selected as a routine endpoint.

5.6.4 Abnormalities

Abnormalities were observed in the embryos reared in the control and tap waters as well as the product waters from the IPR plant. However, the nature of the abnormalities observed in the embryos reared in the IPR product waters were more variable than those observed in the embryos reared in control waters. Also, more abnormalities of the embryos reared in the treatment waters could be classified as teratogenic. The different abnormalities observed in the embryos at 48 hpf have been divided into either lethal, sub-lethal or teratogenic endpoints, based on the information detailed in Table 5.2. The lethal endpoints include no heartbeat, tail not detached, and no somites. The sub-lethal endpoints include no circulation, no pigmentation, no otolith development, no eye development, and no movement. The endpoints classified as teratogenic included enlarged yolk sac (however, some researchers class yolk sac oedema as sub-lethal), curvature of spine or kinks/twisted spine or tail, head malformed, blood clot in the heart, enlarged heart, and no tail.

As previously stated, if death occurred before 48 hpf then it could not be automatically assumed that a developmental toxicant or teratogenic response was responsible. However, with a relatively high survival rate and large sample population it is likely that if teratogenic endpoints are being exerted, then they would have been observed. As stated earlier, teratogens can be divided into three groups; mechanical disruptors, environmental factors and chemical contaminants. Mechanical disruption would be unlikely here as the embryos were exposed under laboratory conditions. The main focus of this project was to determine if any of the chemical contaminants present in the product waters might affect the development of the zebrafish embryos. However, environmental factors could also influence this process because the different samples of product waters were collected from the treatment plant on the different days for each repeat exposure. The nature of effluent is variable as it is affected by outside temperatures and flow rates at the time of collection and the content of the influent. To mitigate against this possibility, 24 hour composite samples were collected, but nevertheless the exposures were carried out over a number of study times which spanned a number of months. Temperature during the exposures was controlled by conducting the exposures in either the incubator or water baths, and ensuring the exposure waters had all equilibrated to the correct temperature prior to the introduction of the embryos. Therefore, there could have been subtle differences in the physical properties between treatments and days; oxygen concentration was one factor which was of particular concern.

When organisms which are dependent on oxygen for survival, as nearly all vertebrates are, are in oxygen depleted environments it is reported to adversely impact cellular energy

generation, leads to the production of reactive oxygen species (ROS) and causes cell damage and apoptosis (Long et al., 2015). Fish that are kept in hypoxic conditions are often observed to swim to the surface of the water where there is more oxygen, increase ventilation capacity to absorb more oxygen or decrease movement to reduce the rate of oxygen consumption (Robertson et al., 2014). Including, these behavioural changes, physiological and biochemical changes are initiated, these can include changes to the gill to increase the surface area, increasing the heart rate and haemoglobin content, modification in the structure or activity of specific ion channels, and activation of anaerobic adenosine triphosphate (ATP) production via glycolysis (Robertson et al., 2014). It has also been reported and detailed in Long et al. (2015), that hypoxia induces significant alterations to gene expression profiles in a number of different species of fish, and hypoxia-inducible factor-1 (HIF-1) has been found to be the fundamental step in the regulation for hypoxia-induced genes (Long et al., 2015). The concentration of dissolved oxygen present in which a water body is considered to be hypoxic varies depending on authors, however, hypoxia seems to be referred to when the dissolved oxygen is 0 to 2.8 mg/l. Long et al. (2015), states that in the aquatic environment hypoxia is reached when dissolved oxygen is <2 mg/l, Wu (2002) reported it to be dissolved oxygen is <2.8 mg/l (Wu, 2002), and Robertson et al. (2014) exposed fish to severe hypoxia and total anoxia with a dissolved oxygen concentrations of 5 and less than 0.5%, respectively (these are approximately, 0.6 and less than 0.06 mg O_2/l). In fish, effects of hypoxia include suppression of development, reduction in growth, disruption to endocrine system, reproductive effects and great numbers of mortalities (Long et al., 2015). Specifically, Long et al. (2015) observed zebrafish larvae that had been exposed to dissolved oxygen levels of 5% for a 24 hour time period to have smaller intestine lumen, larger yolk sac and smaller body length when compared to control fish (Long et al., 2015). However, along with other studies, Long et al (2015) found that developing zebrafish exposed to hypoxic conditions during early stages of development had greater tolerance to subsequent periods of hypoxia (Long et al., 2015).

Prior to 14 days post fertilisation zebrafish use their skin as the main respiratory surface (Robertson et al., 2014).

In studies conducted by Shang & Wu (2004), reported that hypoxic conditions can act as a teratogen in zebrafish embryos, and anoxic (no oxygen) conditions were found to cause 100% mortality within 24 hours (Shang & Wu, 2004). The same study reported a dose-response relationship related to the dissolved oxygen concentration and mortality being 12.3%, 17%, 28.3% and 89.7% mortality at 5.8, 1.0, 0.8 and 0.5 mg O_2/l, respectively, over a 120 hour exposure period (Shang & Wu, 2004). The same researchers also found decreased oxygen levels affected the heart rate, with decreased oxygen levels (0.8 mg O_2/l)

producing significant increases in embryo heart rates compared to that of the controls (5.8 mg O_2/l) up to 96 hour time point, after which they were recorded to decrease compared to the controls. The number of individuals with malformations also increased in the 0.8 mg O_2/l group compared to the control (5.8 mg O_2/l). These malformations included developmental delay, loss of normal synchronization in development (tails were observed to develop faster than the heads), spinal deformity (axial curvature) and failure to develop vascular systems; the latter led to death after several days (Shang & Wu, 2004). It was reported that after 168 hours, the number of abnormalities in the hypoxic group was 18.3%, which was significantly greater than that recorded in the control group (10.3%). Additionally the body length was reported to be significantly reduced in the hypoxic group. As well as the malformations, the levels of sex hormones were affected; testosterone levels in the hypoxic embryos were increased and the estradiol levels were significantly reduced. Apoptotic cells were observed to be significantly reduced in the tail and increased in the head (Shang & Wu, 2004). Robertson et al 2014) found that the cellular responses exhibited by developing zebrafish when exposed to hypoxic conditions differed in relation to the developmental stage and degree of oxygen reduction. These researchers examined the effects of hypoxia of zebrafish embryos at 18, 24 and 36 hpf. A HIF-1 response was not observed in embryos at 18 hpf at low oxygen levels, but was initiated at 24 hpf and 36 hpf, indicating the sensitivity to low oxygen increased with increasing age of the embryo (Robertson et al., 2014). It was also observed by the researchers that embryos exhibited a cellular hypoxia response during early development had increased tolerance to hypoxia as an adult and hypoxia during early development caused increased number of males in the population. This latter finding was linked to increased testosterone production, which is reportedly due to impaired aromatase activity (Robertson et al., 2014).

It has been observed that the concentration of oxygen affect the toxicity of copper in developing zebrafish embryos, and the copper toxicity is altered dependent on the developmental stage (Fitzgerald et al., 2016). It is reported that at early stages of development hypoxic conditions supress the toxicity of copper, via the alteration of HIF signalling pathway, However, after hatching copper toxicity is increased in hypoxic conditions, reported to be due to increased copper uptake under hypoxic conditions (Fitzgerald et al., 2016).

It is reported that zebrafish embryos at 18 hpf, are very tolerant to anoxic conditions. However by 24 hpf, when the heart developing and the heart is beating, the tolerance to anoxia reduces (Robertson et al., 2014). At 36 hpf, when the cardiovascular system is fairly well developed and the red blood cells are being circulated, the embryos are no longer

tolerant to anoxia, this is when early hatching may occur as a response to low oxygen levels (Robertson et al., 2014).

In this current study the embryos were exposed to effluent; as a result there was the concern that decreased dissolved oxygen levels could influence the findings, especially in the product waters from near the start of the treatment plant. It was not possible to measure the dissolved oxygen in the 48 hour observations exposures as these were conducted in 24 well plates, resulting in the volume of water being too small to measure with a standard oxygen probe. However, readings were taken during the extended exposures studies where dishes and beakers were used, enabling the oxygen probe to be used to record levels. Examining these readings it is considered unlikely that the effects of hypoxia observed by Shang & Wu (2004) would have been elicited in these current studies (Table 3.4). The oxygen level did decrease over the 24 hour time period (Table 3.4), and this could have decreased further following another 24 hours, but in the first 24 hours of exposure the levels were not recorded as hypoxic (<2.8 mg O_2/l), or near the level tested in the study carried out by Shang & Wu (2004) of 0.8 mg O_2/l. However, the levels were below the control level used by Shang & Wu (2004) of 5.8 mg O_2/l in the final effluent, and this treatment did have lower oxygen levels than the other treatments. The optimum oxygen level is stated to be between 6 mg O_2/l and saturation (Westerfield, 2007). If further studies were conducted it would be important to develop a reliable technique to easily and safely measure the dissolve oxygen levels in all test vessels.

Both mammals and fish possess the heterodimeric transcription factor hypoxia inducing factor 1 (HIF-1α), which is inducible under hypoxic conditions. It is reported to regulate the transcription of a number of hypoxia related genes involved in erythropoiesis, angiogenesis and glucose transport (Shang & Wu, 2004; Wu, 2002). It was previous highlighted that HIF-1 is the fundamental step in the regulation of hypoxia-induced genes (Long et al., 2015). Long et al. (2015), exposed developing zebrafish (120 hpf) to hypoxic conditions and observed there to be 132 significantly up-regulated genes and 41 significantly down-regulated genes (Long et al., 2015). The majority of the hypoxia induced genes were reported to be involved in biosynthesis of source material of haemoglobin, haemtopoiesis and oxidation-reduction processes (Long et al., 2015). However, at 24 hpf, genes involved in haemoglobin were not observed to be up-regulated following hypoxia (Long et al., 2015). One of the genes which was most greatly inhibited by hypoxic conditions was *hela* (hatching enzyme 1a), its expression was reduced by 51-fold by hypoxic condition (5% dissolved oxygen) (Long et al., 2015). Zebrafish are reported to have four hif1a genes, these include *hif1aa*, *hif1ab*, *hif1a1* and *hif1al2*, however in the study conducted by Long et al (2015), only *hif1a12* was observed to be up-regulated by hypoxia (Long et al., 2015). Indicating that the genes

encoding for hypoxia inducing factor would only be expressed under severe hypoxic conditions, not something that was observed during this current study

One of the abnormalities observed that could have been caused by chemical contamination was the lack of pigmentation. Initially, this abnormality was considered to be a delay in development, until it was observed that the embryos seemed to be developing normally in all other respects apart from the development of pigmentation. In the extended exposure, however, no larvae were observed to be present without pigmentation. Therefore, as the lack of pigmentation was occurring at a low incidence, it could be that this abnormality did not occur to any observable degree in the extended exposure due to random chance, that the embryos were able to compensate and therefore appeared normal, or that unpigmented embryos died before they had reached the larval stage. Pigmentation of zebrafish embryos should be visible from 24 hours post fertilisation (hpf) (Kimmel et al., 1995), with retina pigmentation occurring at 30 hpf and tail pigmentation at 36 hpf (Kimmel et al., 1995). It has been reported by a number of authors that the process of pigmentation can be affected following exposure to certain chemicals. Zebrafish embryos exposed to anilines and phenols were observed to have reduced pigmentation (Schulte, 1997; cited in Nagel, 2002). Another study found a dose dependent hypopigmentation effect in fish embryo cells exposed to p-tert-butylphenol, a substance known to affect pigmentation in humans (Maiwald, 1997 cited in Nagel, 2002). The relevance of hypopigmentation to human health has not been fully established. Brannen et al. (2010) tested 31 chemicals in the zebrafish embryo assay and compared the results to those from *in vivo* mammalian studies. They found that three chemicals affected pigmentation, all-trans-retinoic acid, hydroxyurea and retinol. Along with pigmentation, the other adverse effects these chemicals had in common were effects on the cardiovascular system and fin development (Brannen et al., 2010). Pigmentation was not observed as an endpoint in the corresponding mammalian studies. However, cardiovascular system effects were observed in the studies testing those three chemicals (Brannen et al., 2010). In single chemical exposures, effects on skin pigmentation in zebrafish embryos was found to not be associated with oedema (Ducharme et al., 2013).

Another abnormality observed was oedema of the yolk sac and/or heart and heart malformations. In the same study that found hatching rate to be strongly correlated with a number of morphological endpoints, it was reported that cardiovascular and gross morphology endpoints were well correlated - specifically pericardial oedema. Oedema of or around the heart, and yolk sac oedema, were also found to be strongly associated endpoints. It has been suggested that this may occur because the mechanism of fluid retention is similar in both cases (Ducharme et al., 2013). Oedema is reported to have a knock-on effect to other adverse effects on the circulatory system and kidney function

(Ducharme et al., 2013). However, there was no clear association between hatching rate in my studies with the abnormalities reported to be correlated with hatching rate. This was due to the hatching rate being different in the controls compared to that of all of the other treatments, and the frequency of abnormalities being very low and occurring across all treatments.

Many of the studies that have compared endpoints observed in zebrafish embryos employ single chemical exposures. In this project, the embryos were exposed to large complex mixture of chemicals, which may explain the relatively large number of different abnormalities observed.

5.7 Conclusions

It is considered that the observations made in this group of studies indicate that the product waters from the different IPR treatments did not appear to affect survival of zebrafish embryos.

Variability in the measurement of various endpoints (e.g. heart rate and hatching) means it is difficult to determine if the treatment process is affecting these endpoints.

The change in the type of abnormalities found in a low number of individuals in the controls and along the IPR process warn that development may be impacted by various chemicals in the water.

Pigmentation loss, cardiovascular abnormalities and skeletal deformities appear to be the most common effects observed in process waters.

As there is a large variety of chemical contaminants in effluent, and there were a variety of different abnormalities observed in the treatments and controls, lethal, sub-lethal and teratogenic abnormalities, it was not possible to single out one chemical or groups of chemicals, or even an environmental factor, as the cause of the abnormalities.

CHAPTER 6: Determination of Alterations to Gene Expression Profiles of the Developing Zebrafish Embryos Exposed to the Different Product Waters from the IPR Treatment Process

6.1 Introduction

In the previous chapter, it was reported that there was a low incidence of abnormalities in zebrafish exposed developmentally to the processed water from the IPR plant that were not observed in the controls. The aetiology of these abnormalities was unknown. Therefore analysis of global gene expression of embryos exposed to the processed water undertaken. The rationale for this research was to generate mechanistic insights that could was be used to develop specific hypothesis to be tested empirically taking into account the analytical chemistry data provided by Thames Water Ltd.

6.2 Aims and Objectives

6.2.1 Aims:

1. To determine if any of the product waters from any of the treatment stages at the IPR plant caused significant differences in individual gene expression levels relevant to specific biological pathways and functions.
2. To identify whether changes in gene expression could be associated with the observed changes in phenotype.
3. To determine if ‘culprit chemicals’ measured in the IPR water could be identified that might explain the observed changes in gene expression and phenotype based on current knowledge.

6.2.2 Objectives:

1. To expose zebrafish embryos to each product water from the IPR plant and to an aquarium control and tap water control.
2. To sample zebrafish embryos at different stages of development for gene expression analysis by microarray.

6.2.3 Genomics

Genomic Approaches

DNA encodes genetic information; the entirety of an organism’s DNA is its genome. The study of the genome is known as genomics (World Health Organization, 2002). The term genomics was first used in 1986 by Thomas H. Roderick (McKusick, 1997). Genomics differs from genetics mainly because genetics is concerned with evaluating the properties of an individual gene, whereas genomics examines all genes and their relationships with one another, to determine how they influence in the organism’s biological functions, including growth and development (World Health Organization, 2015). Three disciplines:

transcriptomics, proteomics, and metabolomics, known as 'omic' technologies were derived from genome sequencing projects; they measure fluctuations in gene expression, proteins expression, and the levels of metabolites in a cell, tissue or organism, respectively (Poynton et al., 2008).These techniques generate a huge amount of complex data; as a result, analysis and interpretation of these data is a large undertaking and requires bioinformatics (Snape et al., 2004). Bioinformatics uses an interdisciplinary approach, involving computer science, statistics, and mathematics to develop methods and software applications to analyse biomolecular data (Dale et al., 2012). Bioinformatics is of particular importance for analysing the large datasets generated from genome sequencing, and in this case, experiments using microarrays (Dale et al., 2012). Systems biology combines the omics data gathered from an organism to determine the global response within it, from the genotype to phenotype (Simmons et al., 2015).

The omics approach has been used within toxicology, when it is termed toxicogenomics. Data generated from toxicogenomics has enabled a greater understanding of the mechanism of action of certain chemicals, and it has been used in drug discovery and researching existing drugs (Poynton et al., 2008). In 2004, Snape et al. introduced the term ecotoxicogenomics, which describes the incorporation of the omic technologies into ecotoxicology (Snape et al., 2004). One of the underpinning reasons why ecotoxicogenomics is so important is the need to reduce the number of animals used in ecotoxicity testing. Ecotoxicogenomics helps elucidate the underlying molecular mechanisms of toxicity, to allow the development of predictive models and Quantitative Structure-Activity Relationship-models (QSAR-models) (Snape et al., 2004). As well as Snape et al. (2004) highlighting that data generated from omics technologies can be used to reduce the numbers of animals used in ecotoxicological testing, both Snape et al. (2004) and Van Aggelen et al (2010) stated that it may also be used to inform the researcher on the possible Mode of Action (MOA) of chemicals. Previously, ecotoxicological studies solely focused on apical biomarkers, which often do not provide information about the mechanisms of toxicity, and thus the association with molecular alteration and outcomes in the whole organism (Snape et al., 2004; Van Aggelen et al., 2010). The molecular and cellular biomarkers identified can be associated with population and ecosystems responses, which can be used to inform ecological risk assessments for existing and new chemicals and technologies (Snape et al., 2004).

Genomic responses leading to biological effects can occur either directly or indirectly. The severity of the response is determined by the duration of the exposure and potency of the chemical. A genomic response could be reversed, have a short-lived effect on survival or reproduction, or cause a genotoxic response (Snape et al., 2004). Therefore, investigating

global gene expression can be of greater interest and provide a much greater wealth of knowledge. It is also important to have a large sample size and to examine responses at differing time intervals. Additionally, the assessment of the alteration in gene expression should not just be concerned with the differences in expression of individual genes, but in relation to how the genes respond within given pathways. If the expression of multiple genes has been altered, and analysis shows that those genes act together within a genetic pathway, initiating a specific biological response, more confidence can be had that that specific biological process is likely to be affected by the exposure/conditions tested.

Snape et al (2004) stated that there is the need to develop and refine new technologies to measure gene, protein, and metabolite profiles in populations of wildlife which are exposed to "multiple stressors in complex natural environments" (Snape et al., 2004).

Microarrays

Most references to microarrays refer to an array that measures the expression of cDNA. There are many types of arrays including single nucleotide polymorphism (SNP) arrays and methylation arrays; however, this study used only the cDNA microarray, so only this type of array will be discussed. The rationale behind the use of a microarray is if a compound elicits an effect on an organism, the effect will be initiated at a molecular level and will be apparent as a change in gene expression. Information in DNA is transferred to mRNA, this mRNA is translated to form a specific protein. This alteration can be detected when the chemical/condition being tested acts directly on the nuclear receptors, thereby altering the gene expression. However, some chemicals act directly on proteins and enzymes themselves, and in these incidences it it can be less clear whether we would see an effect on the genome. Transcriptional changes in gene expression (i.e. gene up- or down-regulation) can either be measured on different scales, from an individual genes, small groups of genes, or an organism's entire genome. Individual gene expression is usually investigated using the Quantitative Polymerase Chain Reaction (QPCR), which measures whether there is more, or less, of the gene being expressed, relative to that of the control gene (e.g. a house-keeping gene). The groups of genes, or entire genome, can be investigated using microarrays, whereby the expression levels of a whole array of genes are measured to assess their expression compared to that of a control sample. Any alteration measured at a genetic level does not automatically result in an effect on the organism as a whole, for alterations in gene expression due to an outside stressor can be alleviated via cellular mechanisms. Consequently, very often, toxicogenomic changes are seen as an early warning signal of the potential for adverse effects, and an indication as to what those adverse effects might be, which is why it was used in this study.

Microarrays are tools used in genomics; their use in toxicological studies can lead to the discovery of novel biomarkers and adverse effects (Van Aggelen et al., 2010). Microarrays are comprised of a glass slide onto which known fragments of DNA are spotted onto the surface. They can include a specific array of DNA fragments; for example, if the researcher was solely interested in estrogenicity, then the DNA fragments spotted on to the slide may only be representative of genes known to be involved in estrogenicity. Or they can consist of a wider number of DNA fragments, for example the entire genome of the species or organism of interest. The different DNA fragments, known as probes, are placed in designated positions on the slide, to enable gene identification at the end of the procedure. A RNA sample of interest (known as the 'target sample') is purified, amplified and labelled using a fluorescent dye. The target sample is then placed onto the slide, where it undergoes hybridisation; i.e. is allowed to react with the DNA fragments (probes) attached to the slide to create probe-target interaction. This hybridisation is left to occur under optimum conditions, including high ionic strength buffers, and at elevated temperatures. Following hybridisation, the arrays are washed to remove any cross-hybridisation, where the targets have bound to the incorrect probes. As the target sample has been labelled using a fluorescent dye, this fluorescence can be measured (Dufva, 2009). By measuring the fluorescence, with further analysis, an assessment can be made as to which individual genes have been up-regulated, down-regulated or remained unchanged in comparison with a control sample. The expression of each differentially expressed gene is then assessed to see if they belong to a common pathway, which can inform as to whether there is the potential for a biological function to be affected.

Different types of microarrays

There are three main types of commonly used microarray technologies; spotted microarrays, Affymetrix GeneChips, and other *in situ* synthesised assays (Seidel, 2008). Spotted microarrays consist of probes which are oligonucleotides, cDNA or small fragments of PCR products; these are designed to correspond to the mRNA in the target sample. The probes are spotted onto a glass slides, using fine needles which are controlled by a robotic arm. The needles pick up DNA from microtiter plates and place it onto the surface of the glass slide (Seidel, 2008). After the initial expenditure for the hardware, this is a low cost option, and enables researchers to produce their own customised microarrays. However, they are unstandardised, and can be labour intensive.

Affymetrix GeneChips are the most established commercial array. These consist of multiple short oligonucleotides (usually 25-mers), synthesised *in situ* onto the surface of the chip (Hobman et al., 2007; Seidel, 2008). This method allows a higher density of

oligonucleotides, and uses photolithographic techniques - the same techniques utilised for producing computer chips, hence the use of the name GeneChips (Dale et al., 2012).

Other commercial companies have differing methods of producing microarrays which are either based on the spotted array or Affymetrix methods. These companies include Agilent, Nimblegen, Oxford Gene Technology, Xeotron, Combimatrix, Febit and Nanogen (Hobman et al., 2007). The microarrays used in this project were sourced from Agilent Technologies. Agilent use a process which enables the construction of long oligonucleotides (60-mers) onto glass slides using ink jet printing (Hobman et al., 2007). The ink jet printing technology was developed by Hewlett Packard, and modified to control the liquid precursors of DNA synthesis. Agilent Technologies formed out of Hewlett Packard. The printing technology means that it is a very flexible technology and the arrays can be easily customised to researcher's requirements (Seidel, 2008). However, it is reported that its flexibility results in difficulties in comparing results from different experiments. With the Affymetrix method, whereby the chips are mass produced, and all the same, the comparison from intra- and inter-laboratories is easier than when comparing the results from studies using customised Agilent arrays (Seidel, 2008).

In addition to the different types and methods of production of microarrays, there are also two-colour or one-colour (single channel) arrays. This refers to the number of dyes used to measure gene expression. One-colour measures the intensity, so is therefore an absolute reading of gene expression, whereas the two-colour measures the ratio of the two colours or the relative difference of the gene expression between two samples (Xiao et al., 2006). Figure 6.1 illustrates the basic principles of the two methods: for the purposes of this work, a one-colour (single channel) system was used. The two-colour approach is the most established method and has been used for longer than the one-colour approach.

With the two-colour approach, the mRNA from a biological sample of interest (e.g. the diseased tissue, exposed cells, or tissue from a treated organism) and a control sample are isolated and cDNA is amplified. The target cDNA is labelled with one dye (cyanine 5 dye (Cy5), red dye), and the control cDNA sample is amplified and labelled with another dye (cyanine 3 dye (Cy3), green dye) (Figure 6.1). Both these labelled cDNA samples are then combined and hybridised to one single array. Where the probes on the array are measured to be red, then it can be assessed that that corresponding gene is expressed in the sample of interest. Where the probes on the array are measured to be green, then it is assessed that the corresponding gene is being expressed in the control sample. Where the probes are measured to be yellow then it can be determined that these genes are being expressed in both the sample of interest and the control sample (Dale et al., 2012). For this approach

ratios are generated from direct comparison between two samples, and the experimental design would have to incorporate enough individuals, tissue, samples, or cells, to have adequate number of comparisons.

For the one-colour approach, samples are treated the same way as with the two colour approach, until they reach the labelling stage (Figure 6.1). Instead of two different dyes being used, one dye is used, and with the Agilent system, this is cyanine 3 (Cy3), due to it being less vulnerable to environmental degradation including ozone, pH and organic solvents compared to cyanine 5 (Cy5) (Xiao et al., 2006). The labelled samples (target and control) are then each hybridised to an individual array (Figure 6.1). The intensity of the green colour from the probes is then measured, giving an expression level for each gene. These readings from each array can then be compared to the others to determine the differences in gene expression between samples. It was decided that the one-colour approach was preferable than the two-colour approach, because the one-colour are more cost effective than the two-colour approach, especially with the number of replications (the experiment was repeated three times), time-points (six time-points, 8 hpf to 48 hpf) and treatments (six product waters from the IPR plant, plus a tap water and a control).

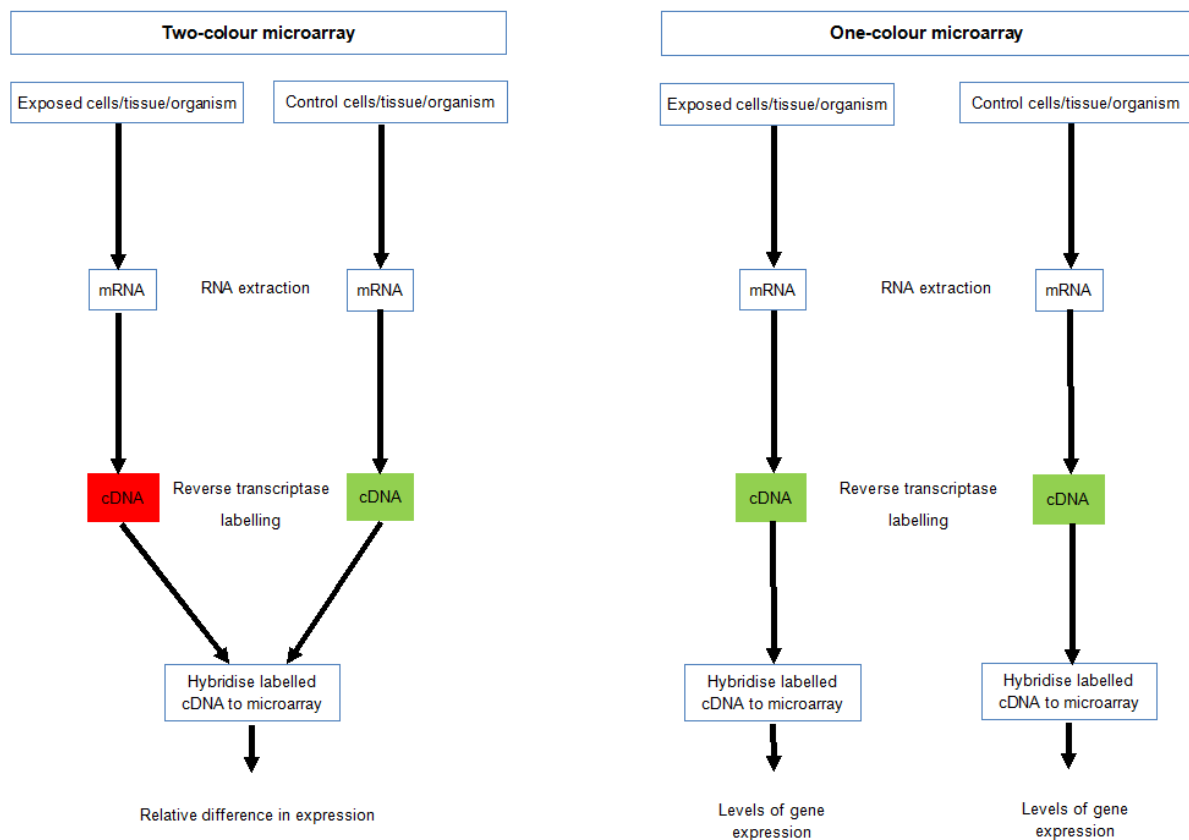

Figure 6.1 Schematic to demonstrate some of the differences between two-colour and one-colour microarrays.

6.3 Methods

6.3.1 Gene Expression Exposures

The aim of this part of the project was to determine if differences in gene expression levels occurred between zebrafish embryos reared in product waters from the IPR plant, relative to gene expression in embryos reared in control water. As developing embryos were being used, and their gene expression levels were being determined, it was considered important to identify time-points that had the greatest amount of genomic activity and which also encompassed critical developmental windows during embryogenesis. The Zfin website was used which hosts the Zebrafish Model Database (University of Oregon, 2015). This website contains a wealth of information on zebrafish, including their development and gene expression. It contains details of specific genes that are expressed during certain stages of development, and at what point in development specific organs and biological systems are forming.

Figure 6.2 below represents the reported number of genes expressed in the developing zebrafish embryo associated with growth of biological systems. It is not an exhaustive list and the data were collated at the end of 2011. However, this information was used to gain a better understanding of the time points that would be most relevant to sample at during development, in order to measure gene expression of the embryos. Early development was chosen because it is sensitive to chemical interference and disruption, leading to organisational and often permanent changes in phenotype (Schug et al., 2011) . However, as genes can be "switched-on" and then "switched-off", it was important to choose time points where large number of genes were being expressed. Figure 6.2 indicates that from 5.25 hpf and 48 hpf, there are a larger number of genes involved in the development of biological systems being expressed. This time period fell into the majority of time points used for observations in the previous developmental observation exposures. The 4 hour time appeared to have fewer expressed genes than the other 6 time points, and 4 hours was considered to be a very short exposure time. For this reason 4 hpf was not included as a sampling point for the gene expression exposures. Instead, it was decided that 8, 12, 16, 24, 36 and 48 hpf would be most relevant, and would provide a good representation of many key stages in the development of biological systems.

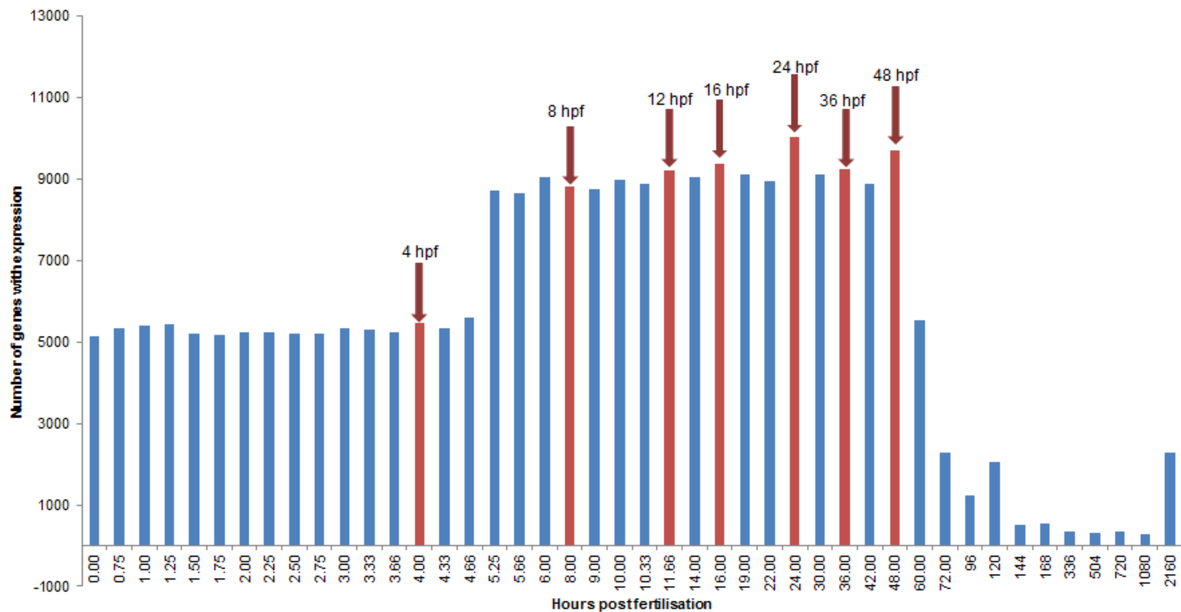

Figure 6.2 The graph represents the number of genes expressed during the development of zebrafish embryos at the key stages of embryo growth. The data were gathered from the Zfin website (University of Oregon, 2015) and were collated on 11/10/2011. The figure does not represent all genes expressed, only genes reported on the website that are associated with developing zebrafish, and is not an exhaustive list of the number of genes being expressed at each time point. The time points used for observations points in the previous zebrafish 48 hour developmental exposure are highlighted in red and by the arrows.

The time points at which various anatomical structures were first evident were also examined using data gathered from Zfin website (University of Oregon, 2015) and available at the end of 2011. It was determined that many anatomical structures first became evident between 14 and 16 hpf, and it was logical to assume that significant gene expression would occur before and during this time, throughout the formation of these structures. Some of the structures remain for the duration of the animal's life, and others develop to form more advanced structures. The largest number of structures to become evident for the first time occur at 14-16 hours. At this time point parts of the brain, endocrine system, immune system, neural tube, olfactory system, pancreas primordium and renal system are evident. Prior to this time, at 11.66-14 hours, parts of the cardiovascular system, immature eye, muscle cells, muscle pioneer, neural rod, and reproductive system are evident. Therefore, before 14 hours, a number of genes will have been expressed that are involved in the development, regulation and maintenance of these structures

From hours 22 to 24 hours additional structures that go on to develop further include mature blood, eye, inner ear, kidney, liver, and pancreatic bud, are evident, and from hours 30 to 48

hours more parts of the heart and liver are evident. At 19-22 hours there is a peak in structures becoming evident; these include the heart, inner ear, and kidney.

The stages of development that appeared as though they could be best for microarray analysis were 12-14, 19-22, 24 and 48 hpf, with greatest importance being given to 24 hours, when maximum gene expression associated with developmental and growth occurs, and many of the organs and biological systems have formed, or are in the process forming. There is an obvious rise in the number of reported genes expressed between 4.5 hours and 5.25 hours (Figure 6.2). However, it was decided that considering exposure time, the embryos at this stage would have only been exposed for a relatively short time. It is important to note that the data on the number of genes expressed collated from the Zfin website represent only the number of genes with expression data, and do not represent the actual total number of genes expressed.

Based on the available information, it was decided that 8, 12, 16, 24, 36 and 48 hpf would be the most relevant time points as they coincided with important developmental stages that had been previously documented, and (with the exception of 4 hours, due to the lack of exposure time) were consistent with observation time points used previously in the developmental exposures.

The detailed material and methods for the gene expression exposures are detailed in the previous General Material and Methods chapter. Any deviations from these methods are detailed below.

All the gene expression exposures were carried out during March 2012.

Gene expression exposure No.1

Composite 24 hour samples were collected from the IPR plant for all treatment processes, except for post AOP2 and final product, because these two treatment processes were shut down at time of sample collection. It was decided that the product water for the rest of the processes should be collected, and that the exposure should go ahead, as the IPR plant was going to be decommissioned at the end of March 2012, and it was unclear if these two processes would be fixed in time.

Due to deterioration in health and egg production in the Founder (F0) fish used to generate embryos in the 48 hour observation studies, it was decided that the F1 generation should be used as breeding adults instead.

Gene expression exposure No. 2

Composite 24 hour effluent samples were collected from all 6 stages of the IPR treatment process.

Gene expression exposure No. 3

Composite 24 hour effluent samples were collected from all 6 stages of the IPR treatment process.

6.3.2 Microarray

In October/November 2012, the embryos (preserved in RNAlater) from these exposures were taken to the Institute of Integrative Biology at Liverpool University and the microarrays were completed with guidance and instruction from Dr Philipp Antczak.

6.4 Results and analysis

The microarrays and the analysis were carried out at the Institute of Integrative Biology at Liverpool University by Dr Philipp Antczak and overseen by Professor Francesco Falciani. I worked alongside Dr Antczak to complete the practical work and he completed the analysis which is detailed below. I worked alongside Dr Richard Evans, Brunel University, to complete some of analysis shown on individual gene expression.

For all the microarrays carried out, Quality Control (QC) files are available and can be obtained from the author.

6.4.1 Normalisation

The data was initially normalised, so that variation between the arrays could be analysed alone, without the variations caused by non-biological sources. Bolstad et al., (2003) describe the existence of two types of variations when analysing multiple arrays; these are interesting variations, and obscuring variations. The interesting variation would be the biological difference between samples arising from differential gene expression in exposed and un-exposed organisms. The obscuring variation arises from the processes involved in conducting the experiment; for example, manufacture of the arrays and sample preparation (labelling, hybridization and scanning) (Bolstad et al., 2003; Potier & Rivals, 2012). Some of these can be minimised using specific experimental designs whereby the same time-point is used, and the experiment conducted by the same person each time; but it is impossible to control for every potential variation, which is why normalisation is used to correct for this (Potier & Rivals, 2012). When normalising microarray data for two-colour arrays there are two steps of normalisation to complete: normalisation between arrays, followed by normalisation within arrays. Normalisation *between* arrays ensures that the intensities have a similar distribution across the group of arrays being analysed, whereas normalisation

within arrays accounts for differences in the signals associated with Cy3 and Cy5 dyes known as dye bias (Carzaniga et al., 2007; G. Smyth, n.d.). As one-colour arrays were utilised for this study, only normalisation between arrays was required. For one-colour arrays, two methods of normalisation *between* the different arrays used can be applied; quantile normalisation and/or cyclic loess normalisation (G. Smyth, n.d.). Both of these methods were carried out, and the resulting normalised data used to create Principal Component Analysis (PCA) plots to make an initial assessment of the data generated from the microarrays.

Quantile normalisation is the most popular method of normalisation for data generated from a one-colour array. It makes the assumption that the distribution of the intensities across the arrays are the same, and without experimental error (Carzaniga et al., 2007). Quantile normalisation can either be conducted by selecting one chip as a baseline against which all other chips are normalised against, or by using a pairwise method (Bolstad, 2001). Quantile normalisation was used to begin with, and the PCA shown in Figure 6.2 and Figure 6.3 clearly illustrates how the developmental stage of the embryos had the strongest influence over the gene expression (i.e. separated the groups) compared to any of the other variables including experiment/exposure (1, 2 or 3) and exposure to the different IPR product waters.

If the tap and control waters are treated as chemical exposures, there were some differences between the three individual experiments (1, 2 and 3); i.e. the experiments themselves influenced the gene expression (Figure 6.4).

For the second method of normalisation, cyclic loess normalisation was used to create the PCA plot shown in Figure 6.5. Cyclic loess normalisation subjects each conceivable pair of arrays to loess normalisation (Smyth, n.d.) which performs locally weighted linear regressions on the whole dataset, and then combines them to produce one smooth curve (Stekel, 2003; Wade, 2007). Therefore, each data point within the dataset was normalised against the corresponding control group. For example, RO 8 hpf Experiment 1 was compared against Control 8 hpf Experiment 1, and MF 36 hpf Experiment 3 was normalised against Control 36 hpf Experiment 3. Subsequently a ratio is calculated, and the influences of the embryo's developmental stage and experiment (1, 2 or 3) were removed, making it possible to see if there were any differences due to exposure to the different IPR product waters. A PCA plot again was used to make initial assessments using this normalisation data (Figure 6.5).

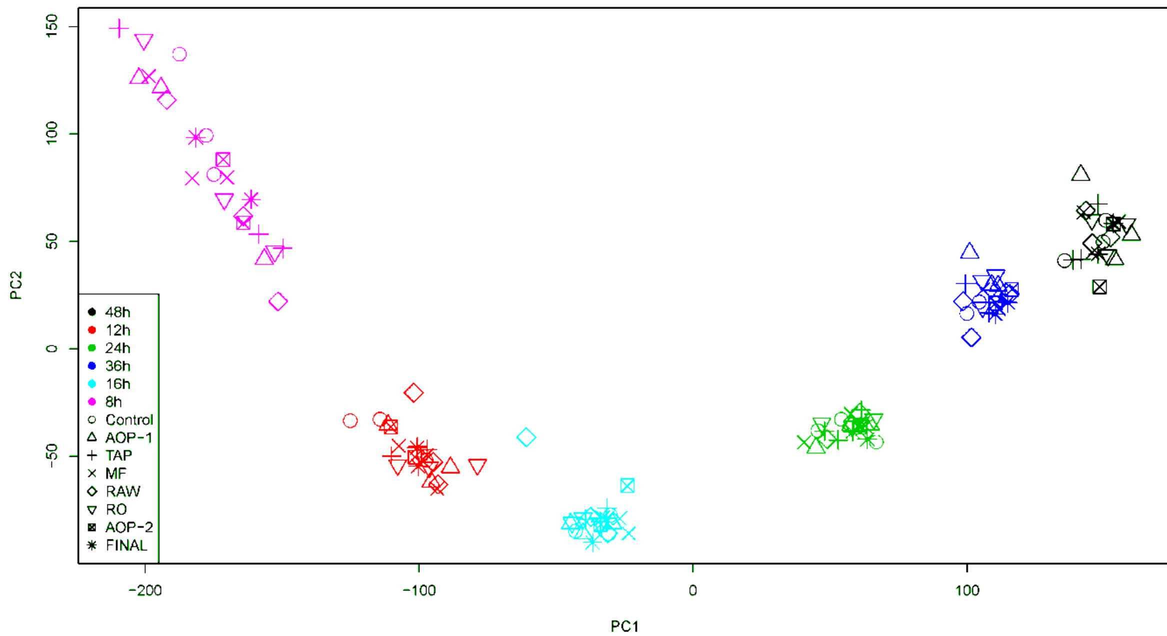

Figure 6.3 Quantile normalised principal component analysis (PCA) plot showing similarities in gene expression in the pooled embryos exposed to at the different time points in the different IPR product waters, tap and control.

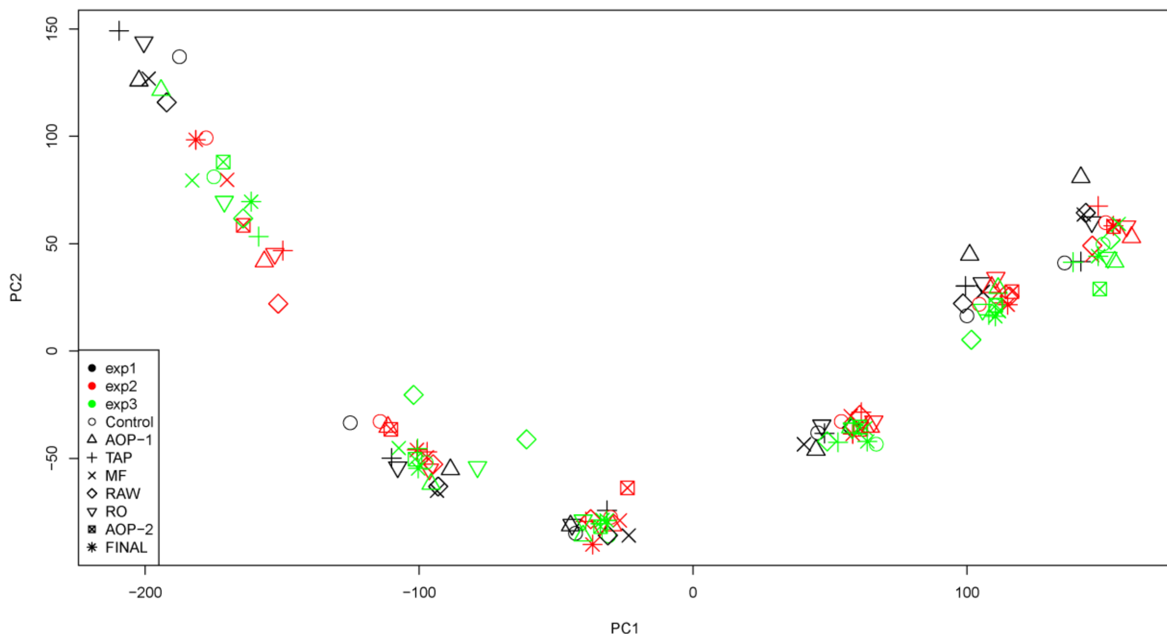

Figure 6.4 PCA plot whereby both the tap and control waters were considered as chemical treatments and the different experiments (1, 2 and 3) are highlighted, showing that the experiment has an influence on the gene expression.

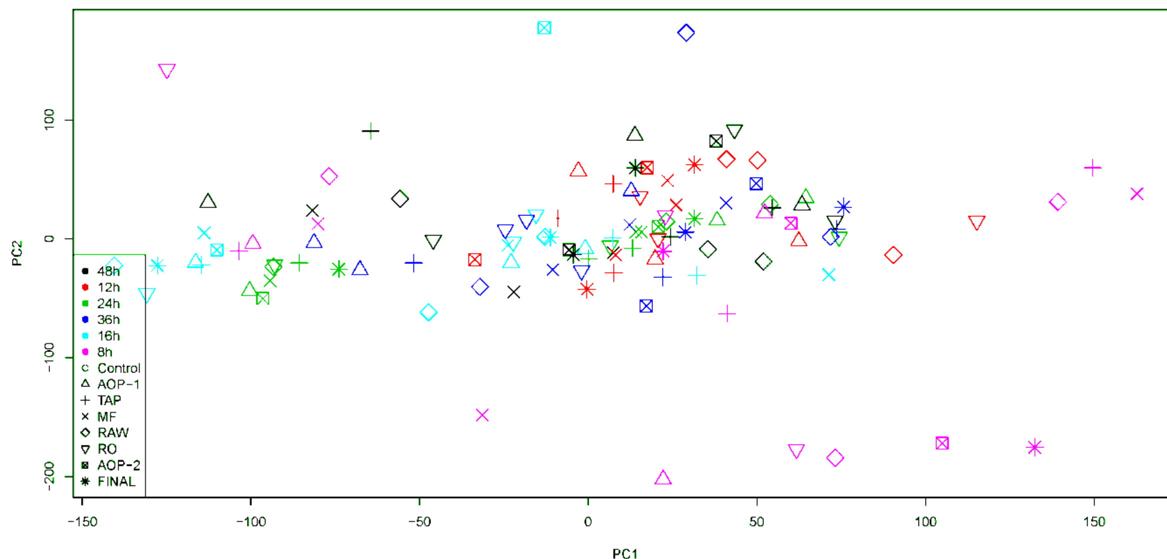

Figure 6.5 PCA plot of data normalised against the control group.

6.4.2 Individual gene expression

A two group comparison using Limma (an R/Bioconductor software package) was carried out on each individual gene expression dataset, against the corresponding control; for example, 8 hour control against 8 hour final product. Within Limma the toptable function was used; this function summarises the results of the model, conducts hypothesis tests, and adjusts the p-values to allow for multiple testing (G. K. Smyth, 2004). The analysis provides log fold changes, average expression, moderated t-test, standard error, P-value, and adjusted P-value (G. K. Smyth, 2004). The package uses a Bayesian model to moderate the standard errors by using all genes, which reduces the error to a common value, resulting in P-values with a normal t-statistic, but the moderated t-statistic has increased degrees of freedom, increasing the reliability. The adjustment made to the adjusted P value is in accordance with Benjamini and Hochberg’s method to control the false discovery rate.

The B-statistic labelled as ‘B’ in Table 6.1 is the log-odds of an individual gene being differentially expressed.

False discovery rates (FDRs) can be calculated from the p-value using the Benjamini-Hochberg procedure; this requires that the p-values are uniformly distributed under the null hypothesis. The method then sorts the p-values into ascending order, and then divides each p-value by its percentile rank to calculate an estimated FDR. Very small p-values near the bottom of the list will end up having a small FDR estimate (Noble, 2009).

Table 6.1 Individual genes which were identified as being differentially expressed compared to the same genes in the control samples. Log fold change (LogFC)>0 denotes that the gene is being up-regulated compared to that of the control and <0 is down-regulated to that of the control.

Treatment	hpf	Probe Name	Systematic Name	logFC	Gene name identified from DAVID	Average Expression	t	P.Value	adj.P.Val	B
Tap	48	A_15_P195156	NM_001037674	0.517261	somatolactin beta	2.790518	8.730334	1.39E-07	0.00605	5.831553
Final Product	16	A_15_P683421	TC381473	0.562369	Unknown	2.336993	9.139347	6.95E-08	0.003034	6.263581
	16	A_15_P661806	NM_001030127	0.360696	zgc:114065	2.509881	7.575909	8.83E-07	0.015055	4.635352
	16	A_15_P132886	NM_205622	0.329937	luteinizing hormone beta 1; luteinizing hormone, beta polypeptide	2.340346	7.484641	1.03E-06	0.015055	4.528206
	16	A_15_P400500	TC376026	0.248182	Unknown	2.382267	7.143164	1.89E-06	0.020599	4.114621
	24	A_15_P679141	NM_001105113	0.499824	novel protein similar to vertebrate phospholipid scramblase 1	2.556971	15.23649	4.61E-11	2.01E-06	10.77498
	24	A_15_P190981	ENSDART00000057296	0.281613	Unknown	2.554738	7.309202	1.57E-06	0.034352	4.585307
	24	A_15_P270651	NP13323810	0.276651	Unknown	2.409246	7.023698	2.60E-06	0.037799	4.210404
	36	A_15_P262686	A_15_P262686	0.421607	Unknown	2.348355	9.18382	6.40E-08	0.002792	6.332795
	48	A_15_P584892	TC395851	0.800674	Unknown	2.547976	11.58461	2.44E-09	0.000106	7.348301
	48	A_15_P195156	NM_001037674	0.500144	somatolactin beta	2.790518	7.550252	9.64E-07	0.021051	4.167615
AOP2	8	A_15_P625245	NM_001128380	0.550568	odorant receptor, family E, subfamily 128, member 7; odorant receptor, family E, subfamily 128, member 6	2.482616	8.548881	1.62E-07	0.00709	3.857985
	16	A_15_P561292	NM_001127254	0.263603	novel immune-type receptor	2.474206	7.733182	6.74E-07	0.029446	4.816688
	48	A_15_P195156	NM_001037674	0.61238	somatolactin beta	2.790518	9.244572	6.28E-08	0.00274	5.754219
RO	48	A_15_P195156	NM_001037674	0.641299	somatolactin beta	2.790518	10.82385	6.61E-09	0.000288	7.583933
	48	A_15_P513717	EH438239	0.371965	Unknown	3.053541	7.174754	1.86E-06	0.020266	4.122775
	48	A_15_P733840	EH438239	0.343911	Unknown	3.103451	6.671604	4.60E-06	0.033488	3.482535
	48	A_15_P101761	NM_001001849	0.206208	transient receptor potential cation channel, subfamily V, member 6	2.636785	7.699042	7.48E-07	0.016322	4.742881
	48	A_15_P150986	NM_001017865	0.150367	si:dkey-24l11.8	3.629266	6.766433	3.87E-06	0.033488	3.606658

Treatment	hpf	Probe Name	Systematic Name	logFC	Gene name identified from DAVID	Average Expression	t	P.Value	adj.P.Val	B
	48	A_15_P631042	NM_181760	-0.14208	annexin A1c	3.921124	-7.23007	1.68E-06	0.020266	4.190432
AOP1	12	A_15_P131961	NM_001145708	0.377019	Cytochrome P450, family 1, subfamily B, Polypeptide	2.531125	7.867428	6.48E-07	0.028271	5.150077
	16	A_15_P164691	NM_001110391	0.172887	cerebellin 4 percursor	2.552454	8.701309	1.37E-07	0.006	6.29359
	24	A_15_P659656	NM_131879	0.40655	Cytochrome P450, family 1 subfamily A	2.972813	7.990429	4.97E-07	0.010857	5.737169
	24	A_15_P100578	NM_131879	0.279395	Cytochrome P450, family 1 subfamily A	3.514583	8.225231	3.39E-07	0.010857	6.02719
	48	A_15_P659656	NM_131879	0.562108	Cytochrome P450, family 1 subfamily A	3.10311	11.16808	4.18E-09	0.000183	7.82275
	48	A_15_P131751	NM_131879	0.510272	Cytochrome P450, family 1 subfamily A	3.109281	9.627507	3.55E-08	0.000516	6.650837
	48	A_15_P100578	NM_131879	0.431093	Cytochrome P450, family 1 subfamily A	3.647145	9.925879	2.30E-08	0.000502	6.899576
MF	12	A_15_P171581	NM_001145708	0.70819	Cytochrome P450, family 1, subfamily B, Polypeptide	2.860979	7.485303	1.23E-06	0.010725	4.692729
	12	A_15_P659656	NM_131879	0.60633	Cytochrome P450, family 1 subfamily A	2.880383	7.812946	7.09E-07	0.007734	5.08635
	12	A_15_P548722	TC414122	0.599259	Unknown	2.405274	8.92135	1.22E-07	0.002444	6.290873
	12	A_15_P100578	NM_131879	0.448848	Cytochrome P450, family 1 subfamily A	3.412419	6.959559	3.06E-06	0.022259	4.022909
	12	A_15_P131751	NM_131879	0.445688	Cytochrome P450, family 1 subfamily A	2.949657	6.695098	4.91E-06	0.030623	3.667686
	12	A_15_P131961	NM_001145708	0.444175	Cytochrome P450, family 1, subfamily B, Polypeptide	2.531125	9.268807	7.23E-08	0.002444	6.630814
	12	A_15_P339770	ENSDART00000114701	0.435986	Unknown	2.633949	8.712291	1.68E-07	0.002444	6.078005
	24	A_15_P659656	NM_131879	0.511047	Cytochrome P450, family 1 subfamily A	2.972813	10.04424	2.18E-08	0.000475	7.995783
	24	A_15_P100578	NM_131879	0.356946	Cytochrome P450, family 1 subfamily A	3.514583	10.50829	1.14E-08	0.000475	8.426655
	36	A_15_P659656	NM_131879	0.502182	Cytochrome P450, family 1 subfamily A	3.011647	8.222874	2.93E-07	0.007741	5.784139
	36	A_15_P131751	NM_131879	0.461879	Cytochrome P450, family 1 subfamily A	3.023627	8.108303	3.55E-07	0.007741	5.650983
	36	A_15_P100578	NM_131879	0.366185	Cytochrome P450, family 1 subfamily A	3.546661	6.863995	3.09E-06	0.044994	4.06378
	48	A_15_P659656	NM_131879	0.62017	Cytochrome P450, family 1	3.10311	12.32167	9.74E-10	4.25E-05	8.545839

Treatment	hpf	Probe Name	Systematic Name	logFC	Gene name identified from DAVID	Average Expression	t	P.Value	adj.P.Val	B
					subfamily A					
	48	A_15_P131751	NM_131879	0.608141	Cytochrome P450, family 1 subfamily A	3.109281	11.47404	2.81E-09	6.13E-05	8.030865
	48	A_15_P100578	NM_131879	0.431144	Cytochrome P450, family 1 subfamily A	3.647145	9.927049	2.30E-08	0.000334	6.905441
Final effluent	12	A_15_P171581	NM_001145708	0.722068	Cytochrome P450, family 1, subfamily B, Polypeptide	2.860979	7.631994	9.59E-07	0.012178	4.871177
	12	A_15_P659656	NM_131879	0.585301	Cytochrome P450, family 1 subfamily A	2.880383	7.541975	1.12E-06	0.012178	4.762099
	12	A_15_P164111	NM_130940	0.568771	similar to bonnie and clyde; bonnie and clyde	2.769646	12.5328	9.78E-10	4.27E-05	9.116653
	12	A_15_P100578	NM_131879	0.458201	Cytochrome P450, family 1 subfamily A	3.412419	7.10458	2.37E-06	0.020691	4.212463
	12	A_15_P131961	NM_001145708	0.431418	Cytochrome P450, family 1, subfamily B, Polypeptide	2.531125	9.002601	1.08E-07	0.002352	6.371895
	24	A_15_P659656	NM_131879	0.407037	Cytochrome P450, family 1 subfamily A	2.972813	7.999995	4.90E-07	0.010687	5.749158
	24	A_15_P100578	NM_131879	0.301522	Cytochrome P450, family 1 subfamily A	3.514583	8.876656	1.21E-07	0.005299	6.786937
	24	A_15_P213061	NM_001030106	0.228872	si:busm 1-132m23.3	2.501567	7.368465	1.42E-06	0.020668	4.925478
	24	A_15_P500697	ENSDART00000146728	0.153671	Unknown	2.794794	6.935972	3.04E-06	0.033141	4.322612
	48	A_15_P659656	NM_131879	0.474143	Cytochrome P450, family 1 subfamily A	3.10311	9.420367	4.82E-08	0.001319	6.471406
	48	A_15_P131751	NM_131879	0.449317	Cytochrome P450, family 1 subfamily A	3.109281	8.477439	2.07E-07	0.003012	5.579879
	48	A_15_P100578	NM_131879	0.402595	Cytochrome P450, family 1 subfamily A	3.647145	9.269723	6.04E-08	0.001319	6.337353

The functions for some gene probes are not known, which is why there are some “unknowns” in the gene identification column.

In the tap water exposure, when the gene expression was compared to that of the aquarium water controls, only one gene was determined as significantly differentially expressed, and that was somatolactin beta, with over expression.

In final product there were ten individual genes which were determined to be differentially expressed compared to the control; all of them exhibited over expression. Six of these genes have not been identified; the others were zgc-114065, luteinizing hormone, a novel protein similar to vertebrate phospholipid scramblase1, and somatolactin beta.

In AOP2 product, three individual genes were determined to be differentially expressed compared to that of the control, all of them with over expression. These genes were an odorant receptor gene, an immune-type receptor gene, and somatolactin beta.

In reverse osmosis product, there were six individual genes determined to be differentially expressed compared to that of the control- all of them with over expression, apart from one which was under expressed. The under expressed gene was annexin A1c, the other five over expressed genes were two unidentified genes, cation channel gene, si:dkey-24I11.8, and somatolactin beta.

In AOP1 product there were three individual genes that were determined to be differentially expressed compared to that of the control - all of them with over expression. These were Cytochrome P450, family 1, subfamily B, polypeptide, Cytochrome P450, family 1 subfamily A, and cerebellin 4 percursor.

In microfiltration product there were four individual genes determined to be differentially expressed compared to that of the control - all of them with over expression. Two of these genes have not been identified and the other two were Cytochrome P450, family 1, subfamily B, polypeptide, Cytochrome P450, family 1 subfamily A.

In final effluent there were five individual genes determined to be differentially expressed compared to that of the control - all of them with over expression. One has not been identified, a gene similar to bonnie and clyde, sibusm 1-132m23.3, Cytochrome P450, family 1, subfamily B, polypeptide, Cytochrome P450, family 1 subfamily A.

It was noted that somatolactin beta was over expressed in the embryos exposed to tap, final product, AOP2, and reverse osmosis product waters; all of these waters have undergone significant treatment. Additionally, it was observed that the same two cytochrome P450 genes were over expressed in the first half of the IPR treatment process in embryos exposed to product waters from AOP1, microfiltration and final effluent.

6.4.3 2-Factor ANOVA Gene Level

Using the normalised data from the quantile and loess methods, a 2-factor ANOVA for gene level was carried out. Examining the influence of the experiment (1, 2 or 3) on gene expression, there were 20746 and 21238 genes differentially expressed using the quantile and loess normalised data, respectively. This indicates that there was a large number of individual genes being differentially expressed in the three different experiments. By assessing the influence of developmental stage of the embryos (hours post fertilisation) on the number of differentially expressed genes using the two methods, it was found that there were 29467 and 27932 differentially expressed genes using the quantile and loess normalised data, respectively, thus indicating that the developmental stage had a greater influence on the number of genes differentially expressed than the experiment (1, 2 or 3). The number of differentially expressed genes based on IPR treatment process (i.e. sample), was only 63 and 34 based on the quantile and loess normalised data, respectively.

6.4.4 2-Factor ANOVA Pathway Level

Another 2-factor ANOVA was carried out, but at the pathway level rather than just individual gene level. Assessing the influence of the experiment (1, 2 or 3) on differentiated expression of pathways in the zebrafish embryos determined that there were 329 and 269 pathway indices differentially expressed using the quantile and loess normalised data, respectively. Examining the influence of the developmental stage of the embryos (hours post fertilisation) on the number of pathways indices using the two methods, there were 392 and 289 differentially expressed pathway indices using the quantile and loess normalised data, respectively. The number of pathway indices differentially expressed based on IPR treatment process (sample) was only 10 and 5 when using the quantile and loess normalised data, respectively. However, from this small number of pathway indices, it was determined that these were all pathways broadly involved in hormone synthesis/metabolism and detoxification, suggesting that the embryos had upregulated their detoxification mechanism when exposed to the different IPR treatment process waters, and these mechanisms may also affect hormone synthesis. This is because steroid hormone synthesis is reported to be controlled by activity of a number of cytochrome P450 enzymes and some steroid dehydrogenases and reductases (Sanderson, 2006). The pathways that are influenced (using the quantile normalised data method) by the IPR treatment process (sample) group are as follows (number in the brackets indicates number of indices among the three different exposures repeated):

1. Steroid hormone biosynthesis (3/3)
2. Tryptophan metabolism (1/3)

3. Retinol metabolism (2/3)
4. Metabolism of xenobiotics by cytochrome P450 (2/3)
5. Drug metabolism-cytochrome P450 (1/3)
6. Drug metabolism-other enzymes /(1/3)

The pathways that are influenced (using the loess normalised data method) by the IPR treatment process (sample) group are as follows (number in the brackets indicates number of indices):

1. Steroid hormone biosynthesis (1/3)
2. Tryptophan metabolism (1/3)
3. Retinol metabolism (1/3)
4. Metabolism of xenobiotics by cytochrome P450 (2/3)

Nonylphenols have been associated with disruptions to the steroid metabolism pathway; this is further discussed in section 6.5.3, Table 6.2 details the concentration of nonylphenols detected in the product waters along the IPR treatment process.

Table 6.2 Concentrations of nonylphenol detected in product waters along the IPR treatment plant.

Product water	Nonylphenol	Average (µg/l)	Minimum-Maximum (µg/l)	Standard Deviation
Final effluent	Nonylphenol (NP)	0.220168	0.2-2	0.18
	Nonylphenol monoethoxylate (NP1EO)	0.3*	0.3-0.3*	0.00*
	Nonylphenol diethyoxylate (NP2EO)	0.202381	0.2-0.3	0.02
	Nonylphenol triethoxylate (NP3E0)	0.30743	0.3-0.6	0.04
Microfiltration	Nonylphenol (NP)	0.234667	0.2-2.5	0.27
	Nonylphenol monoethoxylate (NP1EO)	0.3	0.3-0.3	0.00
	Nonylphenol diethyoxylate (NP2EO)	0.203125	0.2-0.3	0.02
	Nonylphenol triethoxylate (NP3E0)	0.304497	0.3-0.4	0.02
AOP1	Nonylphenol (NP)	0.204878	0.2-0.4	0.03
	Nonylphenol monoethoxylate (NP1EO)	0.301389	0.3-0.4	0.01
	Nonylphenol diethyoxylate (NP2EO)	0.2*	0.2-0.2*	0.00*
	Nonylphenol triethoxylate (NP3E0)	0.307042	0.3-0.6	0.04
Reverse osmosis	Nonylphenol (NP)	0.245283	0.2-2.6	0.33
	Nonylphenol monoethoxylate (NP1EO)	0.3*	0.3-0.3*	0.00*
	Nonylphenol diethyoxylate (NP2EO)	0.2*	0.2-0.2*	0.00*
	Nonylphenol triethoxylate (NP3E0)	0.3*	0.3-0.3*	0.00*
AOP2	Nonylphenol (NP)	0.232813	0.2-1.4	0.17
	Nonylphenol monoethoxylate (NP1EO)	0.3*	0.3-0.3*	0.00*
	Nonylphenol diethyoxylate (NP2EO)	0.201887	0.2-0.3	0.01
	Nonylphenol triethoxylate (NP3E0)	0.307692	0.3-0.5	0.03

*<LoD; data supplied by Thames Water; nonylphenols were not sampled for in the aquarium water

6.4.5 SAM Time-course Analysis

The Significance Analysis of Microarrays (SAM) determines whether alterations in gene expression are statistically significant or not, and works by conducting gene specific t-tests (Zheng et al., 2007). A small positive constant is added to the denominator of the gene-specific t-test; this ensures that genes with small fold changes are not recorded as being significant (Zheng et al., 2007). Additionally, it applies repeated permutations of the data to make an estimate of the percentage of genes significantly related to the response, as well as the genes identified by chance (false discovery rate, FDR). Both the fold change and FDR thresholds can be specified (Chu et al., n.d.; Zheng et al., 2007).

There are two approaches for the analysis of time-course data using SAM; these are 'slope based' and 'signed area' based. The slope based approach is applicable for analysing genes with a monotonous increasing or decreasing expression over time (Chu et al., n.d.). The signed area based approach is more appropriate for the dataset being analysed in this study as it is better at finding genes that increase and then plateau, or their expression returns to baseline levels (Chu et al., n.d.); put simply, the switching-on and -off of genes which would be expected to occur during embryonic development.

For this SAM time-course analysis, the slope and signed area were used for assessing the individual gene expression and pathways, by comparing all combinations of time-courses with two treatments at a time; for example Control 8hpf-48hpf vs Final effluent 8hpf-48hpf, and comparing all treatments as pseudo-one class time-courses. Therefore the individual IPR treatment process stages are used to order the analysis as along the time-course i.e. Final effluent 8hpf → MF 8hpf → AOP1 8hpf → RO 8hpf → AOP2 → FINAL 8hpf → Tap 8hpf →Control 8hpf. In this way each specific developmental stage is compared along the treatment process.

6.4.6 Gene level SAM time-course analysis

This analysis examined the expression of the individual genes. For this analysis, only the loess dataset was used because the quantile dataset showed a strong link to time related genes. The false discovery rates (FDR) were calculated for each combination for the SAM time-course and the smallest FDR was recorded in Table 6.3.

Traditionally, t-tests are designed to compare two sets of results, however in a microarray experiment the t-test is carried out tens of thousands of times. Therefore, based on random

chance, lots of genes will be determined as being differentially expressed (Carzaniga et al., 2007). The example given by Carzaniga et al., (2007) states that when random values are used, the t-test will consider 5% of the genes as significant. If a dataset consists of 10 000 genes this will mean 500 genes will be determined to be differentially expressed. Consequently, a correction factor is used on the p values to help control for these false positives; one method for correction is known as the FDR. FDR works by multiplying each p value by the total number of tests, and dividing by the number of (the remaining) larger p values. This results in a q value, and is the expected number of false positives (Carzaniga et al., 2007). Very often these q values range from 0 to 0.1 (10%), and, the lower the value, the fewer false positives there are, and the more confidence there are in the numbers of significantly difference reported in the gene expression. The FDRs reported in the Table 6.3 indicate that the majority of the genes found using the signed area approach are significant, but none of the genes found using the slope linked approach were significant.

Table 6.3 The minimum false discovery rate (FDR) for the loess normalised dataset using the SAM time-course analysis for gene level. The smallest FDR is indicative of whether there is anything significant or not

Comparison	Minimum FDR signed area approach	Minimum FDR slope approach
MF vs Final	0.08	1.12
Final effluent vs Final	0.11	1.09
Final effluent vs RO	0.14	0.8
MF vs RO	0.15	0.39
RO vs Final	0.28	1.1
Tap vs MF	0.29	1.23
MF vs AOP2	0.35	1.15
Final effluent vs AOP2	0.59	0.74
Tap vs RO	0.62	1.35
AOP1 vs RO	0.71	1.12
AOP1 vs Final	0.75	1.15
AOP1 vs Final effluent	0.78	1.16
Tap vs Final effluent	0.98	0.66
MF vs Final effluent	1.06	1.04
AOP1 vs MF	1.12	1.03
Tap vs Final	1.13	1.19
RO vs AOP2	1.13	1.08
Tap vs AOP2	1.14	1.18
AOP1 vs AOP2	1.16	0.28
AOP1 vs Tap	1.21	1.14
AOP2 vs Final	1.24	1.18

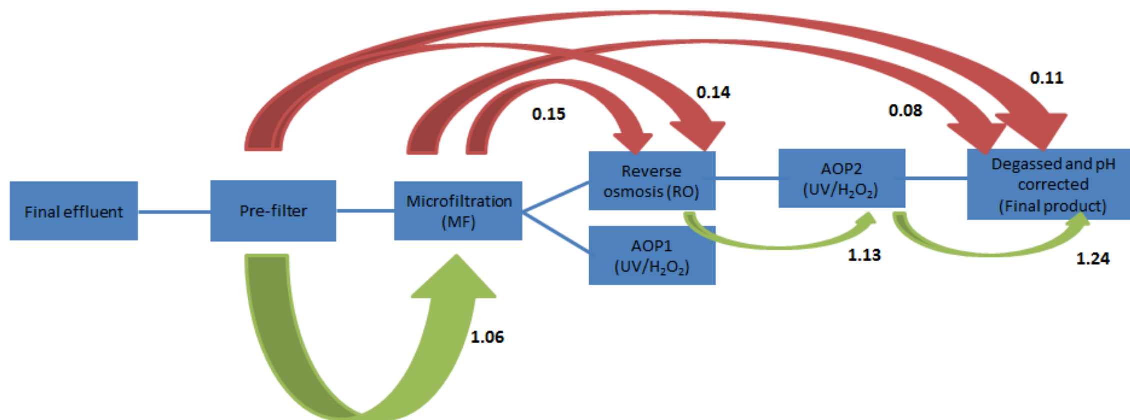

Figure 6.6 Illustration of the IPR treatment plant with the selection of the minimum FDRs for loess normalised dataset using SAM time-course analysis at gene level using the signed approach. The lower FDRs indicate larger difference in the gene expression. The minimum FDRs are displayed in Table 6.3

6.4.7 Pathway Level SAM time-course analysis

This was followed by carrying out the same analysis, but on the pathway level, using both loess and quantile normalised datasets. Loess does not contain any of the control values, as these were used for normalisation, therefore in the Table 6.4 they are recorded as NA. For the pathway level there are very few potentially significant sets. The only potentially significant sets are two in the quantile signed area approach, and one in the Loess slope methods, the FDRs are marked with a * in Table 6.4 and the specific pathways are detailed in Table 6.5.

Table 6.4 The minimum false discovery rate (FDR) for both the quantile and loess normalised datasets using the SAM time-course analysis for pathway level

Minimum FDR	Quantile normalised dataset using the slope approach	Loess normalised dataset using the slope approach	Quantile normalised dataset using the signed area approach	Loess normalised dataset using the signed area approach
Control vs AOP1	0.99	NA	1.28	NA
Control vs Tap	0.96	NA	1.33	NA
Control vs MF	0.59	NA	1.07	NA
Control vs FE	0.75	NA	1.06	NA
Control vs RO	1.03	NA	1.12	NA
Control vs AOP2	1.04	NA	0.93	NA
Control vs Final	0.43	NA	0.95	NA
AOP1 vs Tap	1.2	1.12	0.46	1.26
AOP1 vs MF	1.2	1	1.04	1.25
AOP1 vs FE	1.12	1.07	0.85	0.79
AOP1 vs RO	1.22	1.13	1.18	1.38
AOP1 vs AOP2	1.18	1.05	0.19*	1.09
AOP1 vs Final	1.08	1.1	0.37	0.6
Tap vs MF	1.1	1.26	1	0.6
Tap vs FE	1.27	1.43	0.99	0.77
Tap vs RO	1.23	1.49	1.06	1.37
Tap vs AOP2	1.2	1.38	0.7	1.12
Tap vs Final	0.63	1.21	0.81	1.3
MF vs FE	0.77	1.1	0.91	1
MF vs RO	1.13	1.26	1.06	0.32
MF vs AOP2	1.11	1.26	0.44	1.3
MF vs Final	0.27	1.09	0.12*	0.22
FE vs RO	1.07	1.26	1.02	0.4
FE vs AOP2	0.57	1.19	1.11	0.61
FE vs Final	0.42	0.17*	0.38	0.82
RO vs AOP2	1.39	1.27	1.05	1.33
RO vs Final	1.25	1.18	1.04	0.9
AOP2 vs Final	1.17	1.19	1.19	1.36

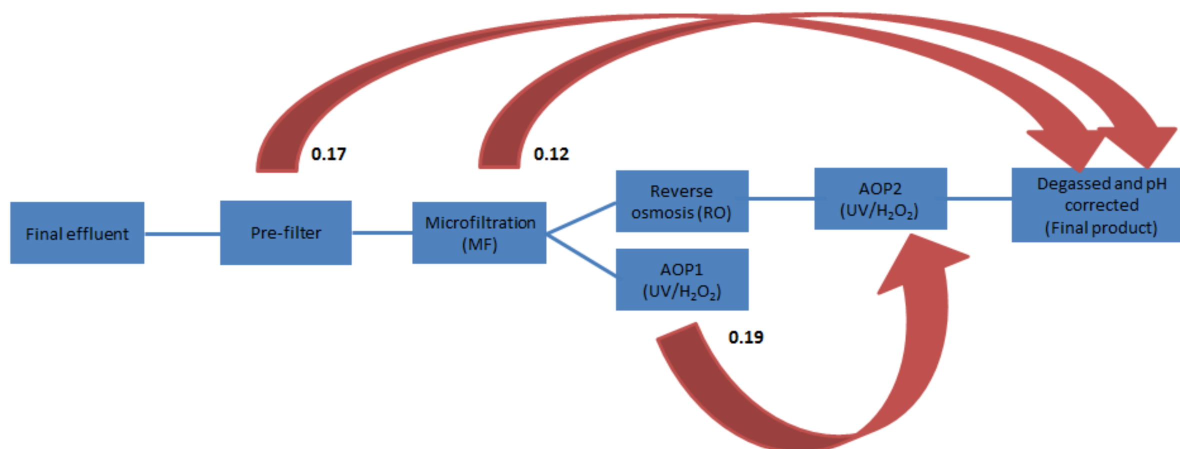

Figure 6.7 Illustration of the IPR treatment plant with the selection of the minimum FDRs for quantile normalised dataset using SAM time-course analysis at pathway level using both the signed and slope approach. The FDRs shown on the figure are significantly different. The minimum FDRs are displayed in Table 6.4.

Table 6.5 The three (highlighted in Table 6.4 above) significantly up- or down-regulated pathways using the two methods of normalising the data and the two methods of analysing them using the SAM approach.

Comparison	Minimum FDR	Normalisation method	Slope or signed area	Pathways up-regulated	Pathways down-regulated
AOP1 vs AOP2	0.19	Quantile	Signed area	Tryptophan metabolism	Metabolism of xenobiotics by cytochrome P450 Retinol metabolism
MF vs Final	0.12	Quantile	Signed area	Metabolism of xenobiotics by cytochrome P450 Retinol metabolism Steroid hormone biosynthesis	Tryptophan metabolism
Final effluent vs Final	0.17	Loess	Slope	None	Fatty acid metabolism Valine, leucine and isoleucine degradation Synthesis and degradation of ketone bodies

6.4.8 Pseudo Time-course

Instead of comparing one treatment against another along the time series (8 to 48 hpf), a pseudo time-pseudo time-course was used to analyse the data. This time-course used the different treatment treatment processes from the IPR plant, starting at the least processed sample and moving along the along the process to the final product, and, finally, the control water,

Figure 6.8.

Final effluent (Raw) → MF → AOP1 → RO → AOP2 → Final product → Control

Figure 6.8 Illustration of the order of the pseudo time-course. MF: microfiltration, AOP: advance oxidation process

The datasets that had undergone quantile and loess normalisation, SAM time course analysis slope approach, and signed area, were analysed using this pseudo time-course method, to determine individual genes (gene level) or entire pathways that had been up or down regulated along the treatment process at the different time intervals.

For both the gene level analysis and pathway analysis, the FDRs were calculated; the smallest values indicating the most significant difference in genomic regulation. Table 6.6 and Table 6.7 show the minimum FDRs for each method of analysis. As previously noted, it is considered that the signed area results are the most relevant when compared to the slope approach as the gene regulation would not necessarily be a gradual up- or down-regulation as the genes during development would be effectively "switching on and off".

Table 6.6 The false discovery rates (FDR) for both the quantile and loess normalised datasets using the SAM time-course analysis (both slope and signed area approaches) for individual gene expression were determined along a pseudo time-course. The table reports the minimum FDR for each time-point sampled.

Hours post fertilisation (hpf)	**Minimum FDR**			
	Quantile time Slope	**Quantile time Signed**	**Loess Time Slope**	**Loess Time Signed**
8 hpf	0.94	Inf	0.3	0.48
12 hpf	0.08	Inf	0.27	0.24
16 hpf	0.42	Inf	0.51	0.31
24 hpf	0.11	Inf	0.24	0.2
36 hpf	1.11	Inf	0.35	0.24
48 hpf	0.16	Inf	0.48	0.44

Table 6.7 The false discovery rates (FDR) for both the quantile and loess normalised datasets using the SAM time-course analysis (both slope and signed area approaches) for pathway level expression were determined along a pseudo time-course. The table reports the minimum FDR for each time-point sampled.

Hours post fertilisation (hpf)	**Minimum FDR**			
	Quantile time Slope	**Quantile time Signed**	**Loess Time Slope**	**Loess Time Signed**
8 hpf	0.89	0	1.12	0.52
12 hpf	0.07	0.01	0.07	0.47
16 hpf	0.25	0	0.24	0.24
24 hpf	0.22	0	0.08	1.17
36 hpf	0.33	0.03	0.8	0.15
48 hpf	0.44	0	0.1	Inf

The differentially regulated pathways listed in Table 6.8 were generated using the pseudo time-course from the dataset that underwent quantile normalisation, and signed area SAM time-course analysis. These pathways were the ones that had the five lowest FDRs, and,

therefore, here the most significantly altered along the IPR treatment process. For example, at 8 hpf, a total of 20 pathways were up-regulated along the treatment process, and the most significantly up-regulated pathway was pyrimidine metabolism.

Table 6.8 The up- and down-regulated pathways with the five lowest FDRs using the quantile normalised dataset signed area approach are displayed in the table below.

Hours post fertilisation (hpf)	Total number of pathways up-regulated	Top five pathways that were up-regulated	Total number of pathways down-regulated	Top five pathways that were down-regulated
8	20	1. Pyrimidine metabolism 2. Protein export 3. Tryptophan metabolism 4. Protein processing in endoplasmic reticulum 5. Peroxisome	155	1. Fatty acid metabolism 2. Valine, leucine and isoleucine degradation 3. beta-Alanine metabolism, 4. Purine metabolism 5. Propanoate metabolism
12	2	1. Metabolic pathways 2. Purine metabolism	176	1.Pyrimidine metabolism 2.O-Glycan biosynthesis 3.Melanogenesis 4.Neuroactive ligand-receptor interaction 5.Folate biosynthesis
16	65	1.Spliceosome 2.Drug metabolism - other enzymes 3.Sulfur relay system 4.Propanoate metabolism 5.Lysine degradation	155	1.Adherens junction 2.Galactose metabolism 3.Pyruvate metabolism 4.Lysosome 5.Arginine and proline metabolism
24	158	1.NOD-like receptor signalling pathway 2.Oxidative phosphorylation 3.Adherens junction 4.Cytokine-cytokine receptor interaction 5.TGF-beta signalling pathway	17	1.Arginine and proline metabolism 2.Lysosome 3.Tryptophan metabolism 4.Metabolic pathways 5.beta-Alanine metabolism
36	151	1.Hedgehog signalling pathway 2.Cytokine-cytokine receptor interaction 3.Selenoamino acid metabolism 4.Basal transcription factors 5.Regulation of actin cytoskeleton	21	1.Glycosphingolipid biosynthesis - lacto and neolacto series 2.Phagosome 3.ErbB signalling pathway 4.MAPK signalling pathway 5.Citrate cycle (TCA cycle)
48	133	1.Circadian rhythm – mammal 2.Metabolic pathways 3.Ubiquitin mediated proteolysis 4.NOD-like receptor signalling pathway 5.Purine metabolism	0	

6.5 Discussion

Once the microarray data had been normalised, it was used to create a PCA plot to gain a better understanding of the variables (experiment, treatment, stage of development) influencing the gene expression. In Figure 6.3 it can be clearly observed that developmental stage has an important effect on separating out the data spatially, indicating it had the greatest influence on the gene expression. Figure 6.4 and Figure 6.5 indicated that the three different independent repeat exposure experiments also influenced the gene expression. This finding could have been because the nature (including chemical composition) of the treated sewage effluent changed between sample times, and therefore the exposure media were not identical despite being collected from the same place albeit on different days. Therefore, the composition and physical properties may vary slightly. There would also be predictable slight variation in the control and tap waters that were used to compare the IPR treatments against. Future studies would take greater account of these differences, especially the tap water control. Additionally, the pairing of individual zebrafish in each breeding group used to obtain embryos was not the same, as they were randomly selected each time. Despite these differences, it is interesting to note that the different experiments could have such a large influence on the gene expression. The data collected from the observational exposures showed some indication that the survival, hatching and abnormalities could vary from one experimental day to another. However, survival in the exposures for gene expression did not significantly vary between experimental days. Nevertheless, it does demonstrate the importance of experimental repeats, and how subtle changes in the water chemistry can impact the results.

In the individual gene analysis it was determined that three genes were significantly over expressed in expressed in certain sections of the IPR plant. Somatolactin beta was over expressed in embryos embryos exposed to tap, final, AOP2, and reverse osmosis product waters; these treatments appear at

appear at the far end of the treatment process (

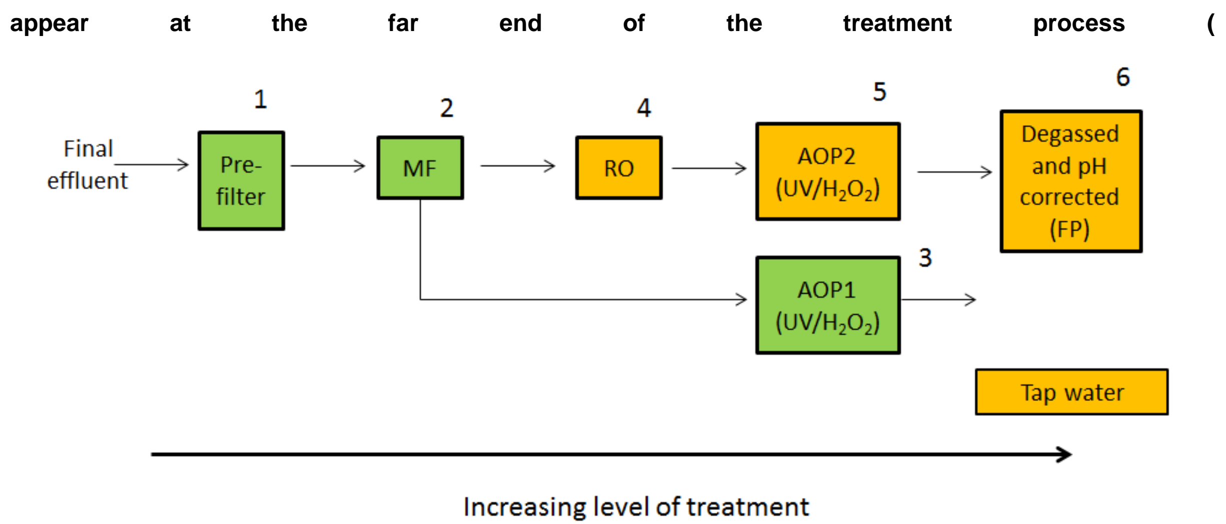

Figure 6.9). There were also two cytochrome P450 genes found to be significantly over expressed in embryos exposed to AOP1, microfiltration, and final effluent product waters, the first three stages of the IPR treatment process (

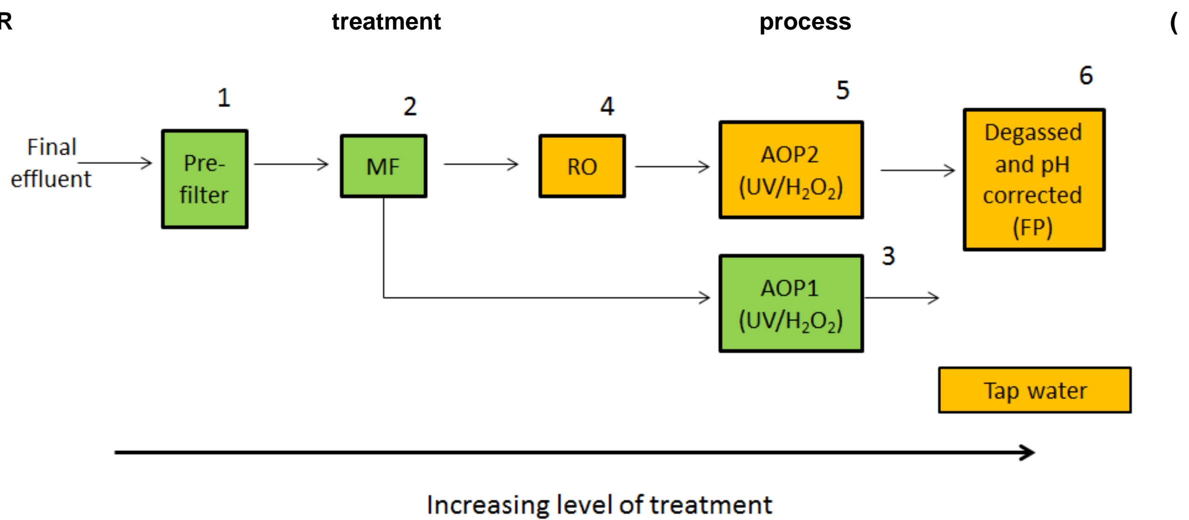

Figure 6.9). The product water from these three treatments all have in common that they have not undergone treatment with reverse osmosis.

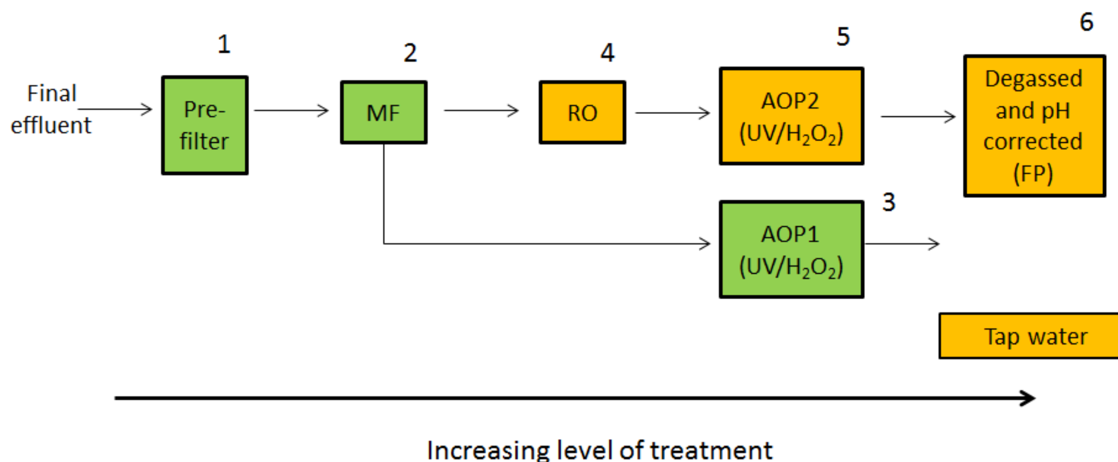

Figure 6.9 An illustration of the stages of treatment of the IPR treatment plant. The treatments highlighted in green (pre-filtered (final effluent), microfiltration and AOP1) all were observed to cause the zebrafish embryos to increase the expression of cytochrome P450 genes and the treatments in orange (reverse osmosis, AOP2 and final product), including tap water, were all found to increase the zebrafish embryo's expression of the gene coding for somatolactin beta.

6.5.1 Somatolactin beta

Somatolactin is reported to be a glycoprotein hormone, exclusive to fish, that is part of the growth hormone/prolactin superfamily (Wan & Chan, 2010). Somatolactin is primarily expressed in the *pars intermedia* of the pituitary gland, separate from the proopiomelanocortin (POMC)-producing cells (Kaneko & Hirano, 1993; Wan & Chan, 2010).

There are two isoforms of somatolactin, known as somatolactin alpha and somatolactin beta (Wan & Chan, 2010), and both these isoforms have been identified in zebrafish (Wan & Chan, 2010; Y Zhu et al., 2004).

The primary function of somatolactin is still uncertain, but it is considered to have several functions that overlap with other members of the growth hormone and prolactin family (Wan & Chan, 2010). These functions include the following:

1. Steroidogenesis and reproductive maturation (Planas et al., 1992; Rand-Weaver et al., 1992).
2. Acid-base balance (Wan & Chan, 2010).
3. Background adaptation (Wan & Chan, 2010).
4. Immune function (Calduch-Giner et al., 1993, cited in Wan and Chan, 2010).
5. Energy mobilisation and stress (Rand-Weaver et al., 1993).
6. Lipid metabolism and pigmentation (Fukamachi et al., 2009; Y Zhu & Thomas, 1997).
7. Regulation of chromatophores (Y Zhu & Thomas, 1997).

The fact that the somatolactin beta gene was differentially expressed became a point of interest once its association with pigmentation was realised. In the previous chapter it was described that some of the embryos exposed to the product waters, and in one incident in the control group, had a lack of pigmentation. Therefore, it was hypothesised that the differential expression of somatolactin beta could be having an influence on the development of pigmentation in the zebrafish. However, this would be considered more likely if the expression of the somatolactin beta gene had been observed to be less than that of the control. Also, researchers have found that decreasing, or preventing, the production of somatolactin does not result in lack of pigmentation. Based on studies on mutant medaka (*Oryzias latipes*) which did not express the somatolactin gene (colour interfere, ci), it was considered likely that somatolactin could act in the proliferation and morphogenesis of epidermal chromatophores, regulation of the body colour, or the secretion of cortisol (Fukamachi et al., 2009). However, when somatolactin gene was silenced in zebrafish (*D. rerio*) during embryonic development, the only effect observed was a delay in the inflation of the swim bladder (Yong Zhu et al., 2007).

As it is considered that somatolactin has overlapping functions with growth hormones and prolactin family functions, it has been stated by Wan and Chan (2010) that the studies undertaken to determine the specific functions of somatolactin, whereby somatolactin has been knocked down during embryonic development, were potentially compromised by the "compensational effects" of the other genes with overlapping functions (Wan & Chan, 2010).

In the previous chapter, the embryos that lacked pigmentation were found in all three types of exposures (developmental observation, extended exposure, and gene expression), and occurred in the control as well as the IPR product waters (specifically AOP2, reverse osmosis, microfiltration, and final effluent product waters). However, gene expression analysis found a increase of expression of somatolactin beta in embryos reared in product waters from reverse osmosis, AOP2, final product and tap waters. Therefore, it is unclear whether the pigmentation effects were as a result of changes in the expression of the somatolactin beta.

6.5.2 Cytochrome P450

Cytochrome P450 mono-oxygenase is a collection of isoenzymes, and they are based on the haem protein. They are situated in the smooth endoplastic reticulum of cells (Timbrell, 2001) and found in most organisms (Bräunig et al., 2015). These enzymes are involved in the phase 1 metabolism reactions and catalyse oxidative transformation, resulting in the

activation or inactivation of numerous endogenous and exogenous chemicals (Bräunig et al., 2015). Cytochrome P450 enzymes are found in the largest concentration in the liver, but are found throughout the organism. The cytochrome P450 mono-oxygenase system requires another enzyme, NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) cytochrome P450 reductase. This enzymes donates electrons to the cytochrome P450 (Timbrell, 2001). Zebrafish are reported to have 94 genes that encode for cytochrome P450 enzymes, and they have been classified into 51 gene families (Goldstone et al., 2010). These are reportedly divided into two main groups: firstly ones that are principally associated with the metabolising endogenous regulatory molecules, these are CYP family 5-51. The second group are mainly associated with oxidation of exogenous chemicals and fatty acids, these are CYP family 1-3, and to smaller degree, CYP family 4 (Goldstone et al., 2010). It is reported that, in zebrafish, five CYP1 genes in four subfamilies have been identified, these are cyp1a, cyp1b1, cyp1c1, cyp1c2 and cyp1d1 (Goldstone et al., 2010). The two cytochrome P450 genes that were over expressed in the embryos reared in the final effluent, microfiltration and AOP1 product waters were from the CYP1 family, cytochrome P450, family 1, subfamily A (cyp1a) and cytochrome P450, family 1, subfamily B, polypeptide 1 (cyp1b1). The protein cyp1a has been induced by exposure to 8-tetrachlorodibenzo-p-dioxin (TCDD) in zebrafish embryo at 36 hpf (Andreasen et al., 2002). Another researcher was able to detect cyp1 activity in zebrafish embryos from 8 hpf (Otte et al., 2010). However, an earlier zebrafish embryo study was not able to detect Cyp1a protein following exposure to TCDD until 72 hpf (Mattingly & Toscano, 2001). In the Zfin database it is stated that cyp1b1 will be active in the embryo from 8-9 hpf into and throughout adulthood, and cyp1a will be active from 4-4.33 hpf into and throughout adulthood (University of Oregon, 2015). The cyp1a gene was over expressed at 12, 24 and 48 hpf, and the cyp1b1 was over expressed just at 12 hpf (Table 6.1). These times are consistent with the findings of Otte et al. (2010) and information reported in the Zfin database.

The aryl hydrocarbon receptor nuclear translocator (ARNTs) belong to the basic helix-loop-helix PAS family of transcription factors (Prasch et al., 2004). This family includes aryl hydrocarbon receptor (AhR), hypoxia inducible factor-1α (HIF1-α), endothelial-specific PAS protein-1 (EPAS-1/HIF2-α), single minded, and others. These proteins are reported to be associated with detection and adaptation to changes to an organism's environment (Prasch et al., 2003, 2004). It is reported that a number of CYP1s are regulated by the AhR (Bräunig et al., 2015). AhR is induced by exposure to potentially toxic xenobiotics, specifically planar congeners of polychlorinated dibenzodioxin (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), a number of polycyclic aromatic hydrocarbons (PAHs) and nitrogen, sulphur or oxygen (NSO) heterocyclic compounds (Bräunig et al.,

2015; Denison & Nagy, 2003; Hinger et al., 2011). In studies referenced by Bräunig et al (2015), zebrafish with the ahr2 gene knockdown demonstrated that cyp1a and cyp1b1, as well as cyp1ca, were principally regulated by the ahr2 gene, and induced by agonists for the receptor.

Studies have shown that the systems involved in how organisms respond to chemical stressors and hypoxia have some molecular targets in common. Also, some substances that are observed to affect one system can, on occasion, affect other systems, a process known as cross-talk. As already mentioned in Chapter 5, hypoxia inducible transcription factor 1 (HIF-1) is important for reacting to depleted levels of oxygen. The HIF1-α gene acts as the sensor and degrades quickly under normal levels of oxygen. In hypoxic conditions HIF1-α dimerizes with HIF1-β (ARNT) before binding to the hypoxia response element on DNA, and various enzymes which are involved in blood supply and energy metabolism are transcribed. These blood supply enzymes include erythropoietin (EPO) and vascular endothelial growth factor (VEGF), and the energy metabolism enzymes include lactate dehydrogenase (LDH). Cross-talk between the AhR and HIF systems has been observed, but the results do not always come to the same conclusion (McElroy et al., 2012).

In embryo zebrafish studies reported in the literature, hypoxic conditions are reported to decrease some of the responses of AhR agonists. TCDD exposure under normal oxygen conditions was observed to increase specific abnormalities in the developing zebrafish (oedema in the yolk sac, pericardium, eyes and head, prevention of swim bladder inflation, shortening of the lower jaw and nose, and reduction in the peripheral blood flow). In contrast, exposure of the embryos to TCDD under hypoxic conditions reduced the occurrence of the abnormalities observed in the TCDD exposed individuals (lack of oedema and the jaw was not shortened) (Prasch et al., 2004). Additionally, benzo(a)pyrene (BaP) was observed to increase the hypoxic initiation of the production of VEGF, EPO, and LDH (Yu et al., 2008). AhR has also been reported to be associated with changes to oestrogen receptor (ER) signalling. This is because a number of AhR agonists have been identified as anti-oestrogens; for example TCDD (McElroy et al., 2012). In a separate study, β-naphthoflavone (BNF) was reported to significantly initiate the expression of cyp1a in zebrafish embryos at 48 hpf. However, the same induction of expression was not observed in HIF1-α in the zebrafish embryos. Under hypoxic conditions, cytochrome P450 aromatase (cyp19a2 or AromB) was down-regulated in killifish embryos but not in zebrafish embryos. When the researchers investigated the effects of hypoxia on cyp1a in zebrafish and killifish embryos, the results were variable. However, in zebrafish, hypoxia was observed not to affect the expression of cyp1a (McElroy et al., 2012). McElroy et al. (2012) stated that, generally, they found that hypoxia reduced the response of chemicals that induced cytochrome P450s and

this was due to down-regulation of metabolic pathways in combination with the down regulation of the target gene itself. In this current study, the cyp1a gene was observed to be upregulated in the treatment where hypoxic conditions were more likely to occur (i.e. final effluent and microfiltration). However, the study by McElroy et al. (2012) found that, if anything, hypoxic conditions would reduce the induction of cyp1a, and not act to enhance its expression. Also, as the HIF genes were not observed to be differentially expressed in the microarray data analysis, it is considered unlikely that low oxygen conditions would have directly influenced the cyp1a up-regulation findings.

6.5.3 Detoxification pathways

The 2-Factor ANOVA at gene level demonstrated that the developmental stage had the greatest influence on the variation in individual genes expressed, relative to individual experiments and the different treatments at the IPR plant. Unsurprisingly, this was also the case for the pathway analysis. However, the pathways that were observed to be differentially expressed, based on the influence of the product waters for both methods of analysis, were detoxification pathways. The pathways found to be differentially expressed were: steroid hormone biosynthesis, tryptophan metabolism, retinol metabolism, metabolism of xenobiotics by cytochrome P450, drug metabolism-cytochrome P450, and drug metabolism-other enzymes. The cytochrome genes cyp1a and cyp1b1 were found to be over-expressed in the embryos reared in product waters from the final effluent; microfiltration and AOP1 stages of the IPR plant are active in some of the same pathways. The gene cyp1a is active in retinol metabolism, steroid hormone biosynthesis, tryptophan metabolism and drug metabolism and cyp1b1 is active in steroid hormone biosynthesis and tryptophan metabolism. As discussed earlier, somatolactin beta has been associated with steroidogenesis and as referenced by Vidal-Dorsch et al. (2013), studies have related steroid metabolism pathway disruptions with exposure to nonylphenol. Nonylphenols were detected along the IPR treatment plant, and were not removed by microfiltration, reverse osmosis, or AOP (Table 6.2) A liver microarray study of mice fed municipal waste water effluents for 90 days found alterations to a number of genes related to steroid hormone biosynthesis and biosynthesis of unsaturated fatty acids as well as purine metabolism. As well as these gene level effects, exposure to the municipal waste water was observed to induce liver and kidney injuries and changes of lipid, nucleotide, amino acid and energy metabolism and disruptions of signal transduction (Y. Zhang et al., 2013).

The alterations in retinol metabolism pathway were also of interest. Retinol, including retinal, retinoic acid and related compounds, are collectively known as retinoids and Vitamin A is used as an overarching term for retinol and its esters. It is reported that, in vertebrates, both

excessive levels and the deficiency of retinoids have been linked with embryotoxicity and/or teratogenicity (Novák et al., 2008; Rolland, 2000). In a review by Novák et al. (2008) it was stated the retinoids are essential for a number of different processes, these include; cell differentiation, proliferation, apoptosis, growth and development, epithelial maintenance, immune function and reproduction. Organisms do not synthesise their own retinoids, instead they must be taken up via the diet. When the levels of vitamin A are sufficient, the retinol is converted into retinyl esters (REs) by lecithin:retinol acyltransferase (LRAT) and stored in liver cells. When the plasma levels of retinol fall, retinyl ester hydrolase (REH) cleaves the REs, releasing the retinol from the liver cells into the plasma. This release system is dependent on cellular retinol binding protein I (CRBP I) and retinol binding protein (RBP) (Novák et al., 2008). As mentioned above, cytochromes are involved of retinol metabolism, once the retinol is converted to retinal and then to the active retinoid, retinoic acid, cytochromes catalyse the inactivation of retinoic acids (Novák et al., 2008). It has been reported that environmental pollutants are known to affect retinoid metabolism, and the alteration of levels of retinoids in organisms has been used as a biomarker. The environmental pollutants observed to alter the level of retinoids have included; PCBs, PAHs, TCDD, pesticides, polluted sediments and effluents (Novák et al., 2008). There is a relationship between polyhalogenated aromatic hydrocarbons and PAHs and alterations in vitamin A metabolism. It is understood that the rate of metabolism and breakdown of vitamin A is accelerated, causing there to be reduced amounts of it circulating in the plasma and stored in the liver (Rolland, 2000). In rats, mice and ring doves, it has been reported that some co-planar PCBs have been observed to reduce levels of stored retinoids. Dioxin and dioxin-like compounds alter the metabolism of vitamin A, by acting via the AhR, as retinoid metabolism involves cytochrome P450 hydroxylation, therefore the induction of cytochrome P450 by the dioxins causes excessive amounts of retinoids to be metabolised, lowering the levels in the plasma and liver stores (Rolland, 2000).

A number of studies have investigated retinol levels in fish inhabiting polluted areas. Lake sturgeon (*Acipenser fulvescens*) from a polluted river were observed to have increased metabolism of retinoic acid (cited by Novák et al., 2008). In a three year mesocosm study, flounder were exposed to polluted harbour sludge. They were observed to have significantly reduced levels of retinol in plasma and liver, and reduced levels of REs in the liver. This reduced retinol liver level was found to be negatively associated with CYP1A protein levels; therefore the association with dioxin-like exposure was made. In the third fish study reviewed by Novák et al. (2008), fish in a river that was receiving pulp mill effluent were observed to have increased number of malformations in the larvae, including eye deformities, and reduced retinol levels in the liver (Branchaud et al., 1995; Novák et al., 2008). Zebrafish

embryos exposed to all-trans-retinoic acid at 10^{-9} to 10^{-6} M were observed to have malformations which included oedema, deformities of the brain (anophthalmy, microcephaly and acephaly), malformed otic placodes and otoliths and shortened and bent tails (Herrmann, 1995). In the developmental exposures carried out for the purposes of this project, malformations included bent tails and spines, malformed heads and oedema. Additionally, it is of note that vitamin A is widely used in cosmetics as an anti-ageing skin treatment and reduces pigmentation.

6.5.4 Time-course analysis

The information displayed in Table 6.3 indicates the differences in genes expressed between two two treatments at the different developmental stages. The smallest FDR values indicate the greatest greatest differences. Error! Reference source not found. **illustrates some of the most significant differences, indicated by the red arrows at the top of the flow chart and FDR values beside the arrow; the green arrows underneath the flow chart indicate where the differences in the genes expressed were not significant. It can be observed from** Error! Reference source not found. **that the largest differences in global gene expression occurs between the treatments that are further apart in the IPR process, for example example final effluent (following a pre-filtration step) and final product (FDR of 0.11). However, However, microfiltration and reverse osmosis are "next" to each other in the IPR treatment process but process but have a large difference in the genes expressed (FDR 0.15), but as discussed in the General the General Introduction chapter, reverse osmosis is known to remove many more contaminants than contaminants than microfiltration.** Error! Reference source not found. **illustrates that in the stages of treatment such as final effluent (following a pre-filtration step) and microfiltration, embryos have fewer differentially expressed genes (FDR 1.06) than compared to that of microfiltration and final product (FDR product (FDR 0.08). This indicates that the product water from the final effluent and microfiltration are microfiltration are much more similar to one another than compared to other treatments further along the further along the process. It also illustrates that these similarities exist between reverse osmosis and osmosis and AOP2 (FDR 1.13) and AOP2 and final product (FDR 1.24). This would suggest that, if the that, if the differences are elicited by the contaminants remaining in the product following the different different treatments, then reverse osmosis seems to cause the largest change in the product water. This**

water. This was also observed in the

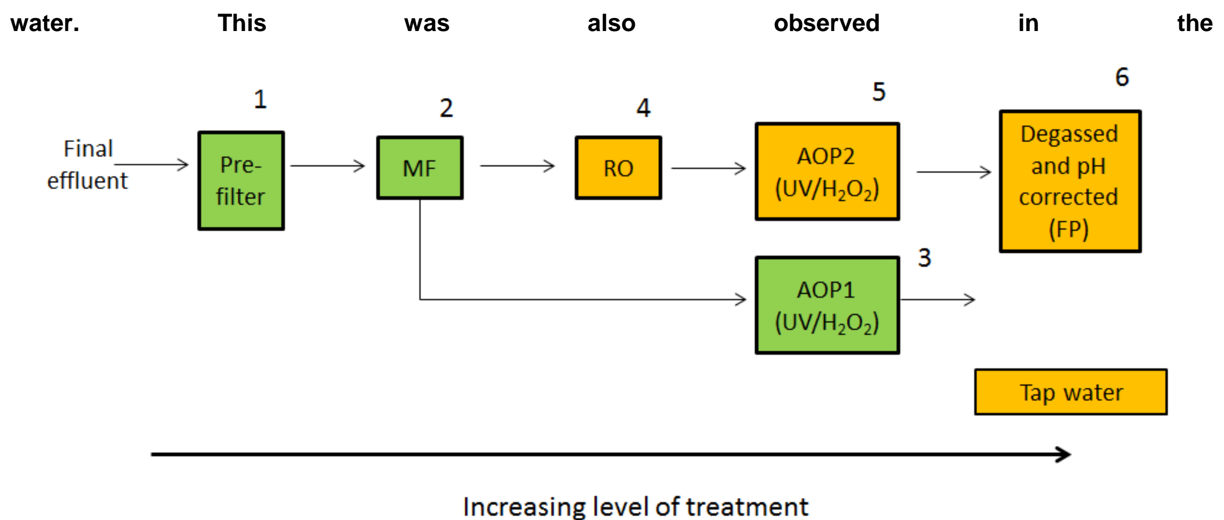

Figure 6.9, with the up-regulation of cytochrome P450, where this up-regulation was observed in the first three product waters, but not any in the product waters following reverse osmosis treatment.

Error! Reference source not found. illustrates similar findings as does **Error! Reference source not found.**; however it is examining the differences between the pathways between two treatments at the different stages of development. Only three treatment combinations were observed to have a significant difference in their expression of the pathways. The first two were between final effluent (following a pre-filtration step) and final product (FDR 0.17) and microfiltration and final product (FDR 0.12). Similarly to the situation with the individual genes (**Error! Reference source not found.**), these differences fall between the first two treatments and the final treatment, suggesting the increased level of treatment is changing the influence on the gene expression in the embryos. Interestingly, it also highlights a significant difference in pathways expressed between the two AOP treatments (FDR 0.19), but the product water from the AOP2 process has also received treatment from reverse osmosis, whereas the product water from the AOP1 treatment has only received treatment via the pre-filtration and microfiltration. Again this suggests that reverse osmosis greatly influences the biological activity of the product water.

Following on from this, a pseudo-time course was assessed; the quantile normalised data using a signed approach had the lowest FDR values and, from these, the top five most significantly differentially expressed pathways were highlighted (Table 6.8). Again there were metabolic pathways and drug metabolism pathways both up- and down-regulated along the treatment process. Another pathway of interest was melanogenesis at 12 hpf being down-regulated along the treatment process. As discussed in Chapter 5, there was lack of pigmentation observed in some embryos in some of the treatments. Two other pathways of

interest were tryptophan metabolism and purine metabolism: these were both up- and down-regulated along the treatment process (Table 6.8).

In a 120 hour zebrafish embryo development study, benz[a]anthracene (BAA) and benz[a]anthracene-7,12-dione (BAQ) were used in the exposure at a concentration of 4 µM (Elie et al., 2015). Both BAA and BAQ are PAHs that are structurally similar. Previous studies had suggested BAA and BAQ share the same mechanism of toxicity, as they caused similar morphological responses and increased the expression of cyp1a by activating the AhR2 in zebrafish (Goodale et al., 2013). Following exposure it was determined which pathways had been altered; this was done by examining the metabolites and linking them to the relevant pathways. The altered pathways included glutathione metabolism, purine metabolism and phenylalanine, tyrosine and tryptophan biosynthesis (Elie et al., 2015). These results were of interest to this project because there was an observed increase in cyp1a gene expression, and both the purine metabolism and tryptophan metabolism pathways had been altered along the treatment process. Purine metabolism is considered to be important in the homeostatic response of mitochondria to oxidative stress. Exposure of the zebrafish to both BAA and BAQ significantly affected the purine metabolism pathway (Elie et al., 2015). The levels of tryptophan metabolites were significantly increased following exposure to both BAA and BAQ. Tryptophan is a precursor of serotonin, which can be subsequently converted to melatonin. Serotonin is a neurotransmitter, and its level was reported to increase following exposure to both BAA and BAQ. Serotonin is responsible for altering a number of behavioural and neuropsychological processes which include sleep, aggression, appetite, memory and rhythmic motor patterns (Elie et al., 2015). It was suggested by Elie et al. (2015) that developmental alterations in serotonin and dopamine, which was also found to be increased following exposure by both PAHs, and the other neuro-transmitter-precursor metabolites (tyrosine, phenylalanine and tryptophan), may be indicators of subsequent effects on neuro-behaviour (Elie et al., 2015).

Of the 29 PAHs that were analysed for along the IPR treatment system between 2008 and 2011, there were only a few incidences where any occurred above the limit of detection (LoD was 0.01 µg/l). In the product water for the microfiltration treatment benzo(ghi)perylene, dibenzo(ah)anthracene and fluorene were detected above the limit of detection at an average (minimum to maximum (standard deviation)) of 0.59 µg/l (0 to 2.2 µg/l (0.95)), 0.92 µg/l (0 to 5.5 µg/l (4.13)) and 1.68 µg/l (0.01 to 11.7 µg/l (4.42)), respectively. Over the sampling programme conducted at the IPR plant by Thames Water Ltd, each of the PAHs detected were sampled for 6 to 7 times, only once or twice being detected above the LoD. Benzo[a]anthrancene (BAA) was measured in the treatments from the IPR plant, but was not detected above the LoD in any of the product waters. In final effluent an average

concentrations of 0.010286 µg/l (0.01 to 0.011 µg/l (0.00)) was reported, but this was below the LoD. In the product water after microfiltration, AOP1 and AOP2, the same concentrations below the LoD were recorded 0.01 µg/l (0.01 to 0.01 µg/l (0.00)). In the aquarium water which was sampled in triplicate in August 2013, concentrations of the PAHs were all below the LoD (1 ng/l). BAA used in the exposures conducted by Elie et al. (2015) was reported to be present at 4 µM, which is equal to 913.16 µg/l; therefore it unlikely that BAA alone was responsible for the effects on the purine metabolism and tryptophan metabolism pathways. However, the nature of the effluent means the contaminants are present in a complex mixture, and therefore it is not possible to assign any effects to one chemical or group of chemicals.

Gene expression levels in fathead minnows exposed to different types of treated municipal waste water effluents were recently reported (Vidal-Dorsch, Colli-Dula, et al., 2013). One effluent had only undergone primary treatment, so had a higher chemical contaminant load than the second effluent, which had undergone secondary treatment. There were distinctly different gene expression profiles in the fish exposed to the two effluents, but similar physiological responses. It was also found that there was a dose-response in relation to the chemical load/degree of treatment. The gene expression in the fish exposed to primary treated effluent elicited a higher magnitude of gene response than the effluent that had undergone secondary treatment. Of the 332 genes altered in common between the two effluents, many were associated with the oxidative stress pathway. The authors also found increased transcript levels of ARNT and cyclooxygenase-1 (COX-1), and it was suggested that this may indicate the activation of a cellular defence responses against oxidative stress. It was also found that cyp2c was up-regulated in both effluents as were transcripts associated with cellular stress and apoptosis, immune response and endocrine function. However, contrary to my studies using zebrafish embryos, Vidal-Dorsch et al. (2013) did not find changes in the expression of cyp1a transcripts in either effluent. It was, however, found that retinol binding protein mRNA was induced in the fish exposed to the primary treated effluent, but not in the effluent that had undergone secondary treatment (Vidal-Dorsch, Colli-Dula, et al., 2013).

The same authors also undertook a gene expression study to investigate a number of specific genes in male hornyhead turbot (associated with responses in reproduction, growth, development, stress responses, contaminant metabolism and detoxification, response to infection and hormone activity) following exposure to two effluents (primary advanced treatment and secondary treated effluents) at two concentrations, 0.5% and 5%, and a negative and positive (estradiol 8 µg/l) controls. As with the previous study in fathead minnows, there was a different pattern in expression between the two effluent exposures.

Additionally in this study there was a difference in patterns of expression also at the two effluent concentrations. Exposure to estradiol caused production of AhR and cyp1a transcripts among others. But this induction was not observed in the two effluent exposures at either concentration (Vidal-Dorsch, Bay, et al., 2013).

Other studies involving exposure to effluents have demonstrated alterations in liver genes involving reproduction, immunity, detoxification and metabolism in the fathead minnow (*Pimephales promelas*) (Garcia-Reyero et al., 2011). In another study, Rainbow trout (*Oncorhynchus mykiss*) were exposed to differently treated effluent (conventional treatment, GAC, ozonation and UV treatment). The fish exposed to effluent that had undergone conventional treatment were observed to have significantly increased liver and heart somatic indexes. However, this effect was removed by the other treatments. Gene expression in the liver was altered in genes associated with xenobiotic metabolism, including cyp1a, and this effect was reduced by GAC or ozone at 15 mg/l. Fish exposed to the ozone treated effluent were observed to have increased levels of gene transcripts encoding heat shock protein 70kDa, which the researchers suggested indicated that ozone-treatment added stress to the fish. An estrogen-response gene was induced by treatment with conventionally treated effluent, but again this effect was removed by the other treatments. Prostaglandin E synthase was induced in the fish exposed to conventional activated sludge and sand filtered treated effluent, but this effect was not observed in the other treatments. It was concluded that GAC treatment was the most effective at removing the effects observed on gene expression (Cuklev et al., 2012).

Information from the literature has suggested that a number of genes and pathways can be induced by sewage effluent; many in common in the ones altered in the current study in zebrafish embryos. However, variations exist in patterns of gene expression that are likely due to the complex nature of effluents from different sources and undergoing different levels of treatments. Also, the species and developmental stage used in the literature vary greatly, which adds a further level of complexity in the understanding and interpretation of genomic data arising from fish exposure studies.

6.6 Conclusions

The first main aim of this section of the project was to determine if any of the product waters from any of the treatment stages at the IPR plant caused significant difference in individual gene expression levels relevant to specific biological pathways and functions. In the process of investigating this research aim zebrafish embryos were exposed to the different IPR product waters during the first 48 hours of their development. It was shown that developmental stage had the largest influence on gene expression in zebrafish compared to

the different experiments and the product waters used to rear the embryos. However, the gene expression was greatly influenced by the experiment (i.e. exposure 1, 2 and 3). This could have been due to use of non-standardised effluents and control waters, indicating how sensitive gene expression is in this model organism.

There were two distinct types of genes expressed in the two halves of the IPR process (pre- and post-reverse osmosis). Firstly, somatolactin beta was up-regulated in the post reverse osmosis and tap water treatments. The other genes were both cytochrome P450 genes, and these were up-regulated in the embryos reared in the treatments pre-reverse osmosis. Cytochrome P450 is important for detoxification, and this finding indicates that embryos were not being challenged with contaminants capable of inducing cytochrome P450 following treatment with reverse osmosis.

The difference in genetic expression was observed to be greater the further the treatments were from one another. Microfiltration and reverse osmosis had different gene expression profiles as did the two AOP treatments, again indicating that reverse osmosis was making a significant difference to the biological activity of the product water.

Examination of the functional pathways being affected indicated that detoxification pathways were being altered. The detoxification pathways are linked to steroid synthesis, another pathway altered. These pathways are also associated with retinol levels and the retinol synthesis pathway, which was also altered.

Only severe hypoxia would have caused a significant alteration in the gene expression, but even then other studies found only one HIF gene altered, therefore not observing any alterations to the HIF genes is not confirmation that hypoxia did not play a role. However, the data available for the dissolved oxygen confirm that severe hypoxia was not a condition likely to have occurred. Although, the slight differences in hatching rates and enlarged yolk sac could be linked to mild hypoxia. Even though the zebrafish larvae with enlarged yolk sacs observed by Long et al. (2015) were exposed to severe hypoxia (dissolved oxygen of 5%), which is unlikely to have been reached in this current study.

Using the pseudo time course approach a number of pathways were altered; these included melanogensis at 12 hpf being down-regulated along the treatment process, tryptophan metabolism, and purine metabolism both up- and down-regulated along the treatment process. The second main aim of this section of the project was to identify whether changes in gene expression could be associated with the observed changes in phenotype. Somatolactin beta was one specific gene to be altered, the significance of the up-regulation this gene in embryos exposed to product water post reverse osmosis and tap water was

unclear. However, somatolactin beta has been linked to a number of biological processes, including pigmentation. However, studies have found the lack of somatolactin beta does not prevent the production of pigmentation in zebrafish. Its altered expression was, however, of interest in relation to the lack of pigmentation observed in a number of embryos discussed in the previous chapter. The phenotype of reduced/absent pigmentation was again linked to the gene expression data as the pseudo time course analysis approach identified an alteration in the melanogensis pathway, this was of interest given the developmental observations of a lack of pigmentation in some embryos.

Malformations observed such as bent tails, malformed heads and oedema have been associated with alterations to the retinoid pathway, the retinol synthesis pathway was observed to be altered.

The third and final aim of this section of the project was to determine if 'culprit chemicals' measured in the IPR water could be identified that might explain the observed changes in gene expression and phenotype based on current knowledge. This was difficult due to the complex nature of the chemicals in known to be present and measured in effluents. However, a few were highlighted as possible "culprits". The induction of cyp1a is associated with exposure to PAHs, but the concentrations of these substances along the IPR treatment process were typically below the LoD. However, given the complex nature of the contaminants in the product waters, it is not possible to target one group of compounds based on these data. Both tryptophan metabolism and purine metabolism had been found by other researchers to be altered by exposure to PAHs. Nonylphenols were detected along the IPR treatment process, not being removed by microfiltration, reverse osmosis, or AOP. It is reported in the literature that nonylphenols are related to disruption to the steroid metabolism pathway. A number of environmental pollutants have been identified in the literature to affect retinoid metabolism, these include PCBs, PAHs, TCDD, pesticides, polluted sediments and effluents. Also, vitamin A is widely used in cosmetics, as an anti-ageing skin treatment and reduces pigmentation. No information was found as to the environmental concentration of the chemicals used in cosmetics that could disrupt the retinoid pathway. However, the literature seems to indicate that a number of different chemicals known to present along the treatment process albeit at low concentrations, can impact this pathway, and could be having a combined effect.

Further work could be done using direct effect analysis to determine the fraction of the product waters causing the induction of the cytochrome genes.

The patterns of gene expression in effluent-exposed fish vary greatly depending on the species, nature of effluent and level of treatment. However, there were a number of

pathways and specific genes that were altered in this study and others described from the literature.

CHAPTER 7: Use of *In Vitro* and Further *In Vivo* Assays to Investigate the Specific Chemicals Responsible for Alterations Observed in the Zebrafish Embryos

7.1 Background

Following on from the work discussed in Chapters 5 and 6, it had been observed that there had been low frequency of a number of different abnormalities, but the lack of pigmentation in the embryos correlated with some of the genomic data. The expression of somatolactin beta gene had been altered; this is a fish-specific gene with multiple functions, but one of them being a role in pigmentation. Analysis of the data from microarrays highlighted a number of pathways, along with biotransformation pathways relevant to pigmentation; melanogenesis (highlighted as a pathway that had been altered along the IPR treatment plant) governs the production of melanin, which determines pigmentation. Based on these data, the question arose as to whether any of the chemicals present in the effluent may be affecting the pigmentation of the embryos.

7.2 Aims and Objectives

7.2.1 Aims:

1. To determine if the IPR product water contains chemicals or chemical groups that could alter melanogenesis.
2. Determine if the extracts from the passive samplers deployed in the product waters along the IPR treatment process are biologically active in a relevant *in vitro* assay.
3. Determine if the chemicals identified can reproduce any of the malformations observed in IPR product water exposures.

7.2.2 Objectives:

1. Conduct a literature search for potential chemicals or groups of chemicals which cause embryos to develop without pigmentation.
2. Examine the analytical chemistry data gathered by Thames Water (gathered over the several years of the operation of the pilot IPR plant), to determine if any of the chemicals known to reduce pigmentation have also been identified in any of the product waters from the IPR pilot plant.

7.3 Introduction

Melanogenesis is the process by which melanin is synthesised; the process that gives the skin, brain, eyes and hair pigment in humans (Lajis et al., 2012). Melanin is classified into three main types depending on the molecular precursor. The types include eumelanin, phenomelanin and neuromelanin. Eumelanin is known as black melanin and is made up of

oligomers of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Pheomelanin is the orange-red melanin and is derived from benzothiazine units. Neuromelanin is thought to be a complex of dihydroxyindole and benzothiazine with other unknown groups (Hong & Simon, 2007).

Melanogenesis takes place in melanocytes by melanogenic cytokines and melanogenic enzymes including tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2). Tyrosinase is an essential, rate-limiting enzyme in the melanogenesis process (Lajis et al., 2012). It catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to DOPAquinone, and the oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone (Ebanks et al., 2009; Sato et al., 2008). Without thiols (sulphur analogues of alcohols), the DOPAquinone is converted to DOPAchrome and then into DHI or indole 5,6-quinone 2-carboxylic acid (DHICA). The other two processes in the melanogenesis pathway are TRP-2 (DOPAchrome tautomerase) which catalyses the conversion of DOPAchrome to DHICA, and TRP-1 (DHICAoxidase), which catalyses the oxidation of DHICA. An essential messenger in the synthesis of melanin is cyclic AMP (cAMP) (Sato et al., 2008). cAMP is produced from a binding reaction between α-melanocyte-stimulating hormone (α-MSH) and its specific receptor (MCIR), which results in the activation of GTP-binding protein (Gs), which stimulates the production of cAMP. cAMP increases the expression of microphthalmia-associated transcription factor (MITF), which then stimulates the expression of the gene that encodes for tyrosinase (Ebanks et al., 2009). Inhibition of tyrosinase has a significant effect on the melanogenesis process and melanin production. Alterations to the production of melanin production cause many incidences of hyperpigmentation, post-inflammatory pigmentation, melisma (formation of darker patches of pigment on the skin), and the skin aging process (Lajis et al., 2012), as well as hypopigmentation. The production of melanin can also be induced by UVB-radiation, in order to protect the skin from damage from UV radiation.

Melanin acts as an antioxidant, it can serve as a metal sink as well as a reservoir for the homeostasis of metal ions such as calcium and zinc. It is not clear what mechanism is involved in metal ion release as a consequence of the degeneration of melanosomes within a tissue (Hong & Simon, 2007). It is also reported that some intermediates produced during melanogenesis are toxic, and if these are not utilised within the process they can become cytotoxic and also cause autoimmunological diseases in mammals (Nilsson Sköld et al., 2013).

7.3.1 Pigmentation in zebrafish

The pigment cells (chromatophores) in zebrafish are derived from the neural crest. They consist of three types: melanophores, xanthophores and iridophores. The melanophores consist of melanin-containing melanocytes and contribute to the lateral dark stripes of the epidermis of the zebrafish. The xanthophores contain pteridine (Nilsson Sköld et al., 2013) and are responsible for the yellow pigment and iridophores are reflective platelets, these two together are responsible for the yellowish-silver inter-stripes on the dark stripes of the melanophores (Choi et al., 2007). There are two other types of chromatophores which are not present in zebrafish; erythrophores containing carotenoids, which are responsible for red colouration, and cyanophores, which are rarer, but are responsible for blue colouring (Nilsson Sköld et al., 2013). The pigmentation of a zebrafish initially begins from the retinal pigment epithelium (RPE), which is then followed by the melanocytes on the dorsolateral skin. Pigmentation of the skin in zebrafish is reported to be visible from approximately 24 hpf, tyrosinase is reported to be detectable from approximately 7 hours prior to when pigmentation is visible in the RPE. Additionally, the enzymatic activity of tyrosinase has been detected 3 hours before pigmentation is visible (Choi et al., 2007). Aggregation of melanosomes within melanophores (i.e. concentration of pigment within centre of cells rather than dispersed throughout) causes the skin to become pale but also more transparent (Nilsson Sköld et al., 2013).

7.3.2 Chemicals that affect pigmentation

There are many chemical compounds and pharmaceuticals which have been found to affect pigmentation, and there are substances on the open market that are supplied specifically to either increase or decrease pigmentation. An example of one of these compounds is hydroquinone; it is a skin lightening substance, although it is cytotoxic and has been found to be harmful to human health, so its use has been restricted.

Choi et al. (2007) exposed zebrafish embryos to a number of chemicals and examined the pigmentation at 35 hpf and 55 hpf. They found that all three chemicals namely, arbutin, kojic acid and 2-mercaptobenxothiazole (MBT) tested in the initial experiment reduced pigmentation, but could be acting differently because arbutin and kojic acid significantly reduced the pigmentation of the body of the zebrafish embryo, but had little effect on the pigmentation of the RPE. In contrast, MBT and 1-phenyl-2-thiourea (PTU) significantly reduced the pigmentation in both the RPE and the body. The authors suggested that this difference could suggest that either the chemicals have different mechanism of action that differentially target the pigmentation of RPE and body, or that the different cell types have different sensitivity or permeability to these chemicals. PTU in this study was used as a positive control and is a known tyrosinase inhibitor. Arbutin is stated to be a naturally

occurring β-D-glucopyranoside of hydroquinone that competitively binds to the L-tyrosine binding site of tyrosinase. Kojic acid is also a tyrosinase inhibitor that is used in topical depigmentation treatment. MBT contains sulphur and is another tyrosinase inhibitor (Choi et al., 2007).

Nilsson Sköld et al. (2013) reviewed the literature to identify the factors that have been found to cause a colour and/or pigment response in fish. The ones that were of relevance to this project are as follows:

- Melatonin - targets Mel1R and causes paleness via aggregation of pigment in melanophores, erythrophores and xanthophores.
- Noradrenaline - targets the α2-adrenoceptors and causes paleness via aggregation of pigment in melanophores.
- Prolactin - targets Prl-R and causes reddish colouration, erythrophore and xanthophore dispersion.
- Prostaglandin (types A2 and B2) - target is unclear, causes melanophore dispersion (darker colouration).
- Somatolactin - target is unclear but causes melanophore aggregation (paler colouration).

7.3.3 Chromatophores as biosensors

Chromatophores have complex links with the nervous system, and hormones and neurotransmitters elicit a response via aggregation or dispersion of the pigment organelles. These processes are important for camouflage and self-defence, courtship and mating as well as thermoregulation and protection from UV radiation (Dukovcic et al., 2010). When fish are stressed, unwell, or experiencing adverse conditions they can appear visibly paler or darker in colour (Dukovcic et al., 2010).

Research has been carried out using fish pigment cell lines, and using these as a biosensor. It has been reported that melanophores from the cuckoo wrasse (*Labrus ossifagus*) are used as a biosensor for monitoring levels of catecholamine in human blood (Dukovcic et al., 2010; Elwing et al., 1990). Additionally, Siamese fighting fish (*Betta splendens*) erythrophores have been reported to be a useful biosensor for bacterial pathogens (Dukovcic et al., 2010; Hutchison et al., 2008) and environmental toxicants (Dierksen et al., 2004; Dukovcic et al., 2010). A study investigating whether the use of melanophores from the Chinook salmon (*Oncorhynchus tsawytscha*) were as responsive to environmental toxicants as erythrophores

from *B. splendens*, found a good correlation between the two assays. When clonidine (a α_2-adrenergic agonist and imidazoline receptor agonist, used to treat blood pressure, anxiety, and ADHD) was used to aggregate pigment cells, the intracellular pigment organelles were observed to move towards the centre of the cell, therefore causing the melanophores and erythrophores to appear smaller (paler in appearance). MSH was used to disperse the melanophores, inducing the intracellular pigment organelles to move towards the periphery of the cell, therefore causing the melanophores and erythrophores to appear larger (darker in appearance). In the *B. splendens* erythrophores assays, adenosine and MSH both caused erythrophores to disperse, and aggregation was observed in exposure to clonidine, dopamine and serotonin. In the *O. tsawytscha* melanophores, adenosine and MSH caused pigment dispersion (i.e. darker in appearance), however; at higher doses adenosine (10^4 µM) caused aggregation of melanophores. Clonidine, dopamine and serotonin all caused aggregation in the *O. tsawytscha* melanophores. Although serotonin was observed to initially cause aggregation within 2 minutes of exposure, there was then a recovery within 25 minutes (Dukovcic et al., 2010). In the tests for environmental toxicants in *O. tsawytscha* melanophores, residual chlorine (sodium hypochlorite) was introduced as a control, to cover the typical levels of chlorine present in drinking water. It was found that this level did not cause any effects on melanophores. Mercuric chloride (24.1 mg/l) and sodium arsenite (1.9 mg/l) both caused pigment aggregation, which was also found to be the case in *B. splendens* erythrophores. However, the melanophores did not show a response to cyanide or paraquat, but aggregation had been observed in earlier studies with *B. splendens* erythrophores (Dukovcic et al., 2010).

Nilsson Sköld et al., (2013) referenced studies that inoculated frogs with bactericidal lipopolysaccharides (LPS) and measured an increase of melanin pigmentation in the testes. This was stated to confirm that there is a relationship between internal melanin, melanocytes and innate immunity (Nilsson Sköld et al., 2013).

7.3.4 Tyrosinase inhibitors

Chemicals used to treat hyperpigmentation are used as skin lighteners both as prescription and “over the counter drugs”; they are present in a large variety of cosmetics. From the literature, inhibition of tyrosinase appears to be the most common/favourable mode of action for these chemicals. Following literature searches, a total of fifty individual chemicals/groups of chemicals were identified as being used, or having properties that could reduce the appearance of hyperpigmentation or lighten the skin. Due to the widespread use of cosmetics, and the nature of their use (skin application), it is highly plausible that active ingredients and/or their breakdown products could enter the waste water treatment system,

and this has been found with other chemicals which are applied directly to the skin (UV-filters from sun lotion, N,N-diethyl-meta-toluamide (DEET) from insect repellents etc. (Brausch & Rand, 2011; Dhanirama et al., 2011)). From the list of fifty, eight have been considered further. These eight were chosen due to their potency and potential high usage. They are as follows:

1. Hydroquinone
2. Kojic acid
3. Retinoids
4. Tretinoin
5. Vitamin B3/Niacinamide
6. Hydroxyanisole
7. 4-n-Butylresorcinol
8. Salicylic acid

Apart from salicylic acid, no data were found in relation to these chemicals being detected in the environment or at the IPR treatment process. However, this is not to say they are absent from the environment and waste water treatment processes, only that there were no data from published sampling campaigns found during the time the literature search was completed. Data on the occurrence of salicylic acid are detailed in Table 7.1 and Table 7.2.

7.3.5 Cyclooxygenase enzyme

Prostaglandins are a class of eicosanoids; there are two types of eicosanoids: prostaglandins + thromboxanes and leukotrienes (Moran et al., 2014). Eicosanoids are oxygenated products of polyunsaturated fatty acids and they act as messengers in the regulation of a number of physiological functions (Moran et al., 2014). Prostaglandins are formed from arachidonic acid, in a reaction catalysed by the enzymes prostaglandin endoperoxide G/H synthases, which are also referred to as cyclooxygenase (COX) enzymes (Grosser et al., 2002; Moran et al., 2014). Firstly, COX acts on the arachidonic acid, the product being prostaglandin G_2 (hydroperoxide). The enzyme has a second active site for hydroperoxidase activity; this transforms any unstable hydroperoxide (prostaglandin G_2) to prostaglandin H_2 (Moran et al., 2014). Prostaglandin H_2 is then converted to a number of regulatory molecules known as prostanoids; these are a sub-class of eicosanoids consisting of prostaglandins, thromboxane A_2 and prostacyclins (Figure 7.1) (Ishikawa et al., 2007; Moran et al., 2014). There are four main prostaglandins; prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Ricciotti & FitzGerald, 2011). Prostanoids are required for numerous physiological processes, including platelet aggregation, reproduction, thermoregulation, wound healing, water balance,

glomerular filtration, mediation of pain sensitivity, inflammation, swelling, and haemostasis (Ishikawa et al., 2007; Moran et al., 2014). In uninflamed tissues, prostaglandin synthesis is relatively low, however there is an immediate increase in production following acute inflammation, then leukocytes and immune cells are introduced (Ricciotti & FitzGerald, 2011). Prostaglandins have been shown to play an important role in the development of males (Kugathas et al., 2015). Epidemiological studies as reported in Kugathas et al. (2015) have highlighted the potential risk to human health during male sexual differentiation posed by inhibiting prostaglandins. Pregnant women who took analgesics such as paracetamol (inhibits the COX enzyme) towards the end of the first trimester, and early in the second trimester, which is considered to be the time-period of human sexual differentiation, were observed to have corresponding increased risk of testicular maldescent (cryptorchidism) in their male offspring (Kugathas et al., 2015).

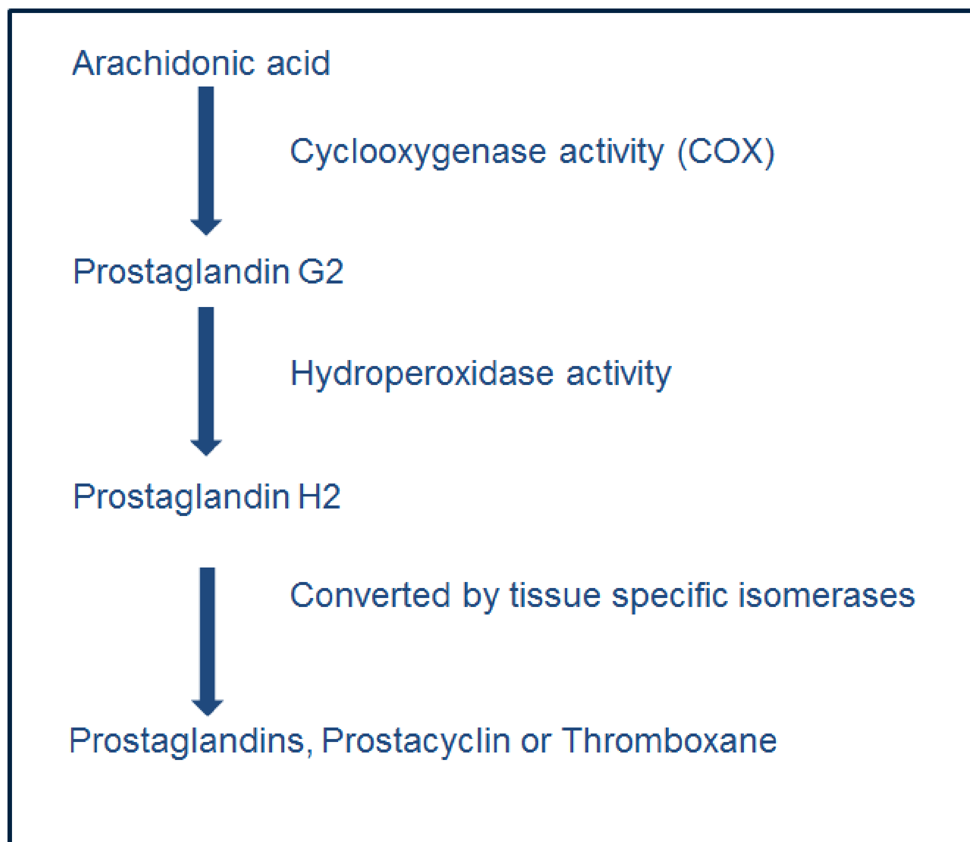

Figure 7.1 The action of cyclooxygenase (COX) on arachidonic acid in the formation of prostaglandins (modified from Moran et al., 2014).

Many pharmaceuticals used to manage pain (for example aspirin, paracetamol and ibuprofen) act by blocking the production of some eicosanoids (Moran et al., 2014). This class of pharmaceuticals is known as the non-steroidal anti-inflammatories (NSAIDs).

Acetylsalicylic acid (active ingredient of aspirin) irreversibly inhibits COX activity, consequently aspirin stops the formation of a number of eicosanoids (Moran et al., 2014).

There are two forms of COX; COX-1 is a constitutive enzyme that is responsible for production of eicosanoids that regulate the secretion of mucin in the stomach. COX-2 is an inducible enzyme that is responsible for the production of eicosanoids that act on inflammation, pain, and fever, Figure 7.2 (Moran et al., 2014). Aspirin inhibits both COX-1 and COX-2, which leads to subsequent side effects such as stomach irritation (Moran et al., 2014).

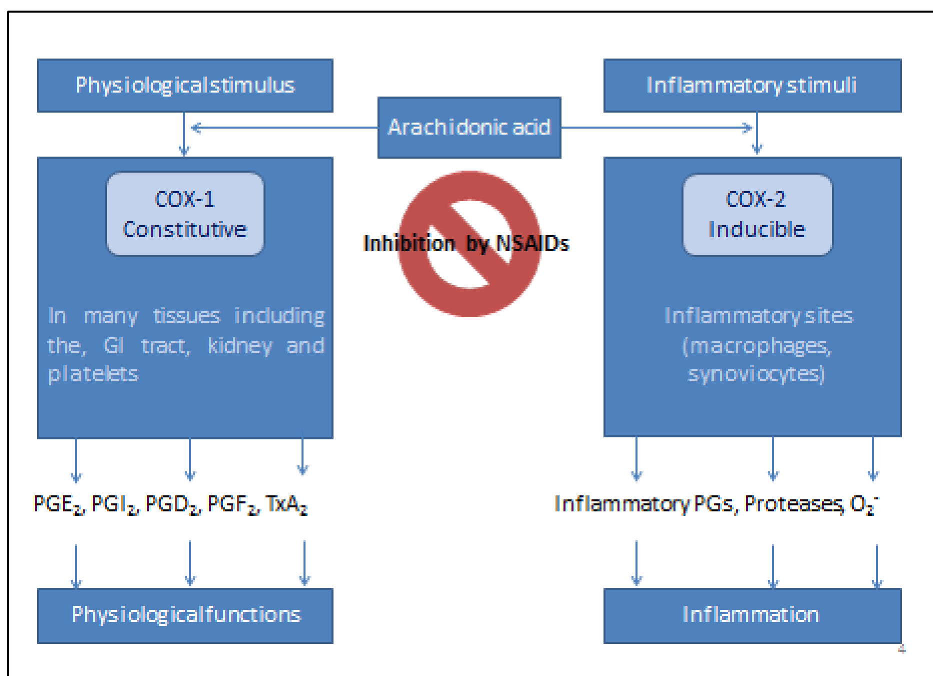

Figure 7.2 The pathways of COX-1 and -2 in the synthesis of prostaglandins. PG: Prostaglandin (Figure modified from (modified Balasubramaniam, 2001).

7.3.6 Alteration to COX and pigmentation

As already discussed, the expression of COX-2 is inducible in cells by factors that stimulate inflammation, such as cytokines, growth factors, hypoxia and UV light. COX-2 expression is up-regulated in the skin following exposure to UV radiation, and this causes the increased production of prostaglandin E2 (PGE_2) (Kim et al., 2012). It is reported that melanocytes have the capacity to produce PGE_2 and express COX-2 mRNA (Gledhill et al., 2010; Kim et al., 2012). It is also reported that PGE_2 produced from melanocytes following exposure to UV radiation can stimulate the release of cAMP and tyrosinase activity (Kim et al., 2012; Starner et al., 2010). The NSAID acetylsalicyclic acid (aspirin), which is well known for its abilities to inhibit both COX-1 and COX-2, has been reported to also inhibit the expression of the rate-limiting melanogenesis enzyme, tyrosinase. The aspirin used in that study was

found to inhibit the synthesis of melanin to a greater extent than arbutin and kojic acid, which are known tyrosinase inhibitors (Sato et al., 2008). A study was undertaken to assess the role of COX-2 in melanogenesis, thus a cell line was used with COX-2 knock-down to test the mechanism. The cell lines used in the study were melan-a, (which is a mouse melanocyte cell line) and hermes-1, which is an immortalised melanocyte cell line. To test the effects of COX-2 knock-down on the melanogenesis pathway, the expression levels of MITF, tyrosinase, TRP-1, TRP-2 and gp100 protein were measured. It was found that in the cells the decreased level of COX-2 was associated with a marked decrease in the expression of MITF, tyrosinase, TRP-1, TRP-2, and gp-100. These results occurred in both the mouse and human cell lines. The activity of tyrosinase was also reduced in melan-a cells transfected with COX-2 siRNA. The authors of that study considered that the results of their study indicated that COX-2 inhibitors could be developed as skin lightening treatments to reduce the amount of melanin pigment (J. Y. Kim et al., 2012).

The anti-fungal agent clotrimazole has also been reported to alter melanogenesis. It was found to reduce melanin content in human and mouse melanocytes, and B16F10 mouse melanoma cells. It was reported that tyrosinase protein levels were reduced, but without changing the expression of tyrosinase mRNA expression. Clotrimazole is an azole antimycotic agent; this group of chemicals are known to have anti-inflammatory and anti-cancer effects. These effects are reported to be mediated via action on the COX-2-PGE2 pathway (Chen et al., 2011).

It has been reported that in mice, PGE_2 applied to their skin caused an increase in the density of melanocytes (Nordlund et al., 1986). Kim et al. (2012), reported on studies which had been carried out to investigate the use of prostaglandin treatment for hypopigmentation disorders, and it has been observed to show positive results (Kim et al., 2012).

7.3.7 COX Inhibitors

The NSAID aspirin was observed to share structural similarities to certain phthalates, and, when tested, these phenolic compounds (phthalates, benzophenones, parabens and alklyphenols) along with paracetamol, aspirin, ibuprofen and indomethacin, all inhibited the synthesis of PGD2 and PGE2 in a mouse Sertoli cell line model (SC5 cells), human mast cells, and in *ex vivo* isolated rat testes. In addition it was determined that the COX enzyme inhibition was the likely mode of action (Kristensen et al., 2011).

Kugathas et al. (2015) investigated twenty-four pesticides to determine whether they could inhibit prostaglandin synthesis. Fifteen of the pesticides (boscalid, chlorpropharm, cypermethrin, cyprodinil, fenhexamid, fluxioxonil, imazalil, imidacloprid, iprodine, linuron,

methiocarp, o-phenylphenol (OPP), pirimiphos-methyl, pyrimethanil and tebuconazole) were observed to inhibit PGD2 synthesis in the SC5 assay, in a dose-dependent manner. OPP was observed to be the most potent pesticide tested, with an IC50 of 175 nM, compared to that of ibuprofen of 128 nM. OPP, cypermethrin, cyprodini, linuron, tebuconazole and imazalil had IC50s ranging from 175 to 2300 nM, and this range was comparable to the known COX inhibitors of ibuprofen with a IC50 of 128 nM and aspirin with a IC50 of 5380 nM (Kugathas et al., 2015).

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Over recent years there has been growing interest into the presence of pharmaceuticals in the environment, and the potential risks they pose to wildlife: NSAIDs have been included in this research. One reason the presence of NSAIDs in the environment has been studied is that they are widely used to relieve pain and inflammation. They are available via prescription, and some are widely available as over the counter medications.

According to the British National Formulary (BNF) website in 2014 in the UK there were 10 NSAIDs registered for prescription use (salicylic acid listed below as number eleven is a breakdown product of aspirin). These are as follows:

1. Diclofenac potassium
2. Diclofenac sodium
3. Fenoprofen
4. Ibuprofen
5. Indometacin
6. Ketoprofen
7. Mefenamic acid
8. Naproxen
9. Aspirin
10. Paracetamol
11. Salicylic acid

The concentrations of some of these NSAIDs in influent, effluent, drinking water, and in environmental waters have been complied, and are detailed in Table 7.1.

Table 7.1 Environmentally relevant concentrations of NSAIDs in the literature

NSIADs	Concentration(s) reported to be present in STW influent (ng/l)	Concentration(s) reported to be present in STW effluent (ng/l)	Concentration(s) reported to be present in drinking water (ng/l)	Concentration(s) reported to be present in the environment (ng/l)
Diclofenac potassium	3000 [a] 330-490 [a] 1300 [a] 470-1900 [a] 2800 [a] 400-1900 [a] 350 [a] 1000 [a] 610 [k] 2430 [k] 730 [k]	2500 [a] 310-930 [a] 1900 [a] 400-1900 [a] 170-350 [a] 290 [a] 359 [b] 429 [c] 424 (2349) [j] 289 (598) [j] 680 [k] 5510 [k] 120 [k]	6 [i]	21 [e] 0-3363 [h] <LOQ (568) [j] <LOQ (195) [j]
Diclofenac sodium		280 [m]	18 [p]	900 [l] 20 [n] 84 [o]
Fenoprofen				
Ibuprofen	3000 [a] 38700 [a] 9500-14700 [a] 2600-5700 [a] 5700 [a] 28000 [a] 2000-3000 [a] 13100 [a] 1430 [k] 1310 [k] 2200 [k]	4000 [a] 10-20 [a] 900-2100 [a] 180 [a] 3000 [a] 600-800 [a] 0-3800 [a] 284&383 [d] 1150 [c] 3086 (27 256) [j] 2972 (4239) [j] 280 [k] 3460 [k]	3 [i]	13 [f] 6.5 [g] 23 [e] 2234-16886 [h] 826 (5044) [j] 297 (2370) [j]
Indometacin		55&105 [d] 166 [c]		20 [e] 66-267 [h]
Ketoprofen	410-520 [a] 5700 [a] 470 [a] 250-430 [a] **2000 [a]** 340 [k] 1750 [k]	8-23 [a] 180 [a] 150-240 [a] 0-1250 [a]		43-1567 [h]
Mefenamic acid	1600-3200 [a] 200 [a]	800-2300 [a] 340 [a] 133 (1440) [j] 340 (396) [j]		26-104 [h] 62 (366) [j]
Naproxen	40 700 [a] 10300-12800 [a] 1800-4600 [a] 950 [a] 4900 [a] 210 [k]	12500 [a] 0-23 [a] 800-2600 [a] 270 [a] 150-190 [a] 589&1790 [d] 2670 [c]		59 [e] 387-3140 [h]
Aspirin	3200 [a]	600 [a]		
Salicylic acid	57000 [a] 330 000 [a]	50 [a] 3600 [a]		27-83 [h]
Paracetamol	6900 [a]	<20 [j]		188 [h] 710 [h] 2813 [h] 55 [q]

a. Referenced in the review by (Fent et al., 2006).

b. Bonnybrook WWTP effluent in Calgary, Canada in 2003, not detected there in 2002 (Mei et al., 2006).
c. Fish Creek WWTP effluent in Calgary, Canada in 2003 (Mei et al., 2006).
d. Bonnybrook WWTP effluent in Calgary, Canada in 2002 & 2003 (Mei et al., 2006).
e. Surface water concentrations, downstream Bow River in 2003 (Mei et al., 2006).
f. Surface water concentrations, upstream Bow River in 2003 (Mei et al., 2006).
g. Surface water concentrations, upstream Elbow River in 2004, not detected in 2003 (Mei et al., 2006).
h. Taken from different sampling points of the rivers of Madrid downstream from the man STPs (Valcárcel et al., 2011).
i. Concentrations in finished drinking water in Germany (Jones et al., 2005, referenced in Watts et al., 2007).
j. Median measured concentration (maximum in brackets) of pharmaceuticals in the UK aquatic environment (Ashton et al., 2004 and Roberts & Thomas, 2006 referenced by Watts et al., 2007).
k. Concentrations measured in a sampling exercise at wastewater plants in Cyprus (Fatta-Kassinos et al., 2011).
l. Maximum measured Rhine River water concentrations in the Netherlands 2009 (van den Brandhof & Montforts, 2010).
m. Maximum measured sewage treatment plant concentrations in 1999 in the Netherlands (van den Brandhof & Montforts, 2010).
n. Maximum measured surface water concentrations in 1999 in the Netherlands (van den Brandhof & Montforts, 2010).
o. Maximum measured surface water concentrations between 2005-2006 in the Netherlands (van den Brandhof & Montforts, 2010).
p. Maximum measured drinking water concentration between 2005-2006 in the Netherlands (van den Brandhof & Montforts, 2010).
q. Median measured concentration (maximum in brackets) of pharmaceuticals in the UK aquatic environment (Bound & Voulvoulis, 2006 referenced by Watts et al., 2007).

As well as concentrations of NSAIDs present in the environment where the data were gathered from the wider literature, some NSAIDs were measured in the sampling campaign run by Thames Water Ltd in the water of the IPR plant. This sampling started in 2008 and ran to 2012, when the pilot plant was closed. The results of this sampling are detailed in Table 7.2

Table 7.2 Concentrations (ng/l) of NSAIDs detected at IPR plant at stages where the POCIS samplers were deployed, post-final effluent (post-pre-filtration), post-microfiltration and post-reverse osmosis.

NSAID	Measurement (ng/)	Post-Final Effluent	Post-Microfiltration	Post-Reverse Osmosis	Aquarium water
Diclofenac	Min-Max	0-1492	0-1580	0-12	nd
	Average	316.22	295.17	1.39	nd
	Standard Deviation	432.76	530.23	3.28	nd
Ibuprofen	Min-Max	0-1532.10	0-338	0-58	10.4*
	Average	910.69	65.36	7.16	10.4*
	Standard Deviation	3494.69	110.76	17.64	N/A
Mefenamic acid	Min-Max	0-456	0-553	0-11	nd
	Average	103.73	98.71	2.23	nd
	Standard Deviation	138-78	178.62	3.96	nd
Aspirin	Min-Max	0.1-10	0-48	10-348	ns
	Average	7.53	9.14	60.2	ns
	Standard Deviation	4.48	18.14	106.82	ns
Salicylic acid	Min-Max	0-252	13-104.67	10-20	ns
	Average	71.53	61.68	11.25	ns
	Standard Deviation	88.27	37.72	3.54	ns
Paracetamol	Min-Max	0-2375.50	0-2.26	0-11	nd
	Average	135.75	0.17	2	nd
	Standard Deviation	559.03	0.63	4.06	nd

nd: Not detected; ns: not sampled; *The aquarium water was sampled in August 2013, but the pharmaceutical screen was only undertaken once, therefore there is not minimum and maximum or standard deviation and the average in not a true average.

Given the possible ability of NSAIDs to affect melanogenesis (via possible interference of arachidonic acid metabolism, tyrosinase and the synthesis of prostaglandins), it was decided to test the hypothesis that NSAIDs present in the IPR water were responsible for the changes in pigmentation observed in the developmental exposures and microarrays. Therefore, the ability of water extracts to inhibit COX activity was assessed *in vitro*, and a number of NSAIDs were tested for their ability to disrupt pigmentation in developmental exposures using zebrafish embryos.

7.4 Methodology

The cell line used in this assay was SC5 mouse juvenile Sertoli cells (Hofmann et al., 1992). These cells produce a high amount of prostaglandins without prior stimulation (Kristensen et al., 2011). Prostaglandin synthesis is dependent on the COX enzyme (Ricciotti & FitzGerald, 2011), therefore measuring the levels of prostaglandin production can indicate whether the COX enzyme is being inhibited.

Once the SC5 cells had been exposed to the NSAIDs or POCIS extract, PGD2 synthesis was determined, using a Prostaglandin D2-MOX enzyme immunoassay (EIA) (Cayman Chemicals). In some experiments using the same assay, Kristensen et al. (2011) also measured PGE_2 synthesis and stimulated the exposed cells with arachidonic acid. This allowed them to more confidently determine whether any observed inhibition of prostaglandin synthesis occurred upstream of both PGD_2 and PGE_2 synthases, and was likely due to the inhibition of COX-1 and COX-2 enzymes. Following exposures to certain chemicals, Kristensen et al. (2011) recorded inhibition of prostaglandin in the EIAs, but further studies to investigate the expression of COX genes found that their expression was unaffected, indicating that COX gene expression was not associated with the prostaglandin inhibition. The authors concluded that their result indicated that the compounds they tested directly affected the activity of the COX enzymes, which is the same mode of action as ibuprofen, aspirin and paracetamol (Kristensen et al., 2011).

The detailed methodology is described in the General Material and Methods chapter. However, here the dilutions of the NSAIDs and extracts will be explained. On each EIA, a standard positive control had to be tested, to ensure that the assay was functioning correctly. For this, ibuprofen was used for the first few EIAs, but other NSAIDs known to present in the environment and effluents were also tested alongside the extracts: these included aspirin, salicylic acid, naproxen, paracetamol, diclofenac and mefenamic acid. From these EIAs with these NSAIDs, IC50s (the concentration of the compound needed to cause 50% inhibition of the production of prostaglandin in the assay) were derived. For the NSAIDs tested, they were initially tested at 8 dilutions and as each assay with the compound was repeated three times depending on the shape of the curve, six dilutions were chosen and repeated a further two times.

Running the extracts in the assay began with aliquots of 1 ml POCIS extracts derived from each of the three replicate discs, and from the eight separate deployments. A pooled sample was made of the extracts by combining the extracts from deployments 1 to 8 for each of the treatments and POCIS blanks. This resulted in a pooled sample for all POCIS extracts, from the POCIS blank, post-reverse osmosis, post-microfiltration and post-final effluent (post pre-filtration). Of these pooled samples, eight dilutions were made, 1:200 to 1:125600. The SC5 cells were exposed to these extracts and the EIA completed to determine the amount of prostaglandin synthesised from the SC5 cells. From these results (see Figure 7.3), four dilutions were chosen for more extensive exposures; these were 1:800 to 1:6400, although extracts from post-reverse osmosis and POCIS blanks were only carried at one dilution (1:200). A further pooled extract was made using the three replicate discs from each separate treatment at each separate deployment, in order to investigate the potential for any seasonal variation in the biological activity.

Following the *in vitro* bioassays of IPR treatment water extracts, developing zebrafish embryos were exposed to three NSAIDs (diclofenac, naproxen and mefenamic acid) and three tyrosinase inhibitors (4-butylresorinol, nicinamide and kojic acid). These exposures took place over a 72 hours period, starting shortly after fertilisation. Environmentally relevant concentrations were chosen for the NSAIDs and concentrations below which were considered toxic were chosen for the tyrosinase inhibitors (Table 7.3 and Table 7.4). The detailed methodology is explained in the General Materials and Methods Chapter.

Table 7.3 Concentrations of NSAIDs used in the zebrafish developmental exposures

NSAIDs (ng/l)		
Diclofenac sodium	Mefenamic acid	Naproxen
30 000	3000	12500
3000	300	5000
300	30	500
30	3	50
3	N/A	5

Table 7.4 The concentrations of tyrosinase inhibitors used in the zebrafish developmental exposures

Final concentration of tyrosinase inhibitors		
4-n-Butylresorinol (μg/l)	Kojic acid (μg/l)	Niacinamide (mg/l)
10	5	10
100	50	100
1000	500	1000

7.5 Results

Figure 7.3 shows the amount of prostaglandin synthesised by the SC5 cells expressed as a percentage of that of the ethanol control (the ethanol control was 20% ethanol v/v), following exposure to the pooled extracts from the POCIS discs deployed at the IPR treatment plant post-reverse osmosis, post-microfiltration, and post-final effluent (post pre-filtration). In Figure 7.3 it can be observed that the extracts of water following reverse osmosis had little effect on the prostaglandin synthesis in the SC5 cells at any dilution tested (levels of prostaglandin was >80% in all dilutions). However, the extracts of water following microfiltration and in the final effluent (i.e. prior to microfiltration) exhibited a clear dose-response, with the most concentrated samples (1:200) causing the levels of prostaglandin to be only approximately 10% of that of the solvent control. Additionally, the activity in these two product waters appeared very similar. The dilutions of 1:800, 1:1600, 1:3200 and 1:3200 were chosen to run in further assays, and these results are displayed in Figure 7.4 and Figure 7.5.

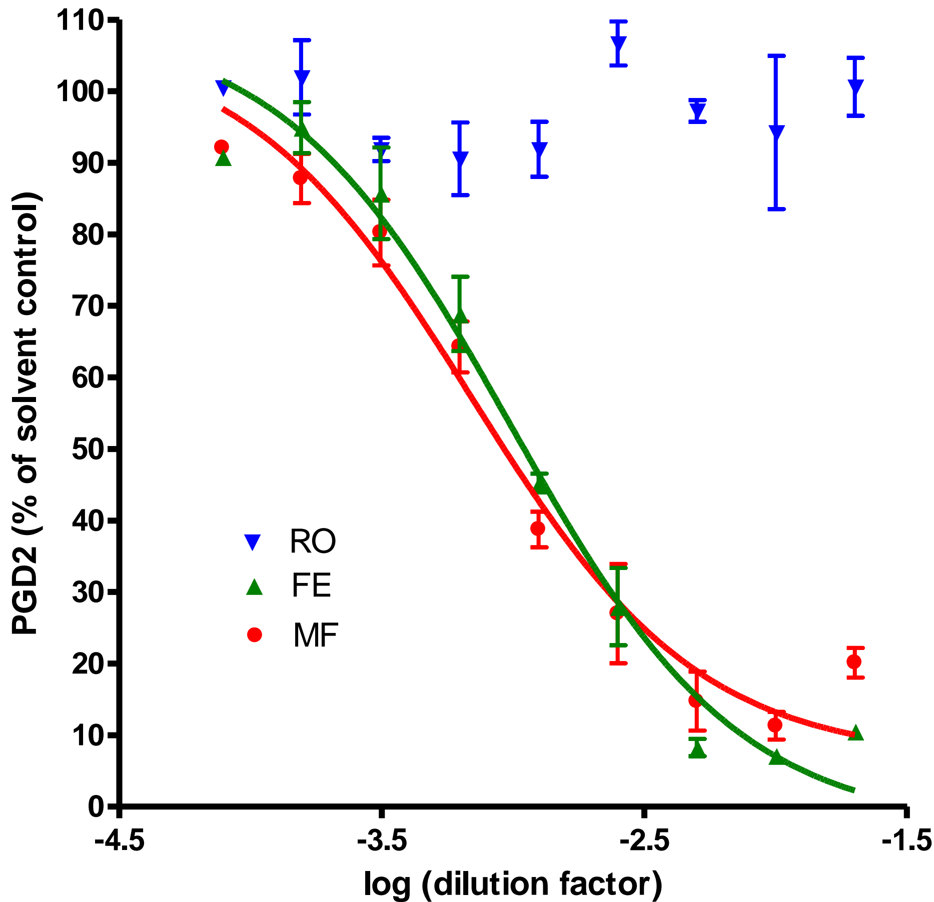

Figure 7.3 The inhibition of synthesis of prostaglandin in the SC5 cell line (%) using the extracts pooled according to replicate and deployment, separated by treatment. POCIS blanks did not inhibit prostaglandin synthesis. RO: post-reverse osmosis; MF: post-microfiltration, and FE: post-final effluent (post-preflitration).

Figure 7.4 shows the amount of prostaglandin synthesised by the SC5 cells (expressed as a percentage of the ethanol control), following exposure to extracts from the POCIS discs deployed in treated product water following microfiltration from the eight separate deployments. There was a clear dose-response, but the different deployments made little difference to the overall potency except on one occasion (MF7). This was also true for the extracts from the POCIS discs deployed in the final effluent product water (Figure 7.5), where COX-inhibitory activity was observed in all cases, with the exception of FE1.Comparing data in Figure 7.3, Figure 7.4 and Figure 7.5, there was a slight difference in the relationship in the extracts from microfiltration and final effluent, for example FE7 had a high activity, whereas MF7 did not.

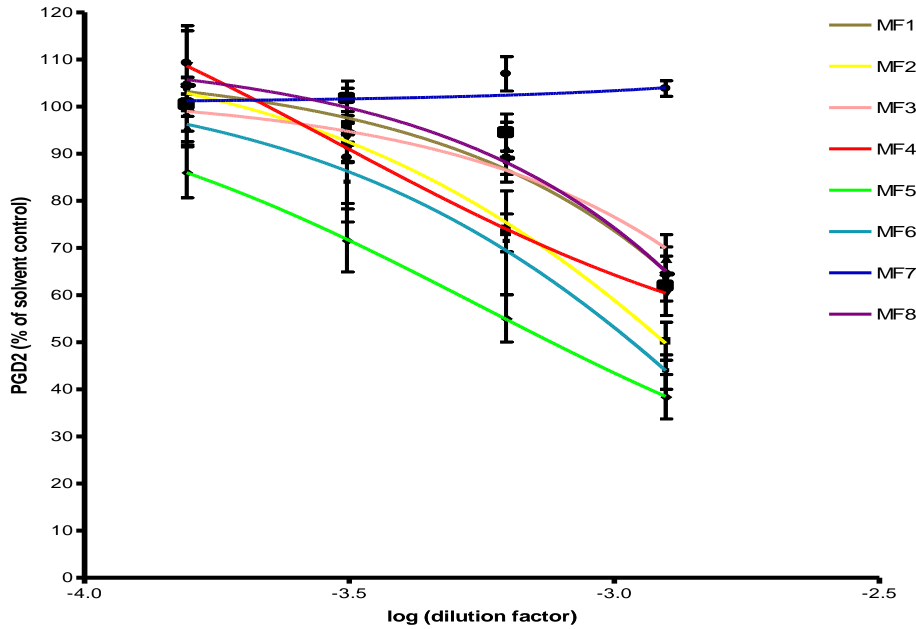

Figure 7.4 The inhibition of synthesis of prostaglandin in the SC5 cell line (%) using the extracts which were pooled from the three replicate POCIS discs from post-microfiltration (MF) taken from the eight different deployments. MF1=Aug-Sept 2011; MF2= Sept-Oct 2011; MF3= Oct-Nov 2011; MF4= Nov-Dec 2011; MF5= Dec 2011-Jan2012; MF6= Jan-Feb 2012; MF7= Feb 2012; and MF8= Feb-Mar 2012.

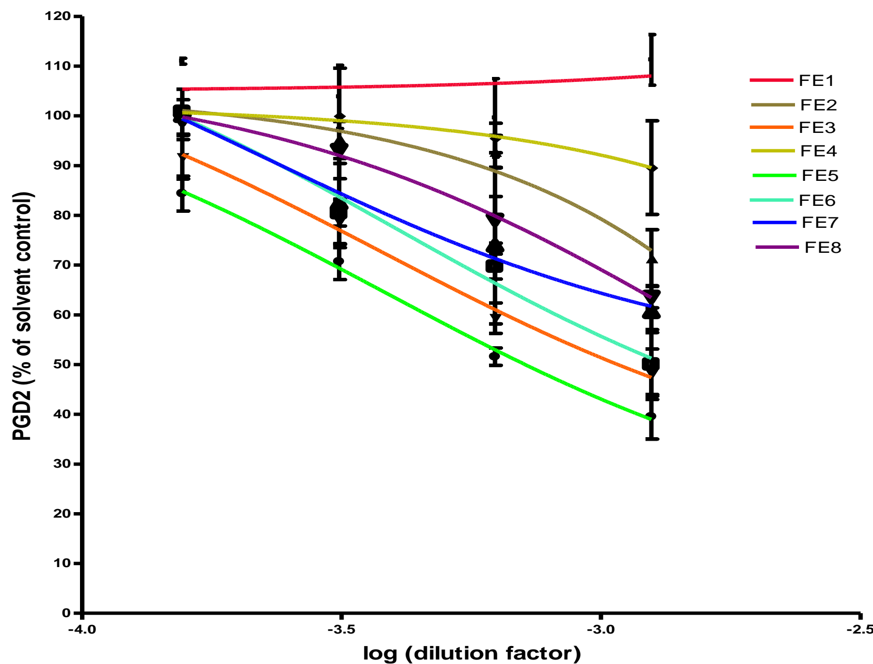

Figure 7.5 The inhibition of synthesis of prostaglandin in the SC5 cell line (%) using the extracts which were pooled from the three replicate POCIS discs from post-final effluent and post pre-filtration (FE) taken from the eight different deployments. FE1=Aug-Sept 2011; FE2= Sept-Oct 2011; FE3= Oct-Nov 2011; FE4= Nov-Dec 2011; FE5= Dec 2011-Jan2012; FE6= Jan-Feb 2012; FE7= Feb 2012; and FE8= Feb-Mar 2012.

In addition to the POCIS extracts, a number of NSAIDs were put through the assay to assess their relative potency and ability to inhibit COX activity (Figure 7.6). From this figure the variation in potencies can be observed. This is further evident in the IC50 derived and displayed in Table 7.5.

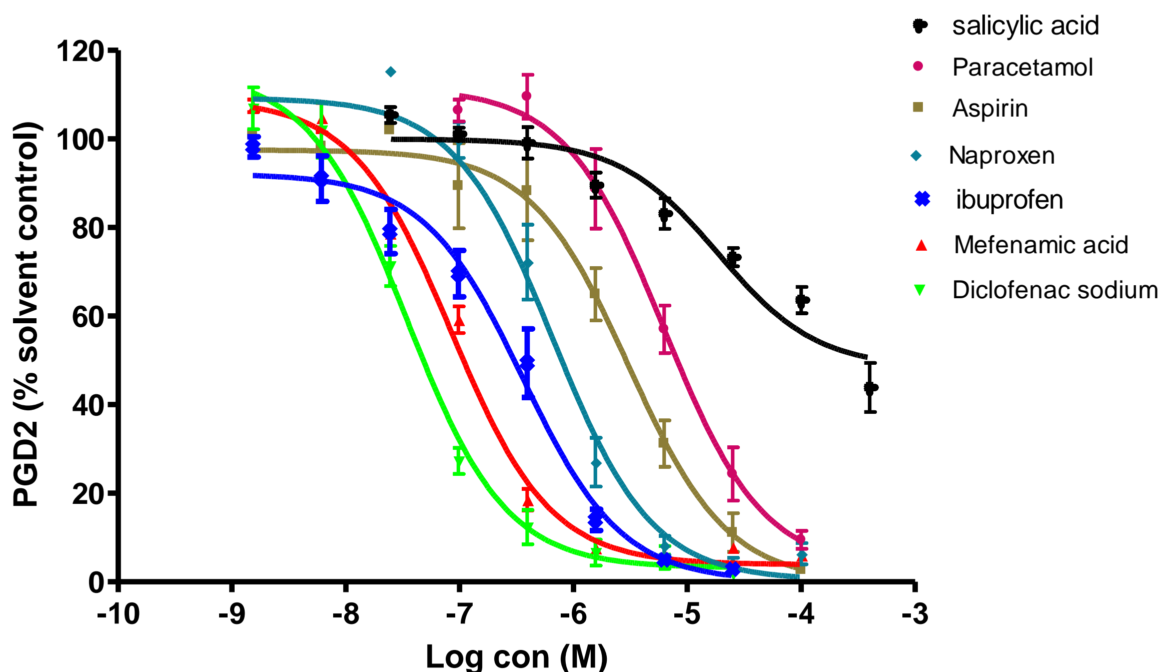

Figure 7.6 The inhibition of synthesis of prostaglandin in the SC5 cell line (%) using the different dilutions of the NSAIDs.

The IC50s in Table 7.5 demonstrate that diclofenac is the most potent NSAID, with an IC50 of 3.451 $\times 10^{-8}$ M (10.19 µg/l) and the least potent is salicylic acid with an IC50 of 1.863 $\times 10^{-5}$ M (2573.18 µg/).

Table 7.5 IC50s of the NSAIDs based on the data presented in Figure 7.6

NSAID	IC50 (M)	IC50 (µg/l)
Diclofenac sodium	3.451e-008	10.19
Mefenamic acid	8.472e-008	20.44
Ibuprofen	3.571e-007	73.66
Naproxen	6.606e-007	152.11
Aspirin	3.055e-006	550.39
Paracetamol	6.241e-006	943.42
Salicylic acid	1.863e-005	2573.18

Table 7.6 IC50 values of a selection of chemicals tested for prostaglandin inhibition using the SC5 cell line. The chemical data supplied by Thames Water Ltd. for the product waters at the IPR treatment plant have been compared to IC50 data generated from this study and by other research to determine if any of the chemicals detected at the IPR plant are likely to be present at biologically active concentrations.

Test compound	Category	IC50 (nM)	Concentration in FE (ng/l)	Concentration in MF (ng/l)
Aspirin	NSAID	1640[a] 3426[b] 3055[c]	7.53	9.14
Paracetamol	NSAID	382[a] 6241[c]	135.75	0.17
Ibuprofen	NSAID	11.2[a] 128[b] 357.10[c]	910.69	65.36
Naproxen	NSAID	660.60[c]	ns	ns
Indomethacin	NSAID	424[a]	ns	ns
Diclofenac	NSAID	34.51[c]	316.22	295.17
Mefenamic acid	NSAID	84.72[c]	103.73	98.71
Salicylic acid	NSAID	18630[c]	71.53	61.68
Diethyl phthalate	Phthalate	19000[a]	<LoD	nd
Di-n-propyl phthalate	Phthalate	2100[a]	ns	ns
Di-n-butyl phthalate	Phthalate	2110[a]	<LoD	nd
Diisobutyl phthalate	Phthalate	1010[a]	2000	nd
Butylbenzyl phthalate	Phthalate	24500[a]	<LoD	nd
Di-n-pentyl phthalate	Phthalate	149000[a]	ns	ns
Di-n-benzyl phthalate	Phthalate	417000[a]	ns	ns
Ethylparaben	Paraben	7590[a]	ns	ns
n-Propylparaben	Paraben	2850[a]	ns	ns
n-Butylparaben	Paraben	2430[a]	ns	ns
Isobutylparaben	Paraben	1090[a]	ns	ns
n-Pentylparaben	Paraben	483[a]	ns	ns
Benzylparaben	Paraben	1300[a]	ns	ns
n-Nonylparaben	Paraben	2910[a]	ns	ns
Benzophenone 3	Benzophenones	197[a]	ns	ns
Benzophenone 7	Benzophenones	1200[a]	ns	ns
Benzophenone 4	Benzophenones	45600[a]	ns	ns
Benzophenone 12	Benzophenones	69400[a]	ns	ns
17β-Estradiol	Estrogenic compound	1.13E+08[a]	1.25 (0.03-7.36)	0.89 (0.03-8)
Diethylstilbestrol	Estrogenic compound	27200[a]	ns	ns
Zearalenol	Estrogenic compound	3180[a]	ns	ns
Genistein	Estrogenic compound	428000[a]	ns	ns
Bisphenol A	Estrogenic compound	2720[a]	12.8 (10-21)	nd
Coumestrol	Estrogenic compound	12500[a]	ns	ns
Flutamide	Antiandrogen	1870[a]	ns	ns
Cyprodinil	Fungicide	803[b]	ns	ns
Imazalil	Fungicide	1510[b]	ns	ns
Pirimphos-methyl	Insecticide	2140[b]	<LoD	<LoD
Pyrimethanil	Fungicide	8270[b]	ns	ns
Imidacloprid	Insecticide	4450[b]	ns	ns
Fludioxonil	Fungicide	30200[b]	ns	ns
Fenhexamid	Fungicide	7370[b]	ns	ns
Iprodione	Fungicide	25200[b]	<LoD	<LoD
Chlorpropham	Herbicide	1340[b]	9.628 (5-25)	11.38 (5-27.9)
O-Phenylphenol	Fungicide	175[b]	ns	ns
Cypermethrin	Insecticide	678[b]	<LoD	<LoD
Boscalid	Fungicide	1550[b]	ns	ns
Methiocarb	Insecticide	7850[b]	ns	ns
Tebuconazole	Fungicide	2320[b]	ns	ns
Linuron	Herbicide	1490[b]	33.75 (4-85)	<LoD

a: Kristensen et al., 2011; b: Kugathas et al., 2015; c: results from this current study; ns: not sampled; nd: not detected; <LoD: below limit of detection. In the aquarium water, all the pesticides sampled were below limit of detection apart from AMPA and metaldehyde, which are not listed in the table. Estradiol (E2) was below the limit of detection (0.03 ng/l), none of the other compounds were sampled.

Table 7.7 IC50s of compounds measured in the final effluent and/or microfiltration product waters relative to actual measured concentrations.

Test compound	IC50 (µg/l)	Concentration in FE (µg/l)	Concentration in MF (µg/l)
Diclofenac sodium	10.19[c]	0.32 (0-1.49)	0.3 (0-1.58)
Mefenamic acid	20.44[c]	0.1 (0-0.46)	0.099 (0-0.55)
Ibuprofen	73.66[c]	0.91 (0-15.33)	0.065 (0-0.34)
Aspirin	550.39[c]	0.0075 (0.0001-0.01)	0.0091 (0-0.048)
Paracetamol	943.42[c]	0.136 (0-2.38)	0.00017 (0-0.0023)
Salicylic acid	2573.18[c]	0.072 (0-0.25)	0.062 (0.013-0.105)
Diisobutyl phthalate	281.13[a]	2	nd
17β Estradiol	30779166[a]	0.00013 (0.00003-0.0074)	0.00089 (0.00003-0.008)
Bisphenol A	620.95[b]	0.013 (0.01-0.021)	nd
Chlorpropham	286.30[b]	0.0096 (0.005-0.025)	0.011 (0.005-0.028)
Linuron	371.15[b]	0.034 (0.004-0.085)	<LoD

a: Kristensen et al., 2011; b: Kugathas et al., 2015; c: results from this current study; nd: not detected; <LoD: below limit of detection

7.5.1 Analytical chemistry of the POCIS extracts

As described in the General Materials and Methods chapter, pooled POCIS extracts were sent to CEFAS laboratories for analysis on the presence of certain pharmaceuticals. Table 7.6 details the results of this analysis.

Table 7.8 The concentrations of pharmaceuticals detected in the concentrated 1 ml pooled POCIS disc extracts (ng/l)

Pharmaceutical	Concentration of pharmaceutical in the extracts from the POCIS discs (ng/ml of extract)			
	Post FE	Post MF	Post RO	POCIS blank
Ibuprofen	nd	nd	nd	nd
Diclofenac	4540	3550	nd	nd
Paracetamol	nd	nd	nd	nd
Erythromycin	289	54	nd	nd
Sulfamethoxazole	nd	164	nd	nd
Acetyl-sulfamethoxazole	nd	33	nd	nd
Trimethoprim	1750	1270	12	nd
Propanonol	817	687	nd	nd
Clofibric acid	nd	nd	nd	nd
Mefenamic acid	879	682	nd	nd
Dextroproxpoxyphene	119	80	nd	nd
Tamoxifen	nd	nd	nd	nd
Clotrimazole	36	20	<LOQ	nd

nd: not detected; LOQ: Limit of Quantitation

The data in Table 7.6 can only be used as qualitative values, and it can only be determined that ibuprofen and paracetamol were not detected in any of the extracts tested, and that diclofenac and mefenamic acid were both detected in the extracts from the POCIS devices deployed in the product waters of the final effluent and microfiltration treatments. The NSAIDs were not detected in the extracts from the POCIS devices deployed in the product water from the reverse osmosis and the POCIS blank extract. To be able to estimate the water concentration from the POCIS concentrations, the specific uptake rate has to be known, and this has to be experimentally derived. An uptake rate for diclofenac was identified, 0.226 l/d (±0.009) (Morin et al., 2013). The equation needed is as follows:

$$Water\ concentration\ = \frac{Concentration\ of\ analyte\ in\ POCIS\ x\ Mass\ of\ POCIS\ sorbent}{Sampling\ rate\ x\ Sampling\ duration}$$

The calculated water concentrations for diclofenac were 165 and 129 ng/l based on the measured concentration of the analytes in the extracts from the POCIS devices deployed in the final effluent and microfiltration product waters, respectively.

A less accurate estimation can be made by considering the general water uptake of the POCIS devices, which were stated to be between 1400 and 8400 ml in a 28 week standard deployment. With an average of approximately 5000 ml, this means that the 1 ml POCIS

extract would be approximately 5000 times more concentrated than ambient water concentrations. However, this is an approximation, and does not take into consideration the individual chemical's uptake rate, but it can provide a guide and tool in which to compare the chemistry data. This calculation was used and the results plotted on Figure 7.7. The results derived from the water sampling and POCIS extracts are very different. For example, the water sampling data determined ibuprofen to be the most prevalent NSAID in the product water from final effluent, but it is not measured in the POCIS extract. Paracetamol was absent in the POCIS extract, but was detectable in all product waters along the process in the water sampling, although at a low concentration. The pattern of removal of mefenamic acid is similar in both. Both methods indicate that the NSAIDs are being removed along the IPR treatment process.

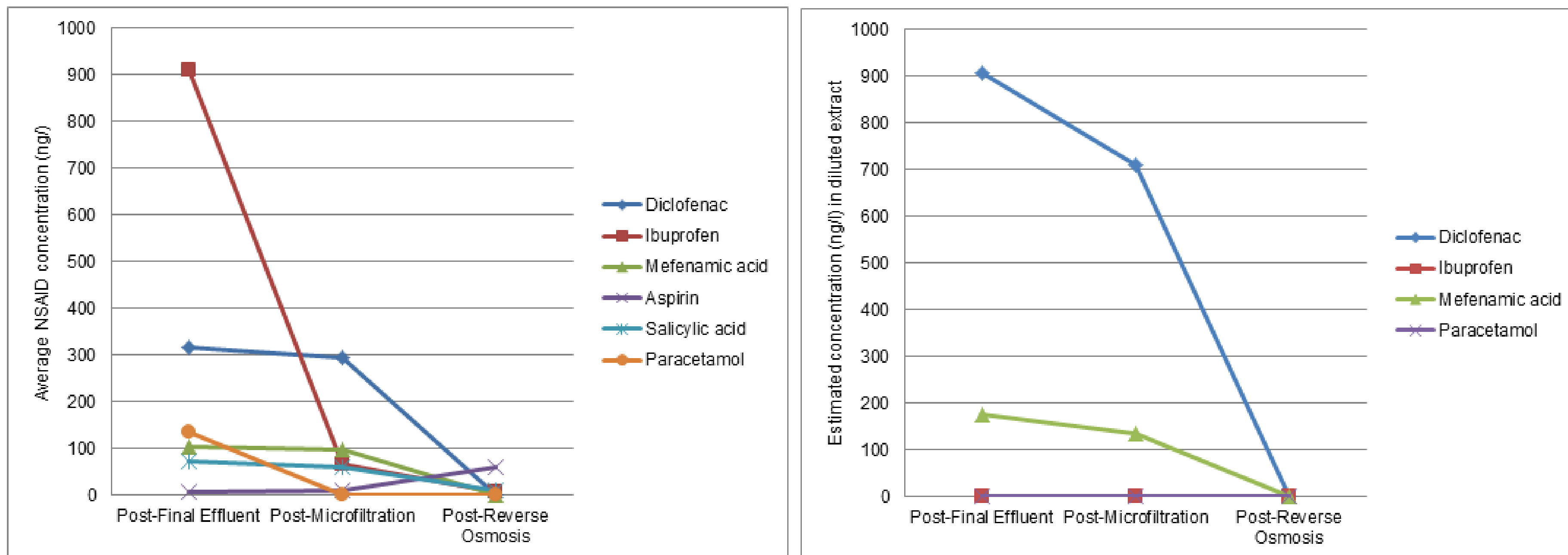

Figure 7.7 The graph to the left shows the concentration of various NSAIDs measured in the product waters following the relevant treatments (final effluent, microfiltration and reverse osmosis) taken from data provided by Thames Water Ltd, which had been collected between the years 2008 and 2012. The graph on the right was created from the chemical analysis data supplied by CEFAS when they examined the POCIS extracts in regarding the presence of a number of pharmaceuticals. For the purposes of this comparison only the relevant pharmaceuticals were used i.e. the NSAIDs. The results supplied by CEFAS are in ng/ml of extract, however to carry out a meaningful comparison, the results were divided by 5000, which was the predicted average concentration that resulted from the use of the POCIS and converted to ng/l.

7.5.2 Additional zebrafish embryo exposures

NSAIDs

Tables 7.7, 7.8 and 7.9 detail the number and type of abnormalities observed at 72 hours in the zebrafish embryos following exposure to the different NSAIDs. There was only one abnormality in the naproxen (Table 7.9) exposure at the highest dose (12500 ng/l). The exposure to diclofenac (Table 7.7) resulted in only three occurrences of abnormal zebrafish embryos, at the two highest doses (300 and 3000 ng/l) and the lowest (3 ng/l). These varied from a slight reduction in pigmentation, stunted growth and a bend in the tail. Mefenamic acid (Table 7.8) had a total of eight abnormal embryos in the exposed embryos and two in the controls. The abnormalities were varied in type. There were no abnormalities observed in any of the solvent controls.

Table 7.9 Number and type of abnormalities observed in the zebrafish embryos exposed to diclofenac for 72 hours (nominal concentrations are shown in ng/l)

Diclofenac sodium (ng/l)	Number of abnormalities at 72 hours		
	Exposure 1	Exposure 2	Total
30 000	0	0	0
3000	0	1 (less pigment)	1
300	1 (stunted growth)	0	1
30	0	0	0
3	1 (bend in the tail)	0	1
Solvent control	0	0	0
0 (control)	0	0	0

Table 7.10 Number and type of abnormalities observed in the zebrafish embryos exposed to mefenamic acid for 72 hours (nominal concentrations are shown in ng/l)

Mefenamic acid (ng/l)	Number of abnormalities at 72 hours		
	Exposure 1	Exposure 2	Total
3000	1 (stunted growth and curvature, redness of the heart, slight oedema) 1 (cloudy)	0	2
300	1 (curvature)	1 (malformed no definition)	2
30	1 (slow circulation)	0	1
3	1 (malformed no definition)	0	1
Solvent control	0	0	0
0 (control)	1 (curvature)	1 (redness of heart)	2

Table 7.11 Number and type of abnormalities observed in the zebrafish embryos exposed to naproxen for 72 hours (nominal concentrations are shown in ng/l)

Naproxen (ng/l)	Number of abnormalities at 72 hours		
	Exposure 1	Exposure 2	Total
12500	0	1 (paler, thinner, oedema of the yolk sac and heart)	1
5000	0	0	0
500	0	0	0
50	0	0	0
5	0	0	0
Solvent control	0	0	0
0 (control)	0	0	0

Tyrosinase inhibitors

Table 7.10 details the number and types of abnormalities observed in the zebrafish embryos exposed to the different tyrosinase inhibitors. In the second exposure, all the control embryos died in the second replicate, and there seemed to be what appeared to be bacterial growth in all the exposures. Ideally this exposure would have been repeated, but major building/renovation work was due to start in our animal facility. There was one individual embryo in the controls that was observed to have a curvature to the spine. This abnormality was common throughout the chemical exposures. There were also a number of abnormal zebrafish embryos in the group exposed to 4-n-butylresorinol in all doses, these were mostly curvature of the spine. This was also true for the group exposed to kojic acid. However, there were no abnormal embryos in either the low dose (10 mg/l) or high dose (1000 mg/l) niacinamide groups.

Table 7.12 Number and type of abnormalities observed in the zebrafish embryos exposed to tyrosinase inhibitors for 72 hours (nominal concentration of chemicals in μg/l or mg/l)

Treatment	Dosage	Number of abnormalities at 72 hours		
		Exposure 1	Exposure 2	Total
Control	0 (control)	1 (curvature)	0	1
4-n-Butylresorinol (μg/l)	10	1 (curvature)	2 (curvature)	3
	100	1 (curvature) 1 (curvature, redness of the heart and stunted growth)	1 (curvature)	3
	1000	3 (curvature)	2 (curvature) 2 (abnormals)	7
Kojic acid (μg/l)	5	1 (curvature)	0	1
	50	2 (curvature) 1 (tail curved upwards)	5 (curvature) 1 (redness of the heart)	9
	500	1 (slight curve) 2 (curvature)	2 (curvature) 1 (curvature and yolk sac oedema)	6
Niacinamide (mg/l)	10	0	0	0
	100	3 (curvature) 1 (stunted, twisted tail)	3 (curvature) 1 (redness of the heart)	8
	1000	0	0	0

7.6 Discussion

The initial assays, using the pooled extracts from the POCIS discs deployed in the product waters from the final effluent, microfiltration, and reverse osmosis treatments, indicated that the product water from reverse osmosis treatment had little effect on the synthesis of prostaglandin in the SC5 cells, regardless of the dilution of the extract. However, the product waters from both the final effluent and microfiltration treatments indicated a significant inhibition of the synthesis of prostaglandin in the SC5 cells in a dose-dependent manner (Figure 7.3). This indicates that reverse osmosis was effective at removing the compounds that were responsible for inhibiting prostaglandin synthesis in the earlier two treatments. Further analysis was undertaken to determine if there were any differences in the activity of the extracts in this assay associated with the different deployments, i.e. the time of year. This was possible, as the POCIS samplers were deployed at the IPR plant in eight separate deployments from August 2011 until March 2012, with each deployment lasting approximately four weeks. There were three replicate discs from each POCIS at every treatment, therefore aliquots of these replicates were pooled and tested in the assay at four

dilutions for the final effluent and microfiltration treatments, but only one dilution (1:200) for reverse osmosis as little effects were observed in the initial assays. The results of these studies are shown in Figure 7.4 and Figure 7.5. In a similar manner to the initial assay, there was a clear dose dependence, and the synthesis of prostaglandin was inhibited in the more concentrated samples for product waters from both final effluent and microfiltration. There was also no clear pattern to suggest that the individual deployments (deployments 1 to 8, August 2011 to March 2012) affected the activity. The 1:200 dilutions of the extract from reverse osmosis (data not shown) were similar to the initial assay, and did not inhibit the synthesis of prostaglandin in the SC5 cells. This confirmed what was indicated in the initial assays, that the reverse osmosis treatment was removing the chemicals responsible for the COX-inhibitory activity.

To further analyse the removal of activity observed following the treatment with reverse osmosis, the concentrations of known COX inhibitors were examined in both the product waters from the IPR plant and the concentrated POCIS extracts.

Table 7.2 illustrates the range of concentrations of different NSAIDs detected in the three product waters of interest between 2008 and 2012. These values are displayed in Figure 7.7 where a reduction in the average concentration of each chemicals can be clearly observed along the treatment. Based on analysis of processed water samples, microfiltration appears to remove a considerable amount of certain compounds of interest, but not others. To illustrate, the average concentration of diclofenac in final effluent is recorded as 316.22 ng/l which only decreases to 295.17 ng/l in the microfiltration, providing a removal rate of 6.66%. This is similar for mefenamic acid, aspirin and salicylic acid, with calculated removal rates (based on the average concentration) from final effluent to microfiltration at 4.84%, -21.38% and 13.77%, respectively. However, for ibuprofen and paracetamol, the microfiltration was much more efficient, with calculated removal rates (based on the average concentration) of 92.82 and 99.87%, respectively. It is noteworthy that the reported standard deviations and minimum and maximums concentrations for all the compounds examined show a large variation (Table 7.2). It is of note that results from Table 7.2 indicate that the concentration of aspirin has increased from post final effluent to post RO, this is considered unlikely as more aspirin would not be produced during the treatment process. The standard deviation of the reported concentration for aspirin at Post RO stage is large, 106.82 and the range of the measured concentration is large, 10 to 348 ng/l. Whereas in the post final effluent and post MF the standard deviations were 4.48 and 18.14, respectively and the reported concentrations ranged from 0.1 to 10 ng/l and 0 to 48 ng/l, respectively. These results indicate an inaccuracy in the measurement rather than an actual rise in concentration.

The small differences in average concentrations of some of the NSAIDs detected (diclofenac, mefenamic acid, aspirin and salicylic acid) go some way to explain the similar biological activity observed in the prostaglandin synthesis assay, when the SC5 cells were exposed to POCIS extracts from the final effluent and microfiltration product waters. However, it should be noted that the efficient removal of ibuprofen and paracetamol by the microfiltration treatment should have reduced the biological activity of the POCIS extracts taken from the microfiltration product water. Moreover, the data from Thames Water Ltd indicated that reverse osmosis had a greater efficiency at removing these compounds than microfiltration (Table 7.2). The reverse osmosis treatment had removal efficiencies (based on average concentrations of chemicals found in the product water from microfiltration treatment) of 99.53, 89.05, 97.74 and 81.76% for diclofenac, ibuprofen, mefenamic acid and salicylic acid, respectively. This level of removal corresponds to the lack of inhibition observed by the extracts taken from the product water following the reverse osmosis treatment.

The chemical data recorded in the extracts themselves were also examined (Table 7.6 and Figure 7.7). The data produced by CEFAS of chemicals in the extracts indicated that the NSAIDs undergo increased removal as the effluent progresses along the treatment process. Using the data from Table 7.6, it can be observed that no ibuprofen was detected in any of the three extracts, which was surprising, because it was the most prevalent in the water sample data supplied by Thames Water Ltd (Table 7.2). It is reported that environmental factors, including water flow, temperature, and the formation of biofilm on the surface of the membrane of the POCIS sampling device, can alter the uptake of the compounds (Alvarez, 2010). Different chemicals have different uptake rates in the membrane, and also degradation could have occurred during storage and extraction. Paracetamol was also undetected in all three POCIS extracts of the product waters. Similarly, this was surprising as paracetamol was detected all three product waters in the water chemistry samples taken by Thames Water Ltd. It is therefore likely that whatever affected the measurement of ibuprofen in the POCIS extracts affected the quantification of paracetamol in the same way.

The ability to estimate the water concentration of chemicals by extrapolation from the concentration determined from the POCIS extracts is limited, because there is the requirement for an experimentally derived sampling rate (R_s) for each chemical of interest (Petty et al., 2004). It is reported that for chemicals without an R_s value, the result should only be reported as mass of chemical sampled per POCIS (ng/POCIS) (Alvarez, 2010). The results reported in Table 7.6 are reported as ng per 1 ml of extract. These results only provide a qualitative value, and can only be used to indicate presence or absence of the chemical (Alvarez, 2010). However, an R_s value was found for diclofenac, and the estimated

concentrations in the product waters from final effluent and microfiltration for this compound were calculated as 165 and 129 ng/l, respectively. These estimated values are within the range detected by Thames Water Ltd. (Table 7.2) of 0 to 1492 ng/l and 0 to 1580 ng/l detected in final effluent and microfiltration, respectively. Based on these estimated concentrations from the POCIS extracts, diclofenac underwent 21.82% removal following microfiltration. This removal rate is greater than what was reported from the water sample data; however microfiltration still indicates a limited ability to effectively remove the level of this compound. There were no NSAID compounds detected in the extract from the post reverse osmosis, or that of the POCIS blank. The lack of NSAIDs detected in the extracts corresponds to the limited biological activity observed to take place in the prostaglandin synthesis assay, when the SC5 cells were exposed to extracts taken from the POCIS discs deployed in the post reverse osmosis product water.

As already discussed by other researchers, using the same prostaglandin synthesis assay in SC5 cells, have found that there are a number of other compounds, rather than just NSAIDs, that are observed to inhibit the synthesis of prostaglandin in the cell line. The IC50s of these have been compared to chemicals occurrence data collected by Thames Water Ltd. (Table 7.6 and Table 7.7). Unfortunately, not all the chemicals that had IC50s derived for them were sampled at the IPR plant. However, Table 7.7 compares the chemicals that possess IC50s and sampling data.

The comparisons made in Table 7.7 indicate that the IC50s for diclofenac, mefenamic acid and ibuprofen are approximately between 10 and 70 fold greater than the levels detected in the effluent, whereas the other chemicals listed the IC50s are hundreds of fold greater than the detected concentrations. Another chemical of interest was clotrimazole, a commonly used antifungal treatment. It belongs to a group of chemicals that have been reported to have anti-inflammatory properties mediated via the COX-2-PGE_2 pathway. Additionally, when tested in melanocytes, it was found to reduce the production of melanin, via what was considered to be indirect action of the tyrosinase, as the gene expression was unaltered (Chung et al., 2015). Clotrimazole was found to be present in the POCIS extracts, and the water samples, with average concentrations (minimum to maximum (standard deviation)) of 17.79 ng/l (0-120 ng/l (27.06)) and 5.23 ng/l (0-32 ng/l (9.56)) in the final effluent and microfiltration, respectively. Clotrimazole was also detected at 1.4 ng/l in the aquarium water, unfortunately there was only one sampling event.

The biological assay indicated that the POCIS extracts from the devices deployed in both the product waters from the final effluent and microfiltration treatments inhibited the production of prostaglandin. Both the chemistry analysis carried out on the water samples, and the POCIS extracts, have shown that there are a number of chemicals present in these two product waters that are known to inhibit prostaglandins, and more specifically PGD_2. However, the occurrence data available for the chemicals known to have these effects seem to show these to be present at concentrations lower than was observed in the activity in the assay, even though these were from concentrated extracts. There are many chemicals that were not sampled and potentially other chemicals present that have not been tested in this bioassay, but could elicit a response. It would be interesting to find out if the combination of these chemicals at low concentrations could cause the effects observed in the bioassay in the final effluent and microfiltration. Considering that melanogenesis and prostaglandin synthesis can impact on one another, it would also be interesting to test a number of known tyrosinase inhibitors in the prostaglandin inhibition assay, to see if they also inhibit prostaglandin synthesis.

The zebrafish embryo assay conducted with the NSAIDs did not demonstrate a consistent number of abnormalities. There was just one abnormality in the embryos exposed to naproxen (Table 7.9) and exposure to diclofenac had just 3 embryos exhibiting abnormalities (Table 7.7). The exposure to mefenamic acid (Table 7.8) produced an increased number of embryos with abnormalities, but there were two individuals with abnormalities in the controls. Therefore, the increased number of abnormalities could be associated with the conditions of the aquarium water for that exposure and/or the quality of the embryos. It would be interesting to look at the gene expression levels of these embryos to determine if it is

different from that of the controls. Overall, in these exposures there was a smaller variety of abnormalities than the exposures to the IPR water, which could be due to the single chemical exposure. The number of abnormalities were too small to apply any meaningful statistics.

The abnormalities observed in the zebrafish exposed to the tyrosinase inhibitors (Table 7.10) were more consistent in type. However, in the second replicate there was high mortality in the treatments and control, which was due to what looked like the appearance of bacterial growth, indicative of a problem with the water quality. However, in the controls there was only one individual with a curvature in the spine. This abnormality was common amongst the fish exposed to chemicals. Skeletal malformations are observed in instances of disruption to the retinol metabolism (Herrmann, 1995; Rolland, 2000).

Main conclusions

Both the extracts taken from microfiltration and final effluent inhibited the synthesis of prostaglandin in the SC5 cell line. However, this inhibition was removed by the reverse osmosis treatment.

NSAIDs were detected in both the water samples and the POCIS extracts. However, it is considered that their concentrations would have needed to be greater than what is reported to be responsible for the activity detected in the assay. However, in the literature there have been other chemicals/environmental contaminants that exhibit the same inhibition of the prostaglandin synthesis in the same assay as the NSAIDs. Some of these were also detected in the water samples; therefore the activity could be attributable to a mixture of chemicals.

The zebrafish embryos exposures using three NSAIDs did not indicate that the NSAIDs were responsible for the lack of pigmentation; however, it would be interesting to test a mixture of prostaglandin inhibitors in the same assay.

Tyrosinase inhibitors also did not induce lack of pigmentation in the zebrafish embryos, but there did seem to be an increase in curvatures in the spine. Due to high mortality in one replicate it would be of interest to repeat this exposure to see if the abnormalities are repeated.

CHAPTER 8: General Discussion

The main aim of the project was to assess whether biological activity was reduced in the product water from recycled water pilot plant at Deephams waste water treatment works. Chemical data from the plant was examined, zebrafish embryos were exposed to these waters, their development and gene expression were examined and *in vitro* tests were carried out on extracts taken from points along the process.

Initially, the chemical data were examined, and it was clear from the data collected over the several years of the running of the IPR plant that chemicals included in the analysis were being reduced, if not completely removed as the treated water progressed along the treatment process. This was not surprising as treatment efficiency of the technologies in place have been studied. However, there was still some residual chemical contamination in the most thoroughly treated product water, and not every conceivable chemical was and could be tested for. This was the reasoning for using a biological assay.

Zebrafish embryos are widely used in research and in toxicology. They have a number of benefits over the use of adult fish; namely it is deemed more ethically sound to use an embryos, and they are not considered a protected animal under the UK Home Office guidelines, until day 5 following fertilisation, and using a developing organism pin points them during this sensitive window.

A number of developmental exposures were undertaken, in which zebrafish embryos were exposed to different product waters from the IPR plant. The exposure design was based on the OECD 236 guidelines (OECD, 2006), and therefore lasted for 48 hours (exposure starting shortly after fertilisation until 48 hours post fertilisation). In 2013 the OECD updated and finalised these guidelines, and recommended an exposure time of 96 hours (OECD, 2013). However, the exposures had already been completed by this time.

In the exposures involving measurement of gene expression, significant differences in survival were observed between the 48 hpf groups reared in final effluent, and those in the rest of the product waters from the treatment process. The survival in the final effluent was reduced, but not significantly from the control. However, there were no differences in survival between the different repeats of the experiments. One explanation for this was that the three repeats of exposures for the gene expression study were all carried out within one month, therefore the adult breeding fish would not be affected in any way by increasing age, and there would be limited seasonal impact on the product waters from the treatment works. The other two types of exposure were repeated over a much longer time period, which could have introduced a level of variation. The survival could also have been adversely impacted in the gene expression exposure because any dead embryos were not removed from the test vessel until the specified sampling time-point. Because the majority of mortalities

observed in the other exposures occurred within the first 12 hours, it is likely that, in the 48 hpf group, the live embryos would be inhabiting the same water as the dead and decomposing embryos for approximately 36 hours.

Hatching rates were a straightforward endpoint to measure, and they are reported to be correlate with twenty different endpoints, including abnormalities such as curvature in the spine, yolk sac size, and yolk sac oedema (Ducharme et al., 2013), and is, therefore, considered to be a useful indicator of toxicity. However, as was highlighted in this study, differences from exposure 1 in the 48 hour developmental exposure, and 3 and 4 (Figure 5.5 and Figure 5.6), show that the hatching figures must be recorded at exactly the same time for all exposures and repeats, to ensure the data are comparable. The results suggested that embryos exposed to the control water had a lower hatching rate than the embryos exposed to the water from the IPR treatment and tap waters; there were fewer types of abnormalities observed in the embryos in the control group. Additionally, the product waters from the treatment plant were treated with chloramine, to prevent biofilm building up in the pipework, and the tap water was chlorinated. These obvious similarities between the tap water and product waters could have increased the hatching rate compared to the control.

The heart rate data recorded did not indicate any differences in the heart rates associated with the treatments in which the zebrafish embryos were reared. However, heart rate was a difficult endpoint to record, and was very variable when the first measurements were recorded, although this improved with later exposures (Figure 5.7). Other authors had also reported the recording of the heart rates being labour intensive, and that the results were variable (Brannen et al., 2010). It is considered that recording heart rates would be only worthwhile if there is evidence to suggest that cardiovascular effects are a potential endpoint of the exposure media.

A low frequency of abnormal embryos was recorded in both control waters and treatments. However, the nature of the abnormalities observed in the embryos reared in the IPR product waters were more variable than those observed in the embryos reared in control waters. Additionally, there were more abnormalities observed in the treatments that were classified as teratogenic abnormalities. Hypoxic conditions have been reported to cause increased abnormal development in zebrafish embryos (Shang & Wu, 2004). However, the data available for the oxygen content of the product waters (Table 3.4), did not indicate that the embryos in this current work would have been exposed to hypoxic conditions. Therefore, it was considered more likely that the abnormalities were being caused by chemical contaminants and biological chance. Abnormalities included oedema of the yolk sac and heart, malformed hearts, bent tails, and lack of pigment. Reduced pigmentation in zebrafish

embryo is reported following a number of chemical exposures to anilines and phenols (Schulte, 1997; cited in Nagel, 2002); p-tert-butylphenol (Maiwald, 1997 cited in Nagel, 2002); all-trans-retinoic acid, hydroxyurea and urea (Brannen et al., 2010).

Two other abnormalities, oedema of the yolk sac and/or heart, and heart malformations were observed. Other studies have found the cardiovascular and gross morphology endpoints in zebrafish embryo exposures correlated well with perocardial oedema (Ducharme et al., 2013). Oedema is reported to have a knock-on effect to other adverse symptoms of the circulatory system and kidney funtion (Ducharme et al., 2013), but oedema has also been associated with disruption to metabolism of retinol (Herrmann, 1995). Disruption to the retinol metabolism is also associated with bent tails and skeletal deformitiies (Herrmann, 1995), which were observed in the following treatments, with the number of incidences of the abnormality recorded in brackets; control (1), Tap (3), final product (1), AOP2 (4), reverse osmosis (3) and microfiltration (3). However, it was difficult to associate the variety of abnormalities to any one specific chemical. This is because, very often, the exposure studies are conducted exposing one single chemical at a time, whereas in the project, the developing zebrafish embryos were being challenged with a large number of different chemicals, eventhough at low concentrations.

Zebrafish embryos exposed to the product waters from the different treatments along the IPR process were used for microarrays. The total exposure was 48 hours, with pooled embryo samples being sampled at 8, 12, 16, 24, 36 and 48 hours post fertilisation. The microarrays consisted of the entire genome. The analysis to determine the gene differentiation indicated that the developmental stage had the strongest influence over the gene expression, but also that there were differences in expression between the different exposures, suggesting that there are large differences in the genes expressed due to the differing nature of the effluents; the breeding adults and laboratory conditions could also have a bearing.

There were differences observed in the individual genes expressed, namely cytochrome P450 (cyp1a and cypb1) and somatolactin beta. The cytochrome P450 is part of detoxification process as well as being involved in other pathways, and this was no longer observed to be up-regulated in the treatment processes past and including reverse osmosis, indicating that reverse osmosis could be removing the contaminants responsible for the induction of these biotransformation genes. The picture with somatolactin beta was less clear; this gene was up-regulated in the embryos exposed to product waters from reverse osmosis, AOP2, final product, and tap water. Somatolactin beta is a fish-specific gene involved in multiple processes, one of which is melanogenesis; the process in which the skin

pigment melanin is synthesised. This was of particular interest due to the lack of pigmentation observed in some of the zebrafish embryos.

Until relatively recently, the analysis of microarray data has only comprised of significance analysis of microarrays (SAMs) (Werner, 2008). This method simply compares lists of genes which were up- and down-regulated: from this data alone it is difficult to get any meaningful insight into the biological processes potentially being affected, and the potential outcome on the organism as a whole. This is because many genes (if not all) possess multiple functions, which are dependent on the context (Falciani et al., 2008; Werner, 2008). It is also important to keep in mind, when assessing these gene lists, that not all alterations in mRNA levels are directly attributable to the experiment itself (Werner, 2008), since there are normal processes still taking place, and, for the purposes of this study, embryo development. Gene ontology can be used; this results in lists of genes being created, but within a meaningful context. The genes are then categorised based on the functional categories. Following on from gene ontology, data from the literature is accumulated and the specific pathways can be determined. Pathways focus on physical and functional interactions between the different individual genes, instead of focusing solely on the genes themselves (Werner, 2008). When looking at the pathways altered in the tissue of organism, or organism as a whole, the pathways can be related to toxic, stress, and adaptive responses (Falciani et al., 2008). However, it is not possible to examine all pathways with microarray data, as metabolic pathways are largely controlled by protein-based events, which are not detectable on the microarray. Additionally, kinase-based signalling cascades do not always involve alterations in the mRNA levels (Werner, 2008). This could have been the case in both the prostaglandin synthesis, and melanogenesis processes. A step further on from investigating pathways is that of networks; commonly, biological processes involve more than one pathway. The pathways interconnect, and, as with the individual genes, act in a context-specific way. This interconnection results in a network; for microarray data, this is mainly a regulatory network. However, unlike the pathways, where a computer programme can be used to link the genes to a specific pathway based on the data present in the literature, networks cannot be easily determined from the literature or pre-compiled pathways. This is because the structures within the networks are not fixed, and alter depending on the context. It is reported that regulatory networks also target and change (normally only a few genes for every pathway are involved), but do so across several pathways within the regulatory network (Werner, 2008). Werner (2008) stated that this explains why only a few genes normally get highlighted as being altered in a given pathway, and complete pathways will never be observed to be co-ordinately up-regulated or down-regulated at the same level as individual genes (Werner, 2008).

The differences in gene expression in the embryos exposed to product waters were greater the further the treatments were from one another. For example, there were larger differences between the genes expressed in microfiltration and final product water, than final product and AOP2 product waters (**Error! Reference source not found.** and **Error! Reference source not found.**). Treatment with reverse osmosis seemed to reduce the differences in gene expression, with microfiltration and reverse osmosis product waters having very different gene expression profiles, as did the two AOP treatments: AOP1 not having been processed by reverse osmosis, whereas the product water from AOP2 had been treated with reverse osmosis.

The pathways altered along the treatment were steroid hormone biosynthesis, tryptophan metabolism, retinol metabolism, metabolism of xenobiotics by cytochrome P450, drug metabolisms-cytochrome P450 and drug metabolism-other enzymes. The cytochrome P40 genes upregulated in the earlier stages of the treatment process (final effluent, microfiltration and AOP1) are active in some of these pathways, cyp1a is active in retinol metabolism, steroid hormone biosynthesis and drug metabolism, cyp1b1 is active in the steroid hormone biosynthesis and tryptophan metabolism. This demonstrates how, as earlier discussed, individual genes are active in multiple pathways. From the literature there seemed to be strong indications that the pathways were interconnected as well. Dioxin and dioxin-like compounds are reported to alter the metabolism of retinoids, by acting via the AhR, this is because retinol metabolism involves cytochrome P450. Induction of cytochrome P450 from chemical exposure can cause excessive metabolism of the retinol thus decreasing its levels in the plasma and liver stores (Rolland, 2000). Some of the retinoids are reported to be used for treatment of hyperpigmentation, and interestingly the tyrosinase inhibitors tested in the zebrafish embryo assay caused spinal curvatures (not in a dose-dependent manner and at a low frequency), which is an abnormality associated with retinol metabolism disruption during development. Lack of pigmentation was reported in zebrafish embryos exposed to the retinoid, all-trans-retinoic acid (Brannen et al., 2010).

As briefly mentioned above, there were some associations between the apical endpoints observed and observed and the genes and pathways observed to be altered. Comparisons were made between these between these and the chemicals detected in the product waters. A group of chemicals known to be known to be present in the product waters were NSAIDs. Because it is known that COX-1 is synthesised synthesised in the skin cells and linked to melanogenesis, it was considered possible that the NSAIDs the NSAIDs present could be responsible for the lack of pigmentation in the embryos. It would have been would have been interesting to test the retinoid pathway, but there was no readily available *in vitro* assay *in vitro* assay to test these, and so the decision was taken to test the extracts from the treatment water in treatment water in a prostaglandin assay. The synthesis of prostaglandin was inhibited in the extracts

extracts taken from the final effluent and microfiltration treatments, but this inhibition was removed by reverse osmosis. It is surprising that there was not a larger difference between the activities of the extracts from the final effluent and microfiltration, as the chemistry data indicated that treatment with microfiltration had removed a production of the NSAIDs concentration. The question was then raised regarding other chemicals present in the extracts that could be responsible for the inhibition. Other authors had found a number of compounds that caused inhibition in the same assay (Kristensen et al., 2011; Kugathas et al., 2015). Some of the chemicals were also present in the product waters, but all at significantly lower concentrations than the IC50 derived for the compounds. There is the possibility that possibility that these, and other unknown chemicals, could be present and have a mixture effect, working effect, working in combination. The RO removed the biological activity, in a similar way demonstrated by demonstrated by the genomic results. However, there were still a variety of abnormalities being observed being observed in the RO water (

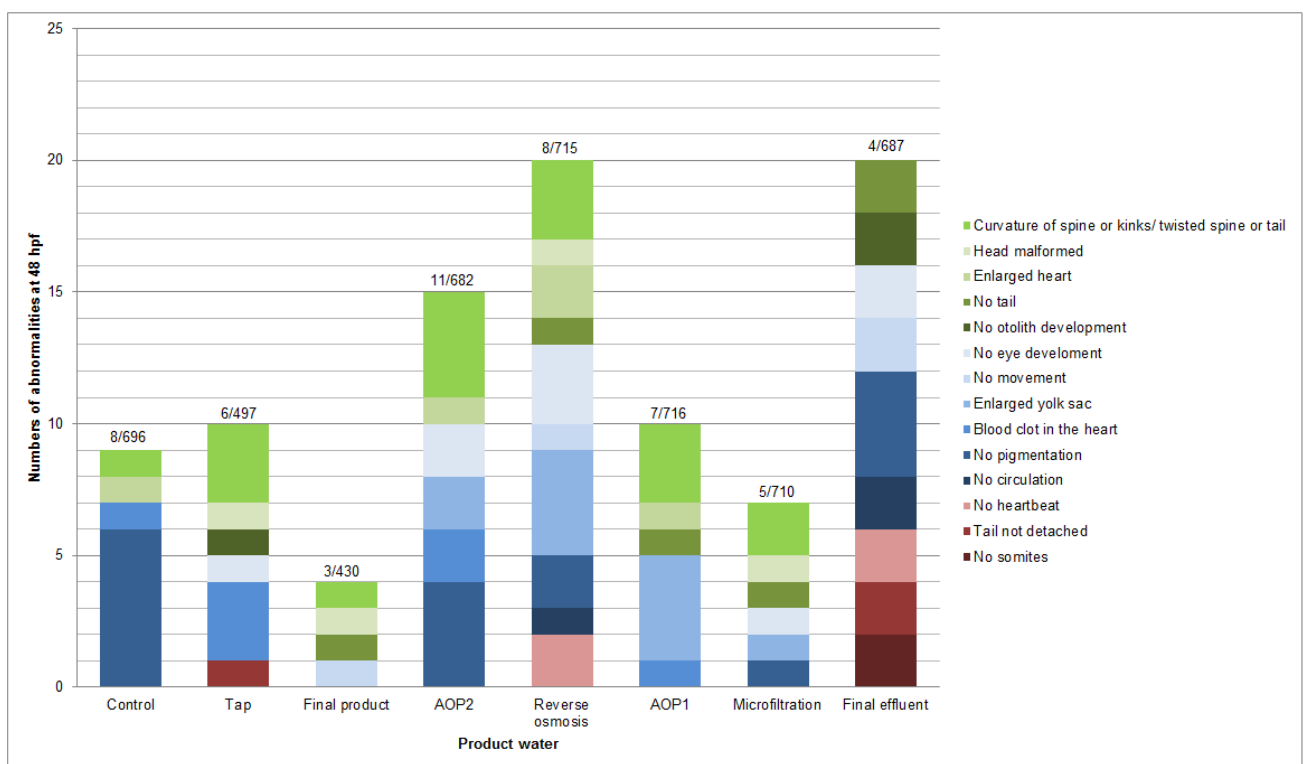

Figure 5.9), therefore, indicating that chemical contaminants are still getting through the reverse osmosis treatment, which is evident from the chemical analysis data completed on the POCIS extracts Table 7.6 with the presence of trimethoprim still in the extract from the reverse osmosis.

Real-time PCR was performed (results not shown) on a number of genes associated with melanogenesis and prostaglandin synthesis, but it is unlikely that the expression levels of these were altered, suggesting that the contaminants could have been acting directly on the protein.

Microarrays produce a simultaneous measure of the expression of thousands of genes expressed in tissues of organisms, or, in the case of this project, the organism as a whole. When these organisms have been taken from the environment, the gene expression profile is not just influenced by the toxicant of interest, but also by genetic make-up, age, diet, salinity, temperature, reproductive stage, etc. In addition to these factors that influence the gene expression profile, there is also inter-individual variability; these, combined, can cause vast variability in gene expression data, and drilling down into the alterations caused by the toxicant in question is difficult (Falciani et al., 2008). In the exposures carried out in this project, we used organisms at very similar ages, as they were collected soon after fertilisation. However, they were from different breeding groups, so there could be slight variations in the age, but the batches of eggs collected from the different breeding groups were mixed together before sorting and exposing to the different treatments. As a result, very slightly younger or older (by minutes) embryos would not be favoured in one specific exposure. The embryos were all from the same strain of zebrafish, TU, reducing, slightly, the variations in genetic make-up. The temperature during the exposures was kept constant. However, the product water from the treatment plant was from treated non-standardised effluent, therefore, introducing variability between experiments. This however, made the experimental design more environmentally relevant because the embryos were exposed to the effluent collected on different days, as if they were exposed in the environment.

It has been reported that microarray technology, and the use of its data, is advancing, and, if a specific alteration to biological pathways in a tissue of an organism can be established, and those pathways can be associated with toxic, stress or adaptive responses, then expression profiles can be used as a diagnostic tool to determine exposure to certain classes of chemicals, and can allow class-prediction, when the samples have been classified to a pre-existing database (Falciani et al., 2008).

When using the gene expression profiles as a potential diagnostic tool, it is also important to take into account that gene expression profiles will vary in relation to the dose of toxicant, length of exposure, and the time taken between exposure and sampling (Falciani et al., 2008). This was evident from the data reported by this project; the gene expression was influenced by the experiment, suggesting that there could have been alterations in the composition of the effluent and water quality. Even if these differences were subtle, the gene expression profiles did differ.

When assessing an exposure that consists of multiple chemical classes, measuring the expression of just one single biomarker or individual gene, cannot provide an integrated assessment of the effects (Falciani et al., 2008). However, as has been observed from the

data gathered in this project, a multi-disciplinary approach allows a greater understanding of the potential effects of environmental pollutants, and specifically mixtures of environmental pollutants containing potentially un-identified compounds, as with the case of effluent. Falciani et al. (2008) stated that the use of molecular biomarkers in conjunction with current biomonitoring approaches (for example, histopathology) can complement the risk assessment, The molecular biomarkers provide valuable information on the mechanisms of toxicity and can also detect subtle changes that are associated with adaptive or compensatory responses to exposure of the toxicant, and then have the potential to be used as a future predictive tool of potential toxicity (Falciani et al., 2008). The alteration in the biotransformation pathways observed in the genomic analysis of the exposed embryos could be an indication of a compensatory response.

When this project was started, data had been collected on a number of chemicals, and there was an understanding on how efficiently they were being the concentration of removed, specifically by the IPR process, but little was understood regarding the biological activity. It was unclear what to expect, which is why the project was begun as non-hypothesis driven. The combination of chemistry data, developmental exposures and genomic analysis from the use of global gene expression was used to inform potential hypotheses. These were the melanogenesis pathway being disrupted by inhibition of the COX enzyme and also disruptions to the retinol metabolism pathway resulting in some of the skeletal and other developmental abnormalities observed. COX inhibition was taken forward as it was feasible to test for this mechanism of action.

This project has highlighted the importance of combining chemical data with biological endpoints, including global gene expression, since chemical screens cannot sample for every conceivable chemical. The use of microarrays can direct attention to an endpoint that would have been missed by any other approach.

8.2 Limitations

The main limitations of this body of work were limited time to complete the initial testing before the closing of the IPR pilot plant, the survival rate of the embryos and variations in the physico-chemical properties between the IPR product waters and the control.

Unfortunately, the survival of the embryos in the treatments and controls were below that which is considered valid by the OECD, which is $\geq$90% (OECD, 2013). The majority of the mortalities occurred within the first 12 hours (Figure 5.1): a number of techniques were tried to improve this but it was not possible in the time allowed. The IPR plant had been in operation since 2008, was due to close in March 2012, and this project was started in May

2011, allowing less than a year to plan and complete the main exposure work. If this work were to be repeated, more techniques to improve the survival would be tried, for example using a variety of embryo mediums, and altering the temperature in the incubator, which was held at 28.5ºC, whereas the OECD guidelines now state 26ºC. It would also be useful to be able to measure the dissolved oxygen within the wells of the 24 well plates; the available oxygen probe was too large to measure within the wells themselves. However, the survival rate did not show any significant differences between treatments and control in the first two study types carried out (developmental exposures and extended exposures), indicating that the water from the treatment processes at the IPR plant did not adversely affect the survival compared to the controls. If survival was decreased in one experiment it was decreased within all treatment groups, indicating that there could have been varying quality of the gametes produced by the zebrafish (breeding groups were chosen at random and were not kept to same individuals in each breeding group).

8.3 Recommendations for further work

It is considered that background differences between the product waters and control were greater enough to have potentially masked some of the genetic alterations caused by the chemical exposure. Therefore, before embarking on similar work in the future it would be of great use to isolate a more appropriate control water. The appropriate control water would have the same physico-chemical properties as the product water being tested, i.e. pH, water hardness etc. Alterations could either be done to the product water, for example pH corrections and adding minerals or a cleaned up version of the product water could be used as the specific control. This would greatly increase the number exposures needed and therefore workload, i.e. having an individual control for every product water tested, but it could greatly increase the informative nature of the gene expression data and would remove background noise.

Two groups of chemicals were highlighted as potentially causing some of the phenotypic differences in the zebrafish exposed to the product water from the IPR plant. These were NSAIDs and chemicals used in cosmetics marketed to reduce skin pigmentation. A number of these chemicals were tested to determine if development of pigmentation in the embryos could be altered. However, no changes in pigmentation following exposure to these chemicals were observed. Further testing at increased concentrations and even mixtures could be informative. Also, effect directed analysis (EDA) could be employed to determine what fraction of the effluent, and therefore the identity of the chemicals responsible for the abnormalities and certain pathway effects.

Some of the abnormalities such as spinal curvatures, cardiac abnormalities and decreased pigmentation have been associated with disputations to retinoid pathway. Additional, the retinol metabolism pathway was shown to be disrupted in the embryos exposed to the IPR product waters. ,. Therefore, further investigation into the effects on the retinoid pathway, and specific retinoid disrupting chemicals would be of interest.

While investigating the potential causes of lack of pigmentation in the zebrafish embryos, a great deal of information was gathered on certain chemicals used in the cosmetics industry used specifically to treat hyper-pigmentation or to reduce levels of pigmentation in the skin. Many of these chemicals act as tyrosinase inhibitors. Literature searches untaken at the time were unable to locate any data of the presence of these chemicals in the environment. As it seems apparent these chemicals are widely used, and have the potential to enter the environment following application to skin and showering and bathing, it would be of interest to determine if they pose a risk and if they are present in the environment. Zebrafish embryos could be used to repeat the exposure studies to determine if the abnormalities observed in the exposures are repeatable.

Inhibition of prostaglandin synthesis was established to occur when SC5 cells were exposed to concentrated extracts taken from both post final effluent and post microfiltration. This inhibition was not observed in the extracts taken from post reverse osmosis. Therefore, it was concluded that chemicals causing inhibition of the production prostaglandins were present prior to treatment with reverse osmosis. QPCR was carried out on the embryos exposed at these treatment points and genes specific to prostaglandin were measured, however, an observable difference in the gene expression of this specific gene was not observed. Therefore, it was presumed that the inhibition was occurring directly at the protein level rather at the gene level, determining this would be of interest. Additionally, it would be of interest to investigate the specific chemicals causing the inhibition of prostaglandin synthesis. This was highlighted as potential point of interest due to the NSAIDs being measured to be present, but at too lower concentrations to elicit the degree of inhibition observed. Suggesting, that other chemicals may have been present in the product waters that also exhibit a inhibiting of prostaglandin synthesis.

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APPENDIX

Pharmaceuticals and oestrogens detected in the aquarium water and tap water

Component Name	Units	Aquarium							Tap						
		1	2	3	mean	min	max	Std dev	1	2	3	mean	min	max	Std dev
Ibuprofen	ng/l		10.4		10.4	10.4	10.4	N/A		11		11	11	11	N/A
Diclofenac	ng/l		nd		N/A	0	0	N/A		nd		N/A	0	0	N/A
Paracetamol	ng/l		nd		N/A	0	0	N/A		nd		N/A	0	0	N/A
Erythromycin	ng/l		1.7		1.7	1.7	1.7	N/A		1.6		1.6	1.6	1.6	N/A
Sulfamethoxazole	ng/l		nd		N/A	0	0	N/A		1.9		1.9	1.9	1.9	N/A
Acetyl-sulfamethoxazole	ng/l		nd		N/A	0	0	N/A		2.8		2.8	2.8	2.8	N/A
Trimethoprim	ng/l		<LOQ		N/A	0	0	N/A		nd		N/A	0	0	N/A
Propanonol	ng/l		nd		N/A	0	0	N/A		nd		N/A	0	0	N/A
Clofibric Acid	ng/l		<LOQ		N/A	0	0	N/A		nd		N/A	0	0	N/A
Mefenamic Acid	ng/l		nd		N/A!	0	0	N/A		nd		N/A	0	0	N/A
Dextropropoxyphene	ng/l		nd		N/A	0	0	N/A		nd		N/A	0	0	N/A
Tamoxifen	ng/l		nd		N/A!	0	0	N/A		nd		N/A	0	0	N/A
Clotrimazole	ng/l		1.4		1.4	1.4	1.4	N/A		1.2		1.2	1.2	1.2	N/A
Estrone (E1)	ng/l	0.03	0.08	<0.03	0.047	0.03	0.08	0.029	0.18	<0.03	<0.03	0.08	0.03	0.18	0.087
Estradiol (E2)	ng/l	<0.03	<0.03	<0.03	0.03	0.03	0.03	0	<0.03	<0.03	<0.03	0.03	0.03	0.03	0
Ethynyl estradiol (EE2)	ng/l	0.04	<.03	<0.03	0.033	0.03	0.04	0.0057	0.05	<0.03	<0.03	0.037	0.03	0.05	0.012

<LOQ: Below Limit of Quantification; N/A: Not Applicable; nd: not detected

Pesticides detected in the aquarium water and tap water

Component Name	Units	Aquarium							Tap						
		1	2	3	mean	min	max	Std dev	1	2	3	mean	min	max	Std dev
2 3 6-TBA	µg/l	<0.006	<0.006	<0.006	0.006	0.006	0.006	1.06×10^{-18}	<0.006	<0.006	<0.006	0.006	0.006	0.006	1.06×10^{-18}
2 4 5-T	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}
2 4-D	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	0.003	<0.002	0.0023	0.002	0.003	0.00058
2 4-DB	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}
Ametryne	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Atrazine	µg/l	<0.005	<0.005	<0.005	0.005	0.005	0.005	0	<0.005	<0.005	<0.005	0.005	0.005	0.005	0
Bentazone	µg/l	<0.005	<0.005	<0.005	0.005	0.005	0.005	0	<0.005	<0.005	<0.005	0.005	0.005	0.005	0
Bromoxynil	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Carbendazim	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Carbethmide	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}	0.003	0.003	0.003	0.003	0.003	0.003	5.31×10^{-19}
Chlortoluron	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}
Clopyralid	µg/l	<0.009	<0.009	<0.009	0.009	0.009	0.009	0	<0.009	<0.009	<0.009	0.009	0.009	0.009	0
Dicamba	µg/l	<0.007	<0.007	<0.007	0.007	0.007	0.007	0	<0.007	<0.007	<0.007	0.007	0.007	0.007	0
Dichloprop	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Diuron	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}
Fenoprop	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Flufenacet	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}
Fluroxypyr	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31×10^{-19}
Ioxynil	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Isoproturon	µg/l	<0.004	<0.004	<0.004	0.004	0.004	0.004	0	<0.004	<0.004	<0.004	0.004	0.004	0.004	0
Linuron	µg/l	<0.004	<0.004	<0.004	0.004	0.004	0.004	0	<0.004	<0.004	<0.004	0.004	0.004	0.004	0
MCPA	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
MCPB	µg/l	<0.007	<0.007	<0.007	0.007	0.007	0.007	0	<0.007	<0.007	<0.007	0.007	0.007	0.007	0

Mecoprop	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Metaldehyde	µg/l	0.016	0.017	0.013	0.015	0.013	0.017	0.00201	0.025	0.015	0.012	0.017	0.012	0.025	0.0068
Metazachlor	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Monuron	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
Pentachlorophenol	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Picloram	µg/l	<0.004	<0.004	<0.004	0.004	0.004	0.004	0	<0.004	<0.004	<0.004	0.004	0.004	0.004	0
Prometryn	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Propazine	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Propyzamide	µg/l	<0.004	<0.004	<0.004	0.004	0.004	0.004	0	<0.004	<0.004	<0.004	0.004	0.004	0.004	0
Quinmerac	µg/l	<0.004	<0.004	<0.004	0.004	0.004	0.004	0	<0.004	<0.004	<0.004	0.004	0.004	0.004	0
Simazine	µg/l	<0.005	<0.005	<0.005	0.005	0.005	0.005	0	<0.005	<0.005	<0.005	0.005	0.005	0.005	0
Tebuthiuron	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Terbutryn	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
Triclopyr	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	0.003	0.002333	0.002	0.003	0.00058
2,3,5,6-Tetrachloroaniline	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
2,3,5,6-Tetrachlorothioanisole	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Aldrin	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Chlorothalonil	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Chlorpropham	µg/l	<0.005	<0.005	<0.005	0.005	0.005	0.005	0	<0.005	<0.005	<0.005	0.005	0.005	0.005	0
DDE -op	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
DDE -pp	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
DDT -op	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
DDT -pp	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Dichlobenil :- {2,6-Dichlorobenzonitrile }	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0

Dieldrin	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Endosulfan A	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
Endosulfan B	µg/l	<0.004	<0.004	<0.004	0.004	0.004	0.004	0	<0.004	<0.004	<0.004	0.004	0.004	0.004	0
Endrin	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
HCH -alpha	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
HCH -beta	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
HCH -delta	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
HCH -epsilon	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
HCH -gamma :- {Lindane}	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
Heptachlor	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Hexachlorobe nzene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Hexachlorobut adiene	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
Isodrin	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Methoxychlor	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Pendimethalin	µg/l	<0.01	<0.01	<0.01	0.01	0.01	0.01	0	<0.01	<0.01	<0.01	0.01	0.01	0.01	0
Pentachlorobe nzene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Propachlor	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
TDE - op	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
TDE - pp	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Tecnazene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Tri-allate	µg/l	<0.006	<0.006	<0.006	0.006	0.006	0.006	1.062x10^{-18}	<.006	<0.006	<0.006	0.006	0.006	0.006	1.062x10^{-18}
Trifluralin	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
Vinclozolin	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
cis-Chlordane	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
cis-Heptachlor epoxide	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
trans-Chlordane	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0

trans-Heptachlor epoxide	µg/l	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}	<0.003	<0.003	<0.003	0.003	0.003	0.003	5.31 x10^{-19}
Pentachlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
AMPA :- {Aminomethyl phosphonic acid}	µg/l	0.23	<0.1	<0.1	0.14	0.1	0.23	0.076	0.22	<0.1	1.35	0.79	0.221	1.35	0.8
Glyphosate	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	0.19	0.128	0.1	0.19	0.049

Organic contaminants detected in the aquarium water and tap water

Component Name	Units	Aquarium							Tap						
		1	2	3	mean	min	max	Std dev	1	2	3	mean	min	max	Std dev
Benzo (A) Pyrene	ng/l	<1	<1	<1	1	1	1	0	<1	<1	<1	1	1	1	0
Benzo(A)pyrene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	0.001	<0.001	0.001	0.001	0.001	0
Benzo(B)fluoranthene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	0.002	0.001	0.002	0.0017	0.001	0.002	0.00058
Benzo(K)fluoranthene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Benzoghiperylene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Fluoranthene	µg/l	<0.005	<0.005	<0.005	0.005	0.005	0.005	0	0.23	0.23	0.29	0.25	0.23	0.29	0.034
Indeno1 2 3 -cdpyrene	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
NDMA	µg/l	<0.0008	<0.0008	0.0013	0.00097	0.0008	0.0013	0.00029	<0.0008	<0.0008	0.0015	0.0010	0.0008	0.0015	0.000404
PAH - Total of 4 in new Reg 200	µg/l	0	0	0	0	0	0	0	0.002	0.001	0.002	0.0017	0.001	0.002	0.00058
PAH'S	µg/l	0	0	0	0	0	0	0	0.23	0.23	0.29	0.25	0.23	0.29	0.035
1,2,3-Trichlorobenzene	µg/l	<0.01	<0.01	<0.01	0.01	0.01	0.01	0	<0.01	<0.01	<0.01	0.01	0.01	0.01	0
1,2,4-Trichlorobenzene	µg/l	<0.01	<0.01	<0.01	0.01	0.01	0.01	0	<0.01	<0.01	<0.01	0.01	0.01	0.01	0
1,3,5-Trichlorobenzene	µg/l	<0.01	<0.01	<0.01	0.01	0.01	0.01	0	<0.01	<0.01	<0.01	0.01	0.01	0.01	0
PCB 008	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 020	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 028	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 031	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 035	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0

PCB 052	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 077	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 101	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 105	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 118	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 126	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB128	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 138	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 149	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 153	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 156	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 169	µg/l	<0.001	<0.001	<0.001	0.001	0.001	0.001	0	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
PCB 170	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
PCB 180	µg/l	<0.002	<0.002	<0.002	0.002	0.002	0.002	0	<0.002	<0.002	<0.002	0.002	0.002	0.002	0
2,3,5,6-Tetrachlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
2,3-Dichlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
2,3-Dimethylphenol :- {2,3-Xylenol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
2,4,5-Trichlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
2,4,6-Trichlorophenol	µg/l	<0.02	0.0403	0.102	0.054	0.02	0.102	0.043	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
2,4-Dichlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
2,4-Dimethylphenol :- {2,4-	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0

Xylenol}															
2,5-Dichlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
2,5-Dimethylphenol :- {2,5-Xylenol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
2,6-Dichlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
2,6-Dimethylphenol :- {2,6-Xylenol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
2-Chlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
2-Ethylphenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
2-Methylphenol :- {o-Cresol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
3,4-Dimethylphenol :- {3,4-Xylenol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
3,5-Dimethylphenol :- {3,5-Xylenol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
3-Chlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
3-Methylphenol :- {m-Cresol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
4-Chloro-2-methylphenol :- {p-	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0

Chloro-o-cresol}															
4-Chloro-3,5-dimethylphenol :- {PCMX}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
4-Chloro-3-methylphenol :- {p-Chloro-m-cresol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
4-Chlorophenol	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
4-Methylphenol :- {p-cresol}	µg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	0.02	<0.02	0.02	0.02	0.02	0
Phenol	µg/l	0.093	0.21	0.17	0.16	0.093	0.21	0.061	<0.05	0.05	<0.05	0.05	0.05	0.05	8.5 x10^{-18}
1,1,1,2-Tetrachloroethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,1,1-Trichloroethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,1,2,2-Tetrachloroethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10-^{17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,1,2-Trichloroethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,1-Dichloroethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,1-Dichloroethylene :- {1,1-Dichloroethene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,1-Dichloroprop	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}

ylene :- {1,1-Dichloropropene}															
1,2,3-Trichlorobenzene	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	0.5	<0.5	0.5	0.5	0.5	0
1,2,3-Trichloropropane	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	0.5	<0.5	0.5	0.5	0.5	0
1,2,3-Trimethylbenzene	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,2,4-Trichlorobenzene	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	0.5	<0.5	0.5	0.5	0.5	0
1,2,4-Trimethylbenzene	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,2-Dibromo-3-chloropropane	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10 $^{-17}$
1,2-Dibromoethane	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10 $^{-17}$
1,2-Dichlorobenzene	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,2-Dichloroethane	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,2-Dichloropropane	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,2-Dimethylbenzene :- {o-Xylene}	µg/l	<0.1	<0.5	<0.1	0.23	0.1	0.5	0.23	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,3,5-Trichlorobenzene	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	0.5	<0.5	0.5	0.5	0.5	0
1,3,5-	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}

Trimethylbenzene :- {Mesitylene}															
1,3-Dichlorobenzene	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	0.5	<0.5	0.5	0.5	0.5	0
1,3-Dichloropropane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
1,4-Dichlorobenzene	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
2,2-Dichloropropane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
2-Chlorotoluene :- {1-Chloro-2-methylbenzene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
3-Chlorotoluene :- {1-Chloro-3-methylbenzene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
4-Chlorotoluene :- {1-Chloro-4-methylbenzene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
4-Isopropyltoluene :- {4-methyl-Isopropylbenzene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Benzene	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Bromobenzene	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	17 x10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}

Bromochloromethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}	<0.1	0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}
Bromodichloromethane	µg/l	2.03	1.75	0.7	1.49	0.7	2.03	0.701	9.77	9.58	9.88	9.74	9.58	9.88	0.15
Bromoform :- {Tribromomethane}	µg/l	0.16	0.124	<0.1	0.142	0.124	0.16	0.025	4.41	3.94	4.68	4.34	3.94	4.68	0.38
Carbon Disulphide	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}
Carbon tetrachloride :- {Tetrachloromethane}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}
Chlorobenzene	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}
Chlorodibromomethane	µg/l	1.77	1.16	0.62	1.18	0.62	1.77	0.58	11.2	11.2	12.4	11.6	11.2	12.4	0.69282
Chloroform :- {Trichloromethane}	µg/l	1.19	1.23	0.48	0.97	0.48	1.23	0.42	7.26	6.27	5.99	6.51	5.99	7.26	0.67
Chloromethane :- {Methyl Chloride}	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0
Dibromomethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}
Dichloromethane :- {Methylene Dichloride}	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0
Dimethylbenzene : Sum of isomers (1,3- 1,4-) : {m+p xylene}	µg/l	<0.2	<0.2	<0.2	0.2	0.2	0.2	3.4×10^{-17}	<0.2	<0.2	<0.2	0.2	0.2	0.2	3.4×10^{-17}
Ethyl tert-butyl ether :- {ETBE}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7×10^{-17}

Ethylbenzene	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10-^{17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Hexachlorobutadiene	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0
Hexachloroethane	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0
Isopropylbenzene	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
MTBE :- {Methyl tert-butyl ether}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Naphthalene	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0
Styrene :- {Vinylbenzene}	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0
Tetrachloroethylene :- {Perchloroethylene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Toluene :- {Methylbenzene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Trichloroethylene :- {Trichloroethene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Trichlorofluoromethane	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
Vinyl Chloride :- {Chloroethylene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
cis-1,2-Dichloroethylene :- {cis-1,2-Dichloroethene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
cis-1,3-Dichloropropylene :- {cis-1,3-	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}

Dichloropropene}															
n-ButylBenzene :- {1-Phenylbutane}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
n-Propylbenzene :- {1-phenylpropane}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
sec-Butylbenzene :- {1-Methylpropylbenzene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
tert-Amyl methyl ether :- {TAME}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
tert-Butylbenzene :- {(1,1-Dimethylethyl)benzene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	1. 7 x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
trans-1,2-Dichloroethylene :- {trans-1,2-Dichloroethene}	µg/l	<0.1	<0.1	<0.1	0.1	0.1	0.1	17x10^{-17}	<0.1	<0.1	<0.1	0.1	0.1	0.1	1.7 x10^{-17}
trans-1,3-Dichloropropylene :- {trans-1,3-Dichloropropene}	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0

Inorganic contaminants detected in the aquarium water and tap water

Component Name	Units	Aquarium							Tap						
		1	2	3	mean	min	max	Std dev	1	2	3	mean	min	max	Std dev
Aluminium	µg/l	20.1	15.7	20.7	18.83	15.7	20.7	2.73	47	35.3	36.2	39.5	35.3	47	6.51
Antimony	µg/l	<0.5	<0.5	<0.5	0.5	0.5	0.5	0	<0.5	<0.5	<0.5	0.5	0.5	0.5	0
Arsenic	µg/l	0.9	1.1	0.9	0.97	0.9	1.1	0.12	0.8	0.7	0.8	0.77	0.7	0.8	0.058
Barium	µg/l	19	19	17.4	18.47	17.4	19	0.92	19.1	22.8	19.6	20.5	19.1	22.8	2.0075
Boron	µg/l	83	87	80	83.33	80	87	3.51	56	59	55	56.67	55	59	2.08
Boron	mg/l	0.083	0.087	0.08	0.083	0.08	0.087	0.0035	0.056	0.059	0.055	0.057	0.055	0.059	0.0021
Bromate	mg/l	0.001	0.001	0.001	0.001	0.001	0.001	0	0.002	0.002	0.002	0.002	0.002	0.002	0
Bromate	µg/l	1.2	1.3	1.2	1.23	1.2	1.3	0.058	1.5	1.6	1.8	1.6	1.5	1.8	0.15
Bromide	mg/l	0.11	0.11	0.11	0.11	0.11	0.11	0.0032	0.017	0.01	0.014	0.013	0.01	0.017	0.00404
Cadmium	µg/l	<0.2	<0.2	<0.2	0.2	0.2	0.2	3.4 x10^{-17}	<0.2	<0.2	<0.2	0.2	0.2	0.2	3.4 x10^{-17}
Cadmium Dissolved	µg/l	<0.2	<0.2	<0.2	0.2	0.2	0.2	3.4x10^{-17}	<0.2	<0.2	<0.2	0.2	0.2	0.2	3.4 x10^{-17}
Calcium	mg/l	115.1	112.2	101.9	109.73	101.9	115.1	6.94	113.4	115.9	116.5	115.27	113.4	116.5	1.64
Chromium	µg/l	<1.2	2	<1.2	1.47	1.2	2	0.46	<1.2	<1.2	<1.2	1.2	1.2	1.2	0
Chromium Dissolved	µg/l	<1.2	<1.2	1.2	1.2	1.2	1.2	0	<1.2	<1.2	<1.2	1.2	1.2	1.2	0
Cobalt	µg/l	<0.7	<0.7	<0.7	0.7	0.7	0.7	1.36 x10^{-16}	<0.7	<0.7	<0.7	0.7	0.7	0.7	1.36 x10^{-16}
Copper	µg/l	<10	<10	<10	10	10	10	0	13	17	18	16	13	18	2.65
Fluoride	µg/l	141	100	116	119	100	141	20.66	157	101	121	126.33	101	157	28.38
Iron	µg/l	<1	<1	<1	1	1	1	0	7.7	8.1	11	8.93	7.7	11	1.8
Iron Dissolved	µg/l	<1	<1	<1	1	1	1	0	<1	1.2	2.9	1.7	1	2.9	1.044
Lead	µg/l	<0.3	<0.3	<0.3	0.3	0.3	0.3	0	<0.3	<0.3	<0.3	0.3	0.3	0.3	0
Lithium	mg/l	0.009	0.009	0.008	0.0087	0.008	0.009	0.00058	0.006	0.01	0.007	0.0077	0.006	0.01	0.0021
Lithium	µg/l	8.8	8.6	7.9	8.43	7.9	8.8	0.47	6.2	9.8	6.9	7.63	6.2	9.8	1.91
Magnesium	mg/l	4.2	4.1	3.7	4	3.7	4.2	0.27	4.1	4.1	3.9	4.033	3.9	4.1	0.12
Magnesium Dissolved	mg/l	4	3.9	3.9	3.93	3.9	4	0.058	3.9	3.8	4	3.9	3.8	4	0.1

Manganese	µg/l	0.7	0.9	<0.7	0.77	0.7	0.9	0.12	<0.7	5.8	1.2	2.57	0.7	5.8	2.81
Manganese Dissolved	µg/l	<0.7	<0.7	<0.7	0.7	0.7	0.7	1.36 x10^{-16}	<0.7	4.2	1.4	2.1	0.7	4.2	1.85
Mercury	µg/l		<0.12	<0.12	0.12	0.12	0.12	0		<0.12	<0.12	0.12	0.12	0.12	0
Molybdenum	µg/l	<1.6	2.3	<1.6	1.83	1.6	2.3	0.404	<1.6	<1.6	<1.6	1.6	1.6	1.6	2.72 x10^{-16}
Nickel	µg/l	<1.6	8	<1.6	3.73	1.6	8	3.70	<1.6	5.4	2.6	3.2	1.6	5.4	1.97
Potassium	mg/l	6.6	6.3	5.7	6.2	5.7	6.6	0.46	6.3	6.5	6.3	6.37	6.3	6.5	0.12
Selenium	µg/l	0.8	1.1	0.9	0.93	0.8	1.1	0.15	1	1.3	1.2	1.17	1	1.3	0.15
Sodium	mg/l	41.5	40.3	36.5	39.43	36.5	41.5	2.61	39.9	40.7	39.2	39.93	39.2	40.7	0.75
Strontium	mg/l	0.36	0.35	0.31	0.34	0.31	0.36	0.025	0.34	0.36	0.35	0.35	0.34	0.36	0.0104
Sulphide	mg/l	<0.01	<0.01	<0.01	0.01	0.01	0.01	0	<0.01	<0.02	<0.01	0.013	0.01	0.02	0.0058
Sulphide	µg/l	<10	<10	<10	10	10	10	0	<10	<20	<10	13.33	10	20	5.77
Total CN Low Level	µg/l	1.4	<1	<1	1.13	1	1.4	0.23	1.4	<1	<1	1.13	1	1.4	0.23
Vanadium	µg/l	<1.6	<1.6	<1.6	1.6	1.6	1.6	2.72 x10^{-16}	<1.6	<1.6	<1.6	1.6	1.6	1.6	2.72 x10^{-16}
Zinc	µg/l	<7	<7	<7	7	7	7	0	<7	<7	<7	7	7	7	0

Nutrients detected in the aquarium water and tap water

Component Name	Units	Aquarium							Tap						
		1	2	3	mean	min	max	Std dev	1	2	3	mean	min	max	Std dev
Alkalinity as $CaCO_3$	mg/l	208	205	210	207.7	205	210	2.52	207	204	213	208	204	213	4.58
Alkalinity as HCO_3 Clean Water	mg/l	254	251	257	254	251	257	3	253	249	260	254	249	260	5.57
Ammoniacal Nitrogen	mg/l	<0.02	<0.02	<0.02	0.02	0.02	0.02	0	<0.02	<0.02	<0.02	0.02	0.02	0.02	0
Ammonium as NH_4	mg/l	<0.03	<0.03	<0.03	0.03	0.03	0.03	0	<0.03	<0.03	<0.03	0.03	0.03	0.03	0
BOD (5 Day using ATU)	mg/l	<1.9	<1.9	<1.9	1.9	1.9	1.9	2.72×10^{-16}	<1.9	<1.9	<1.9	1.9	1.9	1.9	2.72×10^{-16}
Carbon Dissolved Organic	mg/l	1.6	1.5	1.5	1.53	1.5	1.6	0.058	1.8	1.6	1.7	1.7	1.6	1.8	0.1
Carbon Total Organic	mg/l	1.5	1.4	1.6	1.5	1.4	1.6	0.1	1.8	1.6	1.7	1.7	1.6	1.8	0.1
Chloride as Cl	mg/l	59.01	58.35	59.23	58.86	58.35	59.23	0.46	58.22	57.63	59.73	58.53	57.63	59.73	1.08
COD	mg/l	<10	11.1	11.3	10.8	10	11.3	0.7	<10	<10	12.3	10.77	10	12.3	1.33
COD Filtered	mg/l	<10	<10	<10	10	10	10	0	<10	<10	13.2	11.066	10	13.2	1.85
Colour	mg/lPt/Co	1.2	1	1.2	1.13	1	1.2	0.12	0.9	<0.8	<0.8	0.83	0.8	0.9	0.059
Conductivity at 20ºC	µS/cm	672	663	661	665.3	661	672	5.86	669	677	675	673.67	669	677	4.16
Hardness Total as Ca	mg/l	111	109	113	111	109	113	2	110	111	112	111	110	112	1
Hardness Total as $CaCO_3$	mg/l	278	274	282	278	274	282	4	274	277	279	276.67	274	279	2.52
Nitrate as N	mg/l	5.1	5.1	5.3	5.17	5.1	5.3	0.12	5.6	6	6.1	5.9	5.6	6.1	0.27
Nitrate as NO3	mg/l	22.8	22.4	23.3	22.83	22.4	23.3	0.45	24.8	26.4	26.9	26.033	24.8	26.9	1.098
Nitrite & Nitrate Calculation	mg/l	0.46	0.45	0.47	0.46	0.45	0.47	0.01	0.49	0.53	0.54	0.52	0.49	0.54	0.027

Nitrite as N	mg/l	<0.001	0.004	<0.001	0.002	0.001	0.004	0.0017	<0.001	<0.001	<0.001	0.001	0.001	0.001	0
Nitrite as NO_2	mg/l	<0.01	0.01	<0.01	0.01	0.01	0.01	0	<0.01	<0.01	<0.01	0.01	0.01	0.01	0
Nitrogen Total Oxidised as N	mg/l	5.1	5.1	5.3	5.167	5.1	5.3	0.12	5.6	6	6.1	5.9	5.6	6.1	0.27
P SOL Reactive	mg/l	0.98	0.95	0.95	0.96	0.95	0.98	0.017	0.98	0.98	0.95	0.97	0.95	0.98	0.017
pH Value	pH_unit	8.3	8.3	8.5	8.37	8.3	8.5	0.12	7.6	7.8	7.5	7.63	7.5	7.8	0.15
Phosphorus Dissolved by ICP	mg/l	0.99	0.99	0.98	0.99	0.98	0.99	0.0079	1.001	1.029	0.99	1.005	0.99	1.029	0.022
Phosphorus Total by ICP	mg/l	1.02	1.03	1.02	1.023	1.02	1.03	0.0058	1.04	1.04	1.02	1.033	1.02	1.04	0.012
Silica Reactive (SiO_2)	mg/l	12.5	12.2	12.9	12.53	12.2	12.9	0.35	12.3	12.7	12.9	12.63	12.3	12.9	0.31
Solids Suspended 105ºC	mg/l	<1.9	<1.9	<1.9	1.9	1.9	1.9	2.72×10^{-16}	<1.9	<1.9	<1.9	1.9	1.9	1.9	2.72×10^{-16}
Sulphate as SO_4	mg/l	61.9	60.9	61.5	61.43	60.9	61.9	0.503	58.9	59.4	57.6	58.63	57.6	59.4	0.93
Total Nitrogen as N	mg/l	6.3	6.8	7.1	6.73	6.3	7.1	0.404	6.8	7.1	7	6.97	6.8	7.1	0.15
Turbidity (FTU)	FTU	0.07	0.07	0.07	0.07	0.07	0.07	0	0.11	0.07	0.11	0.097	0.07	0.11	0.023

Microbiological tests on the aquarium and tap water

Component Name	Units	Aquarium							Tap						
		1	2	3	mean	min	max	Std dev	1	2	3	mean	min	max	Std dev
22ºC PC neat	no/ml	>300	>300	46	215.33	46	300	146.65	1	0	0	0.33	0	1	0.58
37ºC PC neat	no/ml	26	12	0	12.67	0	26	13.01	0	0	0	0	0	0	0
Cl.perf-Veg&Spores pres. neat	no/100 ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coliform neat	MPN/100 ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E.coli neat	MPN/100 ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enteroccoci presumptive neat	no/100 ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0

PC: Plate Count

MPN: Most Probable Number

Radioactivity tests on the aquarium and tap water

Component Name	Units	Aquarium							Tap						
		1	2	3	mean	min	max	Std dev	1	2	3	mean	min	max	Std dev
Alpha Activity - Total	Bq/l	0.04	<0.04	<0.04	0.04	0.04	0.04	0	<0.04	<0.04	<0.04	0.04	0.04	0.04	0
Beta Activity - Total	Bq/l	0.18	0.15	0.17	0.17	0.15	0.18	0.015	0.18	0.18	0.18	0.18	0.18	0.18	3.4×10^{-17}