A comparison of aquatic species responses to anticancer drug exposure

A thesis submitted for the degree of Doctor of Philosophy

by
Graham Harris

Institute for the Environment
Brunel University

September 2015

Table of Contents

Declaration

The work submitted in this thesis was conducted between 2011 and 2015 at Brunel University (Uxbridge, West London, UK). This work was carried out independently by the author, unless otherwise stated in the text.

Abstract

To date, limited research has been conducted on the effects of cytotoxic drugs on aquatic species. Three of these species, a single celled plant, an invertebrate and a fish, were exposed to six cytotoxic drugs, both singly and as simple mixtures. A range of endpoints were assessed, including growth inhibition in algae, immobilisation in daphnia and the impact on organ size and the expression of the DNA damage repair genes RAD51 and p53 in zebrafish. No effects at environmentally relevant concentrations were observed in any of the three species.

Acknowledgements

I would like to express my gratitude to my fellow Ph.D. students, the post-doctoral researchers, and the technical and academic staff of the Institute for the Environment and the College of Biosciences, Brunel University, for the advice and support they gave me during this project. I would also like to thank the members of the European Union funded Pharmas Project, who also provided invaluable advice and support.

Figures

Tables

Abbreviations

5FU	5-fluorouracil
ABL	Abelson murine leukaemia viral oncogene
ATC	Anatomical Therapeutic Chemical
ANOVA	Analysis of variance
BBM	Bold's Basal Medium
BCF	Biological Concentration Factor
BCR	Breakpoint Cluster Region
bcl-abl	Tumour generating protein
CF	Condition Factor
c-KIT	Proto-tumour generating protein
CMF	Cancer regimen containing: cyclophosphamide, Methotrexate, 5-Flourouracil
CML	Chronic Myeloid Leukaemia
CO_2	Carbon Dioxide

cDNA	complementary Deoxyribonucleic Acid
Ct	Threshold Cycle
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
dpf	days post fertilisation
DSB	Double-Strand Break
ECOSAR	Software: Ecological Structure Activity Relationships
EC_x	Effects Concentration
EDTA	Ethylenediaminetetraacetic acid
ef1α	elongation factor 1 α
ELISA	enzyme-linked immunosorbent assay
EPA	Environment Protection Agency
EU	European Union

FA	Fluoroacetic Acid
FEC	Cancer regimen containing: 5-Flourouracil, Epirubicin, cyclophosphamide
FBAL	Fluoro-β-Alanine
FdUMP	Fluorodeoxyuridine Monophosphate
FdUTP	Fluorodeoxyuridine Triphosphate
FP	Cancer regimen containing: 5-fluorouracil, cisplatin
FUTP	Fluorouridine Triphosphate
gDNA	genomic Deoxyribonucleic Acid
GIST	Gastrointestinal stromal tumours
HBSS	Hanks Balanced Salt Solution
Hpf	Hours post fertilisation
HP & UV	Hydrogen Peroxide & Ultra Violet
HPLC-MS/MS	High Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
HR	Homologous Recombination

HU	Cancer regimen containing: hydroxycarbamide, imatinib
IVM	Institute for Environmental Studies – VU University, Amsterdam
LOD	Limit of Detection
LOEC	Lowest Observed Effects Concentration
$LogDow_{7.4}$	pH dependent octanol:water partition coefficient(Log base 10)
LogKow	octanol:water partition coefficient(Log base 10)
Lux	lumen per square metre
MAS	Mammalian metabolic Activation System
MEC	Measured Environmental Concentration
µM	microMolar
mM	milliMolar
MSDS	material safety data sheets
µg/kg	micrograms per kilograms
µg/l	micrograms per litre
µg/ml	micrograms per millilitre

mg/d	milligrams per day
mg/l	milligrams per litre
mg/kg/d	milligrams per kilograms per day
mg/m^2	Milligrams per square meter (body surface area)
$mg/m^2/min$	Milligrams per square meter per minute
µl	microlitre
Ml	millilitre
mRNA	messenger Ribonucleic Acid
MTS	colorimetric assay for assessing cell metabolic activity
N	Number of components in a sample
NER	Nucleotide Excision Repair
ng/l	nanograms per litre
NHEJ	Non-Homologous End Joining
NHS	National Health Service
nm	nanometre

NOEC	No Observed Effects Concentration
NTC	No Template Control
OECD	Organisation for Economic Co-operation and Development
qPCR	reverse transcriptase-quantitative Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PCT	Primary Care Trust
PDGF-R	platelet derived growth-factor receptor
PEC	Predicted Environmental Concentration
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SCE	Sister Chromatid Exchange
SPE	Solid Phase Extraction
SPE-GC/MS	Solid Phase Extraction-Gas Chromatography/Mass Spectrometry
SPE-LC-MS/MS	Solid Phase Extraction-Liquid Chromatography-Mass Spectrometry/Mass Spectrometry

ssDNA	single strand Deoxyribonucleic Acid
STW	Sewage Treatment Works
Synthesis Phase	S Phase
TBE	Tris, Borate, EDTA solution
TEM	Tunnelling Electron Microscope
tRNA	transfer Ribonucleic Acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UV-Vis	Ultraviolet-Visible
WWTP	Waste water Treatment Plant

1 Introduction

1.1 Pharmaceuticals in the environment

1.1.1 Overview

Pharmaceuticals are widely used chemicals, with the most common being consumed in the 100s of tons per year in European countries such as Germany and the United Kingdom (Fent *et al.*, 2006). Consumption in Europe is expected to increase in the future due to factors such as increasing population (in the near term), increasing life span and the demographic shift to a population with a higher average age (Muenz, 2007; Van der Aa *et al.*, 2011). An ageing population is expected to lead to an increase the use of drugs that treat age-related disorders such as cardiovascular disease and arthritis (Van der Aa *et al.*, 2011). The prevalence of many types of cancers is also associated with age (Finkel *et al.*, 2007).

1.1.2 Environmental entry routes

In developed nations, the main entry route of pharmaceuticals to the environment is via sewage systems (Daughton and Ternes, 1999). When patients are administered drugs, a percentage (varying widely between drugs) of the parent drug and/or active metabolites are then passed in faeces and/or urine (Chu and Sartorelli, 2012). In the case of topical treatments, some may be washed off the patient during bathing. Unused or out-of-date drugs may also enter the sewage system if they are disposed of in residential waste water (Ruhoy and Daughton, 2007; Bound and Voulvoulis, 2005). Entry into the sewage system may be diffuse for those drugs taken at home or point for those administered in a hospital. Point sources have the potential to result in higher levels of pharmaceuticals reaching the environment, due to the greater load they place on the sewage treatment works (STW) (Langford and Thomas, 2009). However, in practice hospital waste is diluted by waste water from other sources before it reaches the STW. In addition, there is a growing trend for out-patient and at home administration of cytotoxic drugs (Mahnik *et al.*, 2004). Drug manufacture also emits significant amounts of parent compounds as point sources and does have the potential to overload STWs (Sanchez *et al.*, 2011; Carlsson *et al.*, 2009). In regions with less rigorous regulations there can also be untreated discharge from these facilities directly into the environment (Larsson *et al.*, 2007). In some cases effluent

containing pharmaceuticals can reach ground or surface waters without passing through STWs. During periods of heavy load, for example during heavy rainfall, sewerage systems may release directly to surface waters (Buser *et al.*, 1999) (Figure 1).

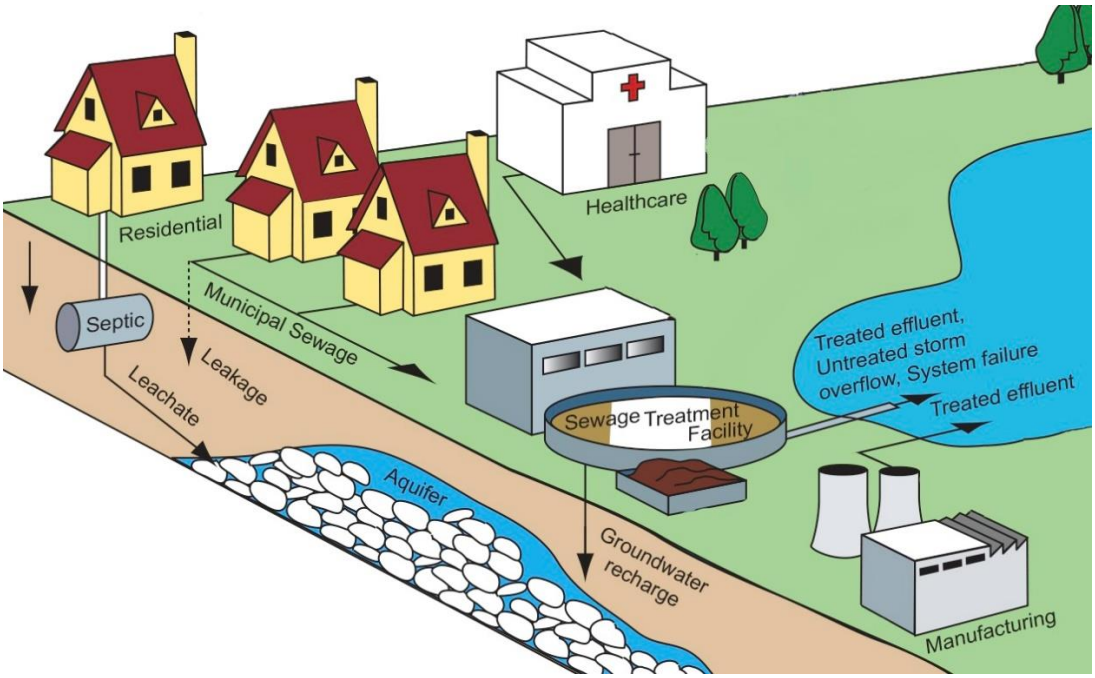


Figure 1: Some of the major sources and entry routes of pharmaceuticals to surface and groundwater (EPA, 2012).

Other routes of entry include:

- Disposal of unused/out-of-date drugs to landfill, with subsequent leaching into groundwater (Metzger, 2004)
- Veterinary administration and subsequent leaching/runoff into ground/surface waters from manure, passed directly by the animal or spread onto land as fertiliser. Discarded veterinary drugs may also enter via wastewater or landfill (Kümmerer, 2010)
- Artificial recharge of aquifers to compensate for unsustainable extraction (Drewes *et al.*, 2003).
- Leakage from sewerage systems and leaching from septic tanks (EPA, 2012).

1.1.3 Environmental fate

The main fates of chemicals entering the STWs and continuing into the aquatic environment are volatilisation, biodegradation and mineralisation, retention in sludge or sediments if lipophilic, or remaining in surface waters if hydrophilic. In the latter case their concentrations are greatly diluted within the surface waters (Pal *et al.*, 2010; Klavarioti *et al.*, 2009). Pharmaceuticals in their parent form tend to be hydrophilic as they are designed to be passed from the body, once their therapeutic effects have occurred (Johnson *et al.*, 2008). Patients excrete varying proportions of drugs both as the parent compound and as one or more metabolites, which may be pharmacologically active. Although a large proportion are removed by STW, As the parent compounds tend to be hydrophilic they are capable of passing through STWs, particularly those that do not employ more advanced treatment methods (Lester *et al.*, 2011; Jelic *et al.*, 2011).

Improvements in analytical chemistry have allowed concentrations of a variety of pharmaceuticals in effluents and surface waters to be detected in the ng/l range, including cytotoxic drugs (Buerge *et al.*, 2006; Kosjek and Heath, 2011). Although present at such low concentrations, effects of certain pharmaceuticals on aquatic organisms have been detected. Synthetic hormones such as ethinyl oestradiol have been shown to have reproductive effects at concentrations below 1 ng/l (Parrott and Blunt, 2005).

1.2 Environmental toxicology of cytotoxic drugs

1.2.1 Overview

Cytotoxic drugs are a class of pharmaceuticals, primarily manufactured for the treatment of cancer. In common with other drugs they are designed to be effectively metabolised by humans (Chabner *et al.*, 2006) and some animals (Cave *et al.*, 2007). They differ from other classes of pharmaceutical in that their mechanisms of actions are intended to be toxic to the patient's cells. There are several classes of cytotoxic drugs, with membership of a particular class based on the mechanism of action of the drug. This Ph.D. project focused on several classes of cytotoxic drugs collectively described as classic cytotoxics. All classic cytotoxics in some way disrupt DNA replication, which prevents cells from progressing

through the cell cycle and producing new cells. This cytotoxicity has a greater effect on cancer cells than normal (non-malignant) cells, as the former replicate at a much greater rate than normal cells. However, the latter are still affected, with clinicians striving to find a dose that kills the cancer whilst keeping side effects at a survivable level (Chabner *et al.*, 2006). As DNA is common to all species (with the exception of some viruses), these drugs are also capable of damaging healthy cells in species other than humans. This is the key reason classic cytotoxics were chosen for assessment in this project.

Cytotoxic drug usage is low compared to some classes of pharmaceuticals, but still significant; it can be in the 1,000s of kilograms per year range in the UK (Figure 2). They were first detected in sewage treatment works (STW) effluent in the United States in the 1970s (Hignite and Azarnoff, 1977), but have since been detected in surface waters worldwide at concentrations in the ng/l range (Lester *et al.*, 2011; Klavarioti *et al.*, 2009; Steger-Hartmann *et al.*, 1997). At the commencement of the project, relatively little research had been conducted into the impact of this class of drug on freshwater aquatic species (Straub, 2010; Zounkova *et al.*, 2010). This class of drugs was, therefore, identified as a potential concern to aquatic organisms, due to the factors outlined above.

1.2.2 Ph.D. objective

This Ph.D. project formed part of one of eight work packages of a European Union (EU) funded project called “Pharmas”, commissioned to evaluate levels of anti-cancer drugs in the environment and the risk they pose to aquatic organisms and human health. These drugs, often termed cytotoxics (i.e. toxic to cells), are widely used in the treatment of cancer and other diseases such as some autoimmune diseases. Whilst likely to be in the environment at very low levels (ng/l), their impacts from chronic exposure to aquatic organisms in surface waters and sensitive human groups (e.g. foetuses and neonates) through drinking water has not been examined to any significant extent.

The objective of the Ph.D. was to determine if diverse aquatic species are adversely affected by the chosen cytotoxic drugs, and if so, at what concentrations? That information could then be used to judge whether or not these drugs pose a threat to aquatic wildlife.

1.2.3 Ph.D. aims

- Assess the effects of cytotoxic drugs on multiple freshwater aquatic species, which are representative of the key trophic levels in the freshwater ecosystem
- Assess the effects of the drugs, both alone and in combination using standardised assays that evaluate endpoints such as growth inhibition, reproduction and morphology
- Assess the effects of the drugs using more sensitive molecular endpoints, such as their ability to induce the expression of RNA coding for DNA repair enzymes.

1.2.4 Hypothesis

The overarching null hypothesis for this project is that:

"The selected cytotoxic pharmaceuticals, at the tested concentrations, will not cause significant effects in the aquatic species tested".

1.3 Cytotoxic drug selection methodology

The target drugs were chosen based on the amount of the drug purchased in the United Kingdom in kilograms per year, their membership of different classes of anti-cancer drug, and the likelihood of them being taken up by aquatic organisms, due to their chemical characteristics.

1.3.1 United Kingdom usage data

There is no single complete source of UK prescription data. Data for drugs that were prescribed in the community (i.e. by National Health Service (NHS) General Practitioners) are freely available on the web from the NHS websites for the four UK countries. However, the majority of antineoplastics (another term for anticancer drugs) are prescribed in hospitals. These data are not currently collected by the NHS, although there are projects in progress to make this information available on a country by country basis (personal

communications, NHS data managers). Instead, a private company, IMS Health, collects purchasing data from each UK NHS hospital. As these data are for drugs purchased by NHS Primary Care Trusts (PCT), rather than drugs prescribed, this is not an exact representation of usage. In addition, there are differences in how PCTs record and store data, leading to potential errors when collating data (Richards, 2009). IMS Health attempts to validate these data with individual PCTs, by sending them the collated figures before publication (personal communication, Peter Stevens, IMS Health). However, a recent study produced for the UK government into international variations in drug usage noted that there were still discrepancies between usage reported by NHS PCTs and sales figures provided by pharmaceutical manufacturers (Richards, 2010). This was confirmed in personal communication with PCT personnel responsible for reporting drug usage (personal communications, David Barber, Lancashire Teaching Hospitals). Finally, these data do not include drugs prescribed by private doctors and hospitals. However, it does include drugs prescribed to private patients in NHS facilities (Richards, 2009).

The data purchased from IMS Health were collated and, along with the data in the proceeding sections, the drugs in Figure 2 were selected based on them being among the most purchased cytotoxic drugs during the five years prior to the data purchase date.

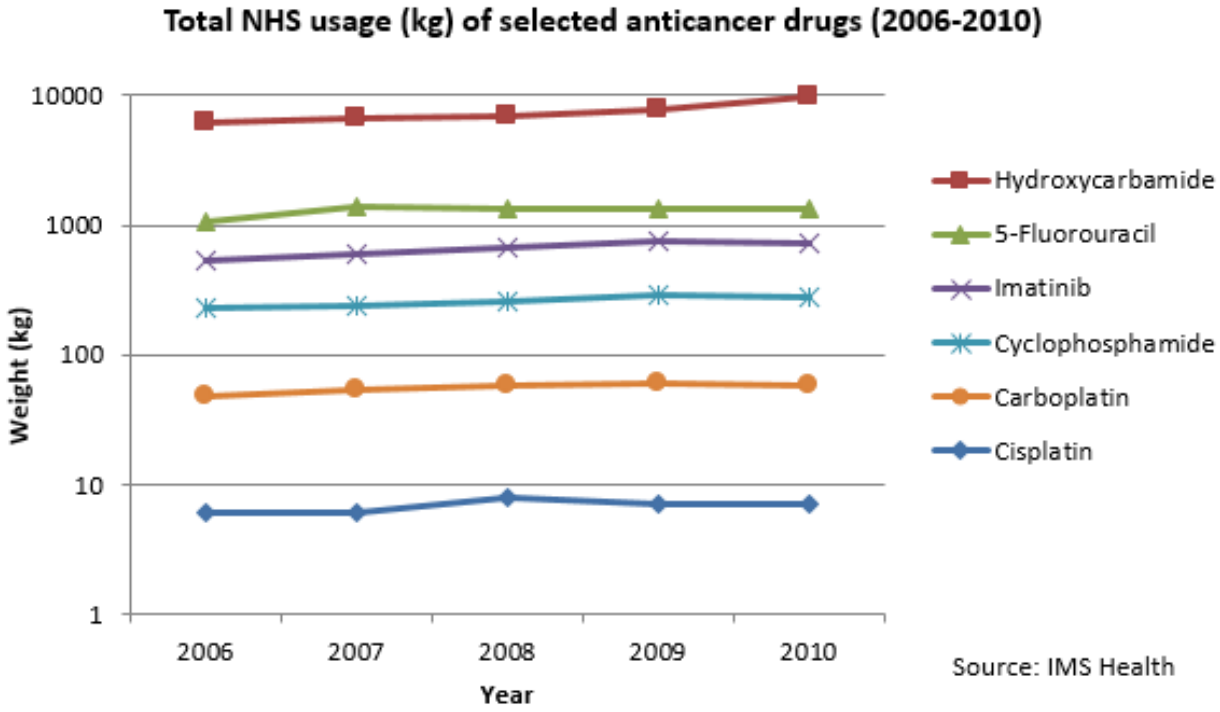


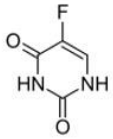
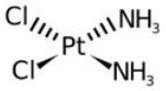
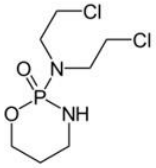
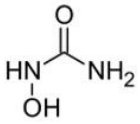
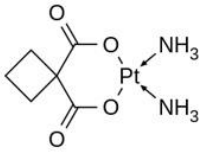
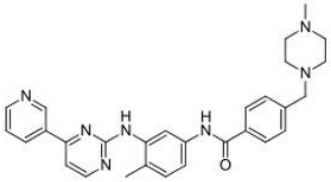
Figure 2: Annual usage by the National Health Service (NHS) in the UK of the cytotoxic drugs chosen for testing. This usage includes NHS community and hospital prescriptions and also private patients treated in NHS facilities, but not private patients treated in private facilities.

1.3.2 Classes of anticancer drug

Cytotoxic drugs are separated into different classes, according to their mechanisms of action. All of the drugs selected for this project, with one exception, can be described as 'classic' cytotoxics as they, in some way, damage DNA. This can either be directly, as in the case of cyclophosphamide, or via the disruption of DNA synthesis, as is one of the mechanisms of action of 5-fluorouracil. The exception is imatinib, which treats cancer by inhibiting proteins that have been shown to be instrumental in oncogenesis; the process by which normal cells are transformed into cancer cells. This drug was included as some research had been done on its effects on algae growth by another Ph.D. student at Brunel, Tara Thrupp. Her algae results have been included in this thesis, and additional experiments were conducted to test it as one component in a mixture of cytotoxic drugs against algae growth and both singly and as a component in a mixture against daphnia inhibition.

According to the World Health Organisation's Anatomical Therapeutic Chemical (ATC) classification system, 5-fluorouracil is a pyrimidine analogue, cyclophosphamide a nitrogen mustard analogue, carboplatin and cisplatin are platinum compounds and imatinib a protein kinase inhibitor. Hydroxycarbamide is in the "Other antineoplastic agents" class. The chemical structures and their general mechanisms of action are shown in Table 1.

Table 1: The cytotoxic drugs selected for assessment in this project, and the classes of drug they belong to according to the ATC classification system.

5-Fluorouracil Antimetabolite		Cisplatin Platinum compound	
Cyclophosphamide Alkylating agent		Hydroxycarbamide Antimetabolite	
Carboplatin Platinum compound		Imatinib Protein kinase inhibitor	

More information on the specific mechanisms of action, doses and side effects of these drugs can be found in section 1.4

1.3.3 Chemical characteristics relevant to the aquatic environment

Each drug has specific chemical characteristics that dictate its fate within the aquatic environment, and these influence their ability to be taken up by aquatic organisms. This information was used by this Ph.D. and the Pharmas project, to select suitable drugs for assessment (Table 2). The information was collected by several members of Pharmas and the selection of drugs made with the support of the Pharmas environmental chemistry experts. The drugs characteristics and their influence on drug selection are defined as follows:

- $LogK_{ow}$: This value defines how lipophilic or hydrophilic a chemical will be. Chemicals with smaller values tend towards hydrophilic, larger values towards lipophilic. Drugs with high $LogK_{ow}$ values were one of the criteria for selection, as these are more likely to bioaccumulate .

- LogDow at pH 7.4: This value defines the same criteria as $LogK_{ow}$ but takes into account the pH, in this case being calculated at a pH 7.4, a value representative of freshwater. This value was required for the blood plasma model calculations in chapter 4.
- BCF: Biological Concentration Factor, defines the extent to which a chemical could build up with an organism, with higher values signifying an increased tendency to concentrate.
- Solubility @ 25 °C and pH 7.4: The ability for the chemical to go into and stay in solution in water.

Table 2: The chemical characteristics of the selected cytotoxic drugs, which influence their environmental fate and their uptake by aquatic organisms. a Pharmas project, personal communication, b calculated using Marvin chemistry calculator www.chemaxon.com, c (Booker et al., 2014), d (Vyas et al., 2014)

Drug	$LogK_{ow}$	LogD at pH 7.4	BCF	Solubility @ 25 °C (mg/l)
5-fluorouracil	-0.69_a	-1.02_b	3_c	$1{,}000_c$
cyclophosphamide	0.63_a	0.1_b	3_c	$4{,}000_c$
cisplatin	-2.19_a	0.04_b	-	$2{,}500_c$
carboplatin	1.06_a	0.01_c	-	$11{,}700_d$
hydroxycarbamide	-1.27_a	-	3_c	$8{,}000_d$
imatinib	-	0.19_c	3_c	$6{,}500_c$

The temperature used for the solubility values is relevant to the laboratory conditions under which the experiments in this project were run. However, it is high compared to many European freshwater ecosystems. Solubility increases with temperature, so this value may be lower in UK surface waters. However, all drugs were highly soluble, going into solution at grams per litre concentrations.

1.4 Mechanisms of Action of the selected cytotoxic drugs.

Each of the target pharmaceuticals has one or more methods of disrupting cancer cells. As their aim is to destroy cancer cells this involves either direct or indirect disruption of DNA replication and/or repair, in order to prevent cell proliferation and to promote their death. As all cells contain DNA, an ideal antineoplastic would be designed to target only those cells displaying neoplasia. In reality, antineoplastic drug design is a compromise, with the parent drug and its metabolites having to meet certain criteria in order for it to be effectively taken up by the patient and transported to the target cells. To achieve this, they are often administered in an inactive form (known as a prodrug), so that they are transported to the sites of the cancer tumours before activation via metabolism into one or more active metabolites. There may also be one or more inactive metabolites formed (either from the parent drug or its active metabolites) that, although not capable of disrupting cancer cells, can cause adverse side effects.

The mechanism of action of many anticancer drugs occurs during specific phases of the cell cycle, and these are therefore known as 'cell cycle-specific drugs' (Figure 3). Other drugs can eliminate cancer cells at any stage of the cell cycle and so are known as cell cycle non-specific drugs (Chu and Sartorelli, 2012).

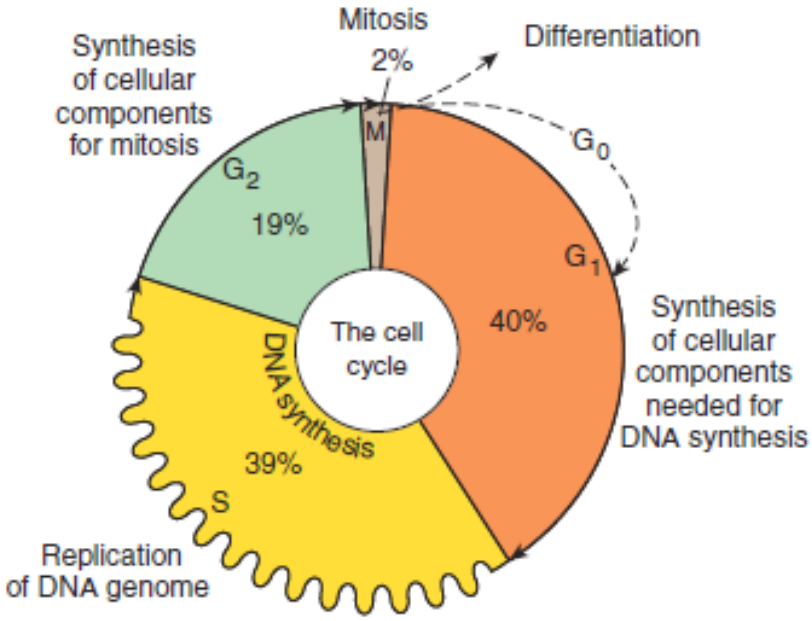


Figure 3: The cell cycle phases that all cells—normal and cancerous—must pass through before and during cell division. The percentages given represent the approximate percentage of time spent in each phase by a typical cell, although the duration of G1 is highly variable. (Chu and Sartorelli, 2012)

Although researchers constantly strive for targeted therapies, cytotoxic drugs have several side effects, ranging from relatively mild (e.g. vomiting) to the severe (e.g. neurotoxicity). This necessitates a "patient-dependent dosage", where the side effects are used as indicators of the specific dose a patient can tolerate. For example, when administering 5-fluorouracil, inflammation of the mouth and diarrhoea are considered reliable warning signs that a sufficient dose has been administered. In addition, intrinsic or acquired resistance to single treatments require the use of combinations of antineoplastics in order to be most effective (Chabner *et al.*, 2006).

The following sections describe the specific mechanisms of action for each of the selected drugs.

1.4.1 5-fluorouracil

A study in the 1950s observed that uracil was utilised in the liver of rats dosed with a carcinogen, whereas no such usage was observed in control animals (Rutman *et al.*, 1954). This suggested that neoplastic cells were incorporating this nucleobase and as such made this compound a potential target for cancer treatments. This led to the development of 5-fluorouracil, which is particularly successful (in combination with other antineoplastics) in the treatment of colorectal and breast cancers. It is administered parenterally, either as an intravenous bolus or infusion (Schilsky, 1998), as oral uptake is highly variable and incomplete (Chabner *et al.*, 2006). As explained in section 1.4, dosage is patient-dependant, but an initial bolus dose may be 500-600 mg/m^2 (the surface area of the patient) (Schilsky, 1998) followed by constant infusions around 15 mg/kg/d for 5 days (Chu and Sartorelli, 2012). It is also administered topically for the treatment of basal cell skin cancers, with a tube of treatment containing 5% 5-fluorouracil (Chu and Sartorelli, 2012; Chabner *et al.*, 2006).

5-fluorouracil is classed as an antimetabolite, belonging to the sub group of pyrimidine antagonists (Chu and Sartorelli, 2012). It is a prodrug, being activated (mainly in the liver) to three main active metabolites that target neoplastic cells via the inhibition of thymidylate synthase and intercalation into RNA and DNA (Figure 4). Inhibition of thymidylate synthase by the metabolite fluorodeoxyuridine monophosphate (FdUMP) depletes the supply of thymine, uracil's DNA nucleobase equivalent. Although the specific

mechanisms are unclear (Longley *et al.*, 2003), this leads to severe disruption of DNA synthesis and repair, resulting in double strand breaks (DSB) and cell death (Yoshioka *et al.*, 1987). 5-FU also causes strand breaks and cell death (called "thymineless death" (Yoshioka *et al.*, 1987)) through the misincorporation of its metabolite fluorodeoxyuridine triphosphate (FdUTP) into DNA in the place of thymine (Longley *et al.*, 2003). As thymine production is blocked, the cell DNA repair machinery is unable to replace FdUTP (Chabner *et al.*, 2006). Finally, the metabolite fluorouridine triphosphate (FUTP) has an additional cytotoxic effect by substituting for uracil in RNA, disrupting RNA processing and mRNA translation (Chu and Sartorelli, 2012). 5-fluorouracil's mechanism of action is cell cycle specific, exerting its cytotoxicity during the S phase when the cells attempt to replicate, but the process is hindered by the effects of the active metabolites (Chu and Sartorelli, 2012).

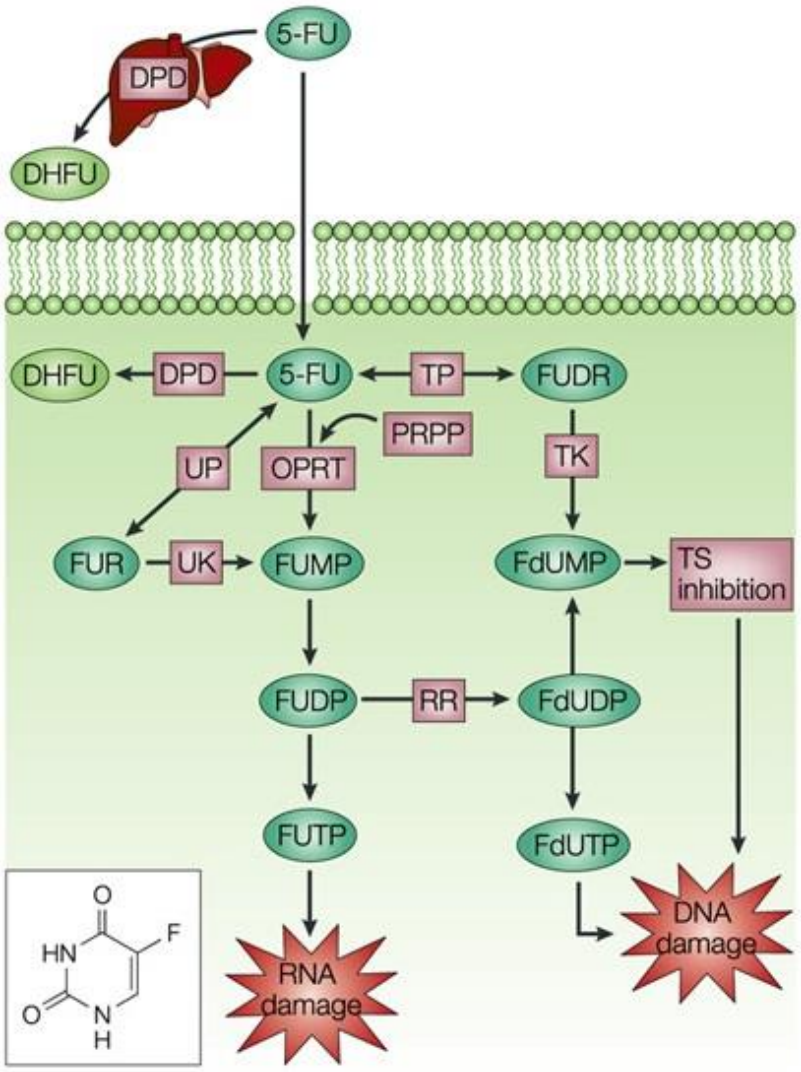


Figure 4: Metabolic pathways for the production of the active metabolites of the mechanisms of action for 5-fluorouracil. 5-fluorouracil is converted to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP, respectively. An alternative activation pathway involves the thymidine phosphorylase catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells. Up to 80% of administered 5-FU is broken down by DPD in the liver. (Longley et al., 2003)

The severe toxic side effects of 5-FU are myleosuppression (bone marrow suppression), cardio and neurotoxicity (Chabner *et al.*, 2006). Research in canine's points to an active metabolite of 5-FU, fluoro-β-alanine (FBAL) and its metabolite fluoroacetic acid (FA) as causative factors in neurotoxicity (Yamashita *et al.*, 2004) and a contributing factor in

cardiotoxicity (Muneoka *et al.*, 2005). Less than 10% of the parent compound is excreted; mainly via the renal pathway (Chabner *et al.*, 2006).

1.4.2 Cyclophosphamide

Developed in the 1950s as a modification of nitrogen mustard, with the aim of improved targeting of cancer cells, cyclophosphamide is still widely used to treat a range of cancers. It is particularly successful in combination with other antineoplastics in the treatment of Lymphomas, due to the sensitivity of lymphocytes to this drug. It is also used to treat breast, ovarian, and bone and soft tissue sarcomas (Emadi *et al.*, 2009). It can be administered orally or intravenously with doses varying widely depending on patient, cancer type and treatment regime. An average dose when used as a single agent would be 3.5–5 mg kg^{-1} d^{-1} taken orally for 10 days and 1 g/m^2 (representing the surface area of the patient) administered intravenously as a single dose (Chu and Sartorelli, 2012). When taken in combination with other drugs, higher doses of 500 mg/m^2 can be administered intravenously every two to four weeks (Chabner *et al.*, 2006).

Cyclophosphamide is a pro-drug that is mainly metabolised in the liver by several cytochrome P450 enzymes (Zhang *et al.*, 2006), a group central to the metabolism of the majority of pharmaceuticals (Guengerich, 2008), with intermediate active metabolites 4-hydroxycyclophosphamide and its tautomer aldophosphamide carried in the circulation to tumour cells, where the latter is spontaneously cleave to phosphoramide mustard (Chabner *et al.*, 2006). Under physiological conditions the nitrogen atom in phosphoramide mustard displaces one of its chloride atoms, forming a highly reactive aziridinium (ethyleneimonium) cation. This cation undergoes a nucleophilic alkylation with the nitrogen-7 (N^7) atom of DNA purine bases, most usually guanine. This frees the nitrogen atom in the phosphoramide mustard to displace the other chloride atom, repeating the above process and resulting in a DNA adduct, a cross-link between purine bases, either in the same strand or both strands of DNA (Emadi *et al.*, 2009) (Figure 5). At near neutral pH, carbonium ions are formed instead of aziridinium, which result in cross-linking in the same manner (Zhang *et al.*, 2006).

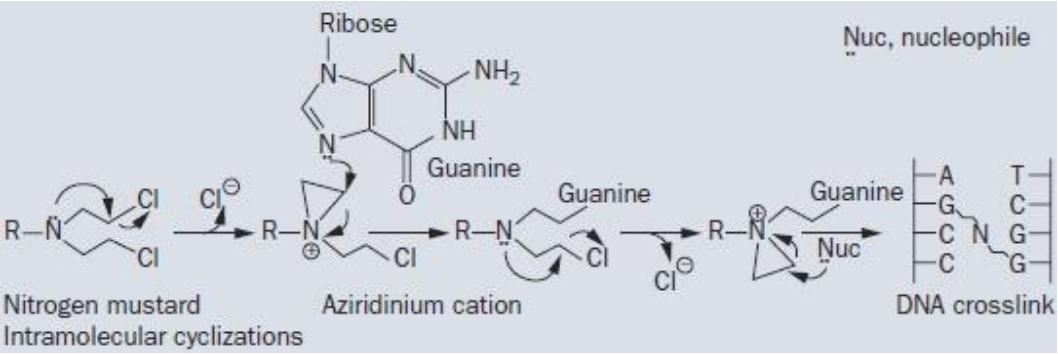


Figure 5: cyclophosphamide metabolism pathway, see text above for explanation of the pathway (Emadi et al., 2009).

As with 5-fluorouracil, the specific mechanisms that lead to the destruction of tumour cells are not fully understood. It is thought that cross-linking prevents DNA replication and causes double strand breaks, that can then lead to apoptosis (programmed cell death) (Chu and Sartorelli, 2012; Schwartz and Waxman, 2001).

Side effects of cyclophosphamide include nausea and vomiting in the short term with the potential to cause myleosuppression (Chu and Sartorelli, 2012), sterility, teratogenic effects and leukaemia (Chabner *et al.*, 2006). Acrolein is an active metabolite, which itself is cytotoxic, having the ability to induce oncosis (accidental cell death) by interrupting the osmotic machinery of the cell, causing it to swell. It is also thought to cause haemorrhagic cystitis (Chabner *et al.*, 2006) and may trigger urinary track cancer due to its urotoxicity (Le Guenno *et al.*, 2011). Excretion of the parent compound is 10-20% in urine and 4% in bile (Zhang *et al.*, 2006), although it is claimed that recovery of the parent compound is minimal after intravenous administration (Chabner *et al.*, 2006).

1.4.3 Cisplatin & carboplatin

Research in the mid-1960s, unrelated to cancer therapies, observed that platinum complexes inhibited cell division in *Escherichia coli* (Rosenberg *et al.*, 1965 in Kelland, 2007). This led to the development of the first of the platinum analogs, cisplatin (Harrap, 1985 in Kelland, 2007). Although effective (especially against testicular cancer), cisplatin can cause severe side effects including nephrotoxicity, gastrointestinal toxicity and ototoxicity (Von Hoff *et al.*, 1979 in Knox *et al.*, 1986). To reduce these, carboplatin was developed in the 1980s which, while less effective at tumour suppression in some cancers (Hartmann and Lipp, 2003), exhibited fewer and less severe side effects, namely

myleosuppression and greatly reduced nephrotoxicity and gastrointestinal toxicity (Chu and Sartorelli, 2012). In addition, carboplatin is significantly less carcinogenic and mutagenic than cisplatin (Sanderson *et al.*, 1996).

Cisplatin and carboplatin employ the same mechanism of action (Knox *et al.*, 1986), similar to an alkylating agent such as cyclophosphamide (Chu and Sartorelli, 2012). However, the bonds that they form, whilst covalent, do not alkylate DNA (Chabner *et al.*, 2006). Carboplatin is administered intravenously (Macmillan Cancer Support, 2010) and enters cells passively by diffusion and actively via copper transporters (Kruh, 2003). Before carboplatin can bind to DNA it must first lose its bidentate cyclobutanedicarboxylate group through two hydrolysis reactions (Chabner *et al.*, 2006), becoming a positively charged molecule (Liu *et al.*, 2005). It is possible for carboplatin (and cisplatin) to form a monoadduct with a guanine after the first hydrolysation, but this is an infrequent occurrence (Fichtinger-Schepman *et al.*, 1985). The major adducts are intrastrand cross links, mostly between neighbouring guanines but also between a neighbouring guanine and an adenine[1]. There is evidence that the latter is the major cause of cytotoxicity, although why was not stated in the literature reviewed (Eastman and Barry, 1987; Fichtinger-Schepman *et al.*, 1995). However, unlike cyclophosphamide, intrastrand links with non-adjacent guanine/adenine and interstrand links appear to be minor occurrences (Fichtinger-Schepman *et al.*, 1985). Cisplatin and carboplatin are cell cycle nonspecific drugs, whose mechanisms of action are active throughout the cell cycle.

Nausea and vomiting are usual with clinically significant side effects reported as myleosuppression, neurotoxicity (manifesting as peripheral sensory) and nephrotoxicity (Chabner *et al.*, 2006). However, other sources state the latter two as absent or greatly reduced when compared to cisplatin (Knox *et al.*, 1986; Liu *et al.*, 2005). Being relatively un-reactive compared to cisplatin, a significant proportion of the parent compound of

[1] The following naming conventions can be applied to intrastrand cross links. Cross links between adjacent cytosines are known as 1,2-intrastrand adducts; between non-adjacent, 1,3-intrastrand adducts. To describe specific links, the convention for adjacent links is, for example, 1,2-d(GpG) for two guanines and 1,2-d(ApG) for an adjacent guanine and adenine. For non-adjacent links 1,3-d(GpXpG) (Jiang *et al.*, 2004).

carboplatin is excreted in the urine, ~75% carboplatin compared to 3% cisplatin (Chabner *et al.*, 2006).

1.4.4 Hydroxycarbamide

After experiments on mice identified hydroxycarbamide (also known as hydroxyurea) as a potent treatment for leukaemia, it was first used as an anti-cancer drug in the 1960s (Fishbein and Carbone, 1963). It is mainly used to treat chronic myeloid leukaemia (CML), although it has also been used against melanoma and other solid tumours (Belt *et al.*, 1980). It has been superseded in efficacy by imatinib, but is still used for its cost-effectiveness (Reardon *et al.*, 2012). It is also used for the treatment of a range of other ailments, including sickle cell anaemia (Platt, 2008), which accounts for some of the amount purchased shown in Figure 2. When used as an anticancer treatment it can be administered orally or intravenously, with maximal tolerated dose reported as 800mg/m^2 every 4 hours over a 72-hour period via the oral route and 3.0 mg/m^2/min over the same time period via intravenous infusion (Belt *et al.*, 1980).

Hydroxycarbamide exerts its mechanism of action by inhibiting DNA synthesis, and thereby preventing cells replicating. This inhibition is achieved by inhibiting the enzyme ribonucleotide reductase, which produces the deoxyribonucleotides (dNTP) that are needed to synthesise DNA (Elledge *et al.*, 1992). Hydroxycarbamide has a hydroxamic acid at its core, which binds to heavy metals (Figure 6). As ribonucleotide reductase contains iron, hydroxycarbamide can bind to it, thus applying its inhibiting effect (Platt, 2008). Without a steady supply of dNTP, synthesis of DNA during the replication (S phase) of the cell cycle is disrupted, causing highly cytotoxic double strand breaks (Koç *et al.*, 2004).

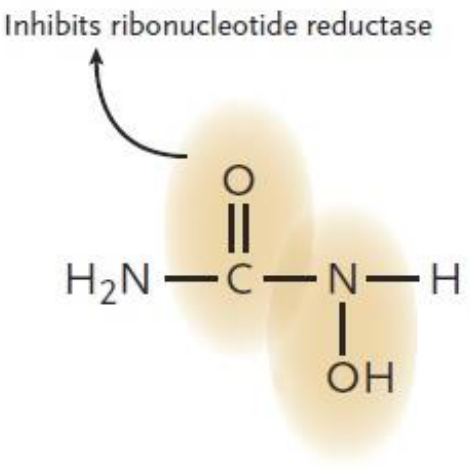


Figure 6: The Hydroxamic acid at the core of hydroxycarbamide, which binds to the iron in the enzyme ribonucleotide reductase (Platt, 2008)

Dose-limiting side effects of hydroxycarbamide include myleosuppression, granulocytopenia and gastrointestinal toxicity (Belt *et al.*, 1980). Excretion of parent compound is urinary and varies widely from approximately fifteen to sixty percent (Belt *et al.*, 1980) hydroxycarbamide is a cell cycle specific drug, with its mechanism of action being felt during the S phase of the cell cycle (Figure 3).

1.4.5 Imatinib

One of the aims of the project was to assess classis cytotoxics; those that exert their mechanism of action by in some manner damaging DNA in tumour cells. Imatinib differs from the other drugs assessed in this project in that, instead of damaging DNA, it inhibits the proliferation of tumour cells. It achieves this by blocking the activity of three tyrosine kinases. Due to this mechanism of action, imatinib is known as a cytostatic, rather than cytotoxic drug. The proteins it inhibits are the tumour generating protein (oncoprotein) *bcl-abl*, platelet derived growth-factor receptor (PDGF-R) and the proto-oncoprotein c-KIT. (Gambacorti-Passerini *et al.*, 2003). Developed in the early 1990s, it is primarily used to treat certain forms of leukaemia, such as chronic myeloid leukaemia (CML), and gastrointestinal cancers, replacing hydroxycarbamide due to its increased efficiency.

The *bcl-abl* oncoprotein is formed by the translocation of the ABL gene to another chromosome, where it fuses with the BCR gene. This oncoprotein has been shown to be the cause of almost all cases of CML and is implicated in several other leukaemias, to a lesser extent. It transforms bone marrow stem cells into tumour cells by deregulating

tyrosine kinase. This protein normally activates stem cells in a regulated manner. Deregulation by *bcl-abl* results in a rapid proliferation of cells, one of the hallmarks of cancer. The ability to inhibit *bcl-abl* makes imatinib a highly specific treatment for these types of cancer. Its ability to block the proto-oncoprotein c-KIT also makes it an effective treatment for a specific gastrointestinal cancer, GIST (Gambacorti-Passerini *et al.*, 2003). Its third target, PDGF-R, has been implicated in the causation of brain tumours (glioblastoma), a rare skin cancer (dermatofibrosarcoma protuberans) and soft tissue tumours (fibromatosis). Imatinib has also been used again prostate cancer, in combination with another anti-cancer drug, docetaxel (Homsi and Daud, 2007).

Imatinib is administered orally, in doses of 400 to 600 mg per day. Due to the specificity of its mechanism of action, it has relatively few side effects. Those documented include nausea, vomiting, muscle pain (myalgia), fluid retention (oedema) and diarrhoea (Homsi and Daud, 2007). As its mechanism of action does not involve DNA damage, or interruption of DNA synthesis, its status in relation to the cell cycle is not relevant.

1.5 The selected cytotoxic drugs in combination - Drug regimens

Cancer treatment has often been shown to be more effective if two or more drugs are used in combination. These are described as drug regimens, and a selection that contain some of the drugs tested in this project, along with the cancers they are used to treat, are contained in Table 3.

Table 3: Anticancer drug regimens that contain some of the cytotoxic drugs selected for assessment in this project. The target cancers represent those sited in the literature, rather than a complete list of cancers the regimen is used to treat.

Regimen	Components	Target cancers	Source
CMF	cyclophosphamide Methotrexate 5-Flourouracil	Breast cancer	(Di Lauro *et al.*, 2015)
FEC	5-Flourouracil Epirubicin cyclophosphamide	Breast cancer	(Pietri *et al.*, 2015)
HU	hydroxycarbamide imatinib	Meningioma (Brain and nervous system)	(Reardon *et al.*, 2012)
FP	5-fluorouracil cisplatin	Nasopharyngeal carcinoma (head and neck cancer)	(Zhou *et al.*, 2009)

These regimens use these drugs in the tens to hundreds of mg range, and can be administered in combination or serially via oral, intravenous or via injection routes. Whilst the mixtures experiments conducted in this project did not consider these or any other drug regimens when designing the mixtures, this information is included as evidence that these drugs have been shown to act in combination, in a therapeutic context.

1.6 Target Cytotoxics in the environment

Information on measured levels of some of the cytotoxic drugs chosen for assessment in this project was found in the literature. These concentrations were measured in hospital effluents, STWs and surface waters in several European countries. Concentrations ranged from µg/l in hospital effluents to ng/l in surface waters. These results have been summarised in Table 4. It should be noted that, where total platinum was measured, it could be possible that other sources of platinum were contributing to the MEC.

Table 4: Measured concentrations of cytotoxic drugs (using a variety of analytical methods) in European hospital effluents, STWs and surface waters. <x.xx indicates the Limit of Detection (LOD) for the test protocol.

Author	Drugs, methodology, compartment	MEC (µg/l)
Martín *et al.*, 2011	• 5-fluorouracil • SPE – capillary electrophoresis • hospital oncology ward effluent (Switzerland)	2 – 12.2
Mahnik *et al.*, 2004	• 5-fluorouracil • SPE – capillary electrophoresis • hospital oncology ward effluent (Switzerland)	20-122
Kovalova *et al.*, 2009	• 5-fluorouracil • HPLC-MS/MS • hospital effluent (Switzerland)	0.027
Lin *et al.*, 2006	• cyclophosphamide • SPE-LC-MS/MS • influent & effluent WWTP (Switzerland)	0.002 – 0.011
Mark-Kappeler *et al.*, 2011	• cyclophosphamide • SPE-GC/MS • surface waters (Romania)	<0.030 – 0.0648 (±8.0)
Steger-Hartmann *et al.*, 1997	• cyclophosphamide • SPE-GC/MS • WWTP influent & effluent (Germany)	0.02 – 4.5
Buerge *et al.*, 2006	• cyclophosphamide • SPE-LC-MS/MS • Surface waters (Switzerland)	0.00005 – 0.00017
Zaharie, 2006	• cyclophosphamide • SPE-GC/MS • Surface Waters (Romania)	0.0461 (±7.2) – 0.0648 (±8.0)
Vyas *et al.*, 2014	• All platinum sources	0.03 – 100

	• Filtered and digested in aqua regia ICP-MS • Hospital effluent (United Kingdom)	
Kümmerer and Helmers, 1997	• cisplatin & carboplatin • HP & UV decomposition-adsorptive voltammetry • Hospital effluent (Germany)	0.021 – 0.045 & 0.096 – 0.165
Kümmerer *et al.*, 1999	• All platinum sources • HP & UV decomposition-adsorptive voltammetry • Hospital effluent (European hospitals)	0.010 – 3.58

MECs, whilst useful, only provide a snapshot of the drug concentration at the time the measurement was taken. This may not be representative of an average concentration, which would require multiple measurements to be taken over a greater temporal and spatial range, in order for an accurate average concentration to be calculated. A complementary technique for assessing environmental concentrations is to predict their presence using modelling. It should be noted that, due to the complexity of the environmental interactions a chemical undergoes, assumptions have to be made in the design of the model and some abstraction has to be accepted. However, this method can provide a more representative environmental concentration. These types of measurement are known as predicted environmental concentrations (PEC), and ones found in the literature for the selected cytotoxic drugs are contained in Table 5.

Table 5: Predicted Environmental Concentrations (PEC) of the chosen cytotoxic drugs collected from the literature. If a named model was used, this is stated in the table. Booker et al. (2014) did not name the model, so the equation is shown instead, containing the following abbreviations Cons = per capita consumption, Exc = percentage parent compound excreted, Biodeg = percentage drug intact after STW treatment, HDil = STW dilution factor 200 litre/capita/day

Author	Drugs, model, compartment	PEC (ng/l)
Straub, 2010	• 5-fluorouracil • Local PEC EMEA Ph II, refined • Surface waters	2.65
Besse *et al.*, 2012	• 5-fluorouracil • European Medicine Agency (adapted, refined) • Surface waters	7.91
Booker *et al.*, 2014	• 5-fluorouracil • $PEC = \frac{Cons \times Exc \times Biodeg}{HDil \times 10}$ • Effluent & Surface waters (N.W. England)	8.9 & 0.9
Franquet-Griell *et al.*, 2015	• 5-fluorouracil • European Medicine Agency (adapted, refined) • Surface waters (N.E. Spain)	0.01
Besse *et al.*, 2012	• cyclophosphamide • European Medicine Agency (adapted, refined) • Surface waters	>1.74 (>LOD)
Booker *et al.*, 2014	• cyclophosphamide • $PEC = \frac{Cons \times Exc \times Biodeg}{HDil \times 10}$ • Effluent & Surface waters (N.W. England)	40.9 & 4.1
Franquet-Griell *et al.*, 2015	• cyclophosphamide • European Medicine Agency (adapted, refined) • Surface waters (N.E. Spain)	0.11

Besse *et al.*, 2012	• cisplatin* & carboplatin† • European Medicine Agency (adapted, conservative*, refined†) • Surface waters	0.52 & 1.91
Booker *et al.*, 2014	• carboplatin • $\mathrm{PEC} = \frac{Cons \times Exc \times Biodeg}{HDil \times 10}$ • Effluent & Surface waters (N.W. England)	2.2 & 0.2
Franquet-Griell *et al.*, 2015	• carboplatin • European Medicine Agency (adapted, refined) • Surface waters (N.E. Spain)	0.01
Besse *et al.*, 2012	• hydroxycarbamide • European Medicine Agency (adapted, refined) • Surface waters	78.07
Booker *et al.*, 2014	• hydroxycarbamide • $\mathrm{PEC} = \frac{Cons \times Exc \times Biodeg}{HDil \times 10}$ • Effluent & Surface waters (N.W. England)	4.7 & 0.5
Franquet-Griell *et al.*, 2015	• hydroxycarbamide • European Medicine Agency (adapted, refined) • Surface waters (N.E. Spain)	32.1
Besse *et al.*, 2012	• imatinib • European Medicine Agency (adapted, refined) • Surface waters	4.99
Booker *et al.*, 2014	• imatinib • $\mathrm{PEC} = \frac{Cons \times Exc \times Biodeg}{HDil \times 10}$ • Effluent & Surface waters (N.W. England)	4.6 & 0.5

Franquet-Griell *et al.*, 2015	• imatinib • European Medicine Agency (adapted, refined) • Surface waters (N.E. Spain)	2.34

In summary, the current literature suggests that the cytotoxic drugs chosen for study in this project can be present in hospital effluents in the low to medium µg/l range and in wastewater effluents and rivers in the low to sub ng/l range. Nothing is known about the rate of loss of these drugs by processes such as photolysis and biodegradation in rivers. However, due to the constant input into rivers in wastewater effluents, these drugs can be considered persistent in the environment; although their levels will fluctuate, based on factors such as time of day and weather conditions. Aquatic species in surface waters in Europe will therefore be exposed more or less continuously to these drugs, albeit at very low concentrations.

1.7 An overview of the selected test species

One of the aims of this project was to test multiple species, which represented key tropic levels in freshwater ecosystems. Additional criteria were defined, including one that required the species to have been approved as part of standardised environmental toxicology tests, such as those published by the Organisation for Economic Co-operation and Development. The other criterion was that the species should ideally have all, or at least a majority, of its genome mapped. This was to allow the identification of DNA repair enzymes, using the annotated genes from other species.

Based on these criteria, the following species (Figure 7) were selected:

- Raphidocelis subcapitata: A single celled green alga, a plant that represents the Primary Producer trophic level
- Daphnia magna: A herbivorous crustacean, an invertebrate representing the Primary Consumer trophic level

- Danio rerio: An omnivorous fish, commonly known as the zebrafish, a vertebrate representing the Secondary Consumer trophic level.

Figure 7: Test species selected for this Project. Left: the plant R. subcapitata, Middle: the invertebrate crustacean D. magna, Right: the vertebrate fish D. rerio

These species are described in more detail in the relevant chapters of this thesis.

1.8 Anticancer ecotoxicology research in the literature at the commencement of this project

Freshwater aquatic organisms have the potential to be significantly impacted by exposure to cytotoxic drugs as they spend all or some of their lifecycle in surface waters that, in catchments with urban populations, receive a relatively consistent input. At the beginning of the project, a search of the literature identified research that had already been conducted on some of the selected cytotoxic drugs, using the chosen test species (Table 6). However, none of the literature found reported significant effects occurring at, or near, the concentrations detected or predicted in surface waters (Tables 4 and 5).

Table 6: A selection of literature that examined the impact on aquatic organisms of exposure to a range of cytotoxic drugs and concentrations. > indicates values for which no significant impact was observed at the highest level tested. LOEC = Lowest Observed Environmental Concentration

Author	Drugs, species, tests	LOEC (mg/l)
(Zounková *et al.*, 2007)	cyclophosphamide, 5-Fluorouracil, cisplatin Algae OECD 201 (growth inhibition), Daphnia OECD 202 (immobilisation)	Algae: 500, 0.01, 1 Daphnia: >1000, 10, 0.5
(Grung *et al.*, 2008)	cyclophosphamide Algae OECD 201 (growth inhibition), Daphnia OECD 211 (reproduction)	Algae: >100 Daphnia: 100
(Straub, 2010)	5-Fluorouracil Daphnia OECD 211 (reproduction) zebrafish FETAX (embryo exposure)	Daphnia: 10 zebrafish: >32

This information, along with research conducted on these species that was published during the project, and research found in the literature that was conducted on other species, is discussed in more detail in the relevant chapters of this thesis. At the time this project was initiated, the small amount of relevant literature available suggested 5-fluorouracil to be considerably more potent than the other drugs that had been tested, and that cyclophosphamide was extremely weakly active. All reported effect concentrations were well above reported environmental concentrations. This is in contrast with some other human pharmaceuticals, which cause effects on aquatic organisms at concentrations near to, or even at, environmental levels; the contraceptive steroid ethinyl oestradiol being a good example (Parrott and Blunt, 2005).

1.9 Ph.D. thesis overview

The research conducted in this project was broken into the following work packages, represented by the following chapters in this thesis:

- Chapter 2: Effects of the selected cytotoxic drugs on the growth of the green algae *R. subcapitata*, exposed singly and as simple mixtures.
- Chapter 3: Effects of the selected cytotoxic drugs on the mobility of the crustacean *D. magna*, exposed singly and as simple mixtures.
- Chapter 4: Effects of three of the selected cytotoxic drugs (5-fluorouracil, cyclophosphamide and cisplatin) on the morphology of the fish *D. rerio*, exposed singly and as simple mixtures.
- Chapter 5: Effects of three of the selected cytotoxic drugs (5-fluorouracil, cyclophosphamide and cisplatin) on the levels of mRNA expression of certain mRNAs that code for selected proteins of the fish *D. rerio*, exposed singly and as simple mixtures.
- Chapter 6: A comparison of the results from the different species and general conclusions.

2 Impact of cytotoxic drugs on algal growth

2.1 Introduction

Commonly known as a green alga, *Raphidocelis subcapitata* (formerly named as *Selenastrum capricornutum* and then *Pseudokirchneriella subcapitata*) is a photosynthetic, unicellular eukaryotic organism, belonging to the phylum Chlorophyta, in the Viridiplantae Kingdom. It is a free floating freshwater species and has a broad geographical range, having been identified in Europe (John *et al.*, 2011), North America (Fawley *et al.*, 2006) and South America (Menezes, 2010). A cell is crescent shaped and is approximately 2 µm wide and 15 µm long (Figure 8). Each cell is capable of producing two, four or eight daughter cells, roughly every 18 hours under ideal conditions.

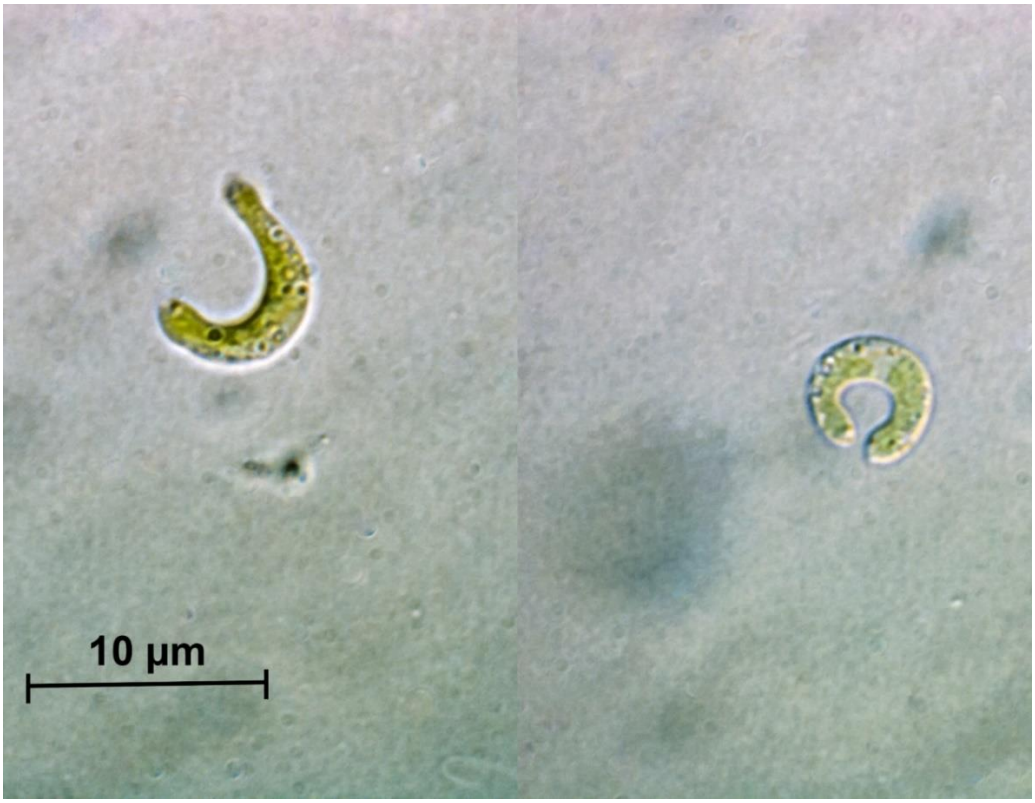


Figure 8: Examples of Raphidocelis subcapitata taken under a light microscope at x1000 magnification. Internal organelles are visible, particularly in the right hand cell. The light green organelles are likely to be chloroplasts, the organelle responsible for photosynthesis, and the green hue due to the photosynthetic pigment chlorophyll. The scale is approximate and for illustrative purposes only.

As primary producers, algae form part of the base of the aquatic food web. Any external factor that perturbs this group is likely to have knock on effects at higher trophic levels.

Therefore, it is important that primary producers are considered when investigating the effects of anticancer drugs. The Organisation for Economic Co-operation and Development (OECD) guidelines for testing chemicals has approved a selection of primary producers, including *R. subcapitata*, as suitable for this purpose. The guidelines also stipulate how these tests should be conducted (outlined in section 2.2).

The short life cycle of ~18 hours means that approximately four generations of *R. subcapitata* are exposed during the 72-hour exposure period that the OECD guidelines propose. This allows the test to be classed as chronic, something that becomes increasingly difficult and more expensive to achieve at higher trophic levels. Chronic testing better assesses the effects experienced in the environment, where exposure to anticancer drugs is generally of this nature.

2.1.1 Aims

- Calculate effects concentrations for 10% and 50% algae growth inhibition (EC_{10} & EC_{50}), when the algae are exposed to individual cytotoxic drugs, which are listed in Table 7.
- Expose algae to the two simple mixtures defined in section 2.2.2 and compare results with each other and with the results of the single chemical exposures.

2.1.2 Hypothesis

"The selected cytotoxic pharmaceuticals, at the tested concentrations, will inhibit alga growth, with the effects being additive".

2.2 Methods & materials

OECD 201 "Freshwater Alga and Cyanobacteria, Growth Inhibition Test" (OECD, 2011) defines the testing procedure for determining the effects of a substance on the growth of a selection of approved single cell algae and bacteria species. Batches of a selected organism are exposed to a concentration range of the target substance for a period of 72 hours. At the end of this period the numbers of algae cells present are compared to those in an appropriate control, and a percentage growth inhibition calculated. From this Effects

Concentration (EC_X) values can be determined, where $_X$ is the percentage growth inhibited. As direct assessment of the number of organisms is difficult, proxy measurements using parameters such as fluorescence or optical density are used.

Organisations that regularly employ this procedure culture batches of test organisms in dedicated facilities, configured to the required temperature and light regimes. As these facilities were not available at Brunel University, AlgalToxkit F™ kits were purchased from MicroBioTests Inc., Belgium. The kit contained the test organism and the OECD defined medium for their culture (made up in deionised water). The test was run in a standard incubator and used optical density as a proxy for organism number. The kit includes 25 ml 10 cm long path spectrophotometer cells to allow optical density to be measured with a spectrophotometer. This kit has been independently tested by the AstraZeneca aquatic ecotoxicoloy laboratory at Brixham, United Kingdom and the results were found to be comparable to self-cultured organisms (Daniel *et al.*, 2004).

2.2.1 Exposures to single cytotoxic drugs

The tests were run following the supplied protocol, with the modifications detailed below. The hydroxycarbamide and imatinib tests were conducted by Miss Tara Thrupp, another Ph.D. student in the Institute for the Environment. All other tests were conducted by the author. The algae medium was supplied in four vials, containing the required minerals for optimal algal growth. These were added to 1 litre of deionised water, pH balanced to 8.1 (± 0.2) and aerated with a filtered aquarium pump for 15 minutes prior to use. The algae species *R. subcapitata* are supplied immobilised in a matrix formed into beads, which allows storage for up to three months. Using the supplied solution, the algae are removed from the matrix and re-suspended in algae medium. A starting algae cell density of 1 million cells per ml is required. The initial aliquot of algae is placed in a 25ml 10 cm long path cell and the optical density measured using a Jenway 6300 spectrophotometer (Jenway Ltd, England). This value is converted into cells per ml using the supplied graph (specific to each batch of algae) and the aliquot diluted to the required cell density.

The standard kit allows for a control and five concentrations to be tested, with three replicates of 25 ml each per treatment for statistical robustness. The desired concentration ranges were calculated with a ten-fold increase between either 0.001 and 100 or 0.01 and

1000 mg/l in five steps for range finder tests. For definitive tests the fold change and range were defined based on the toxicity of the chemical. The pharmaceuticals and their concentrations tested are listed in Table 7.

Table 7: Pharmaceutical information and definitive exposure concentration ranges (mg/l) for the algae single chemical exposures.

Drug	CAS number	Supplier	Supplier drug name	Definitive range (fold change)
5-fluorouracil	51-21-8	Sigma-Aldrich	5-fluorouracil	0.01 - 1000 (10)
cyclophosphamide	6055-19-2	Sigma-Aldrich	cyclophosphamide monohydrate	100, 400 - 3200 (2)
cisplatin	15663-27-1	Sigma-Aldrich	cis-Diammineplatinum(II) dichloride	1 – 16 (2)
hydroxycarbamide	127-07-1	Sigma-Aldrich	hydroxyurea	0.01 – 100 (10)
imatinib	220127-57-1	Cayman Chemical	imatinib (mesylate)	0.01 – 100 (10)
carboplatin	41575-94-4	Sigma-Aldrich	carboplatin	0.01 – 100 (10)

Instead of following the dilution series method detailed in the protocol, a concentrated stock solution was made up in algae medium and diluted to the desired to a total volume of 100 ml per concentration in the series, with a further 100 ml aliquot of algae medium only for the control. 1ml of algae was then added to the control and each of the five chemical concentrations. 25 ml aliquots were placed in long path cells and the cells arranged in a random order in a holding tray. The holding tray is positioned in an incubator next to a sideways light source of 10,000 lux at the side of the tray of long cell paths at 23° C (± 2° C) for 72 hours, with optical density readings taken every 24 hours. The tests were repeated twice for each drug, apart from hydroxycarbamide, which was not repeated.

The optical density readings were entered into excel spreadsheets provided with the kit. These converted optical density into cell number and calculated a percentage growth

inhibition per concentration. These data were then used to calculate the $EC_{10, 20}$ and $_{50}$ values in weight per volume (mg/l), as required by the OECD guidelines.

2.2.2 Exposure to mixtures of cytotoxic drugs

The protocol defined in 2.2.1 was followed but further adapted to allow assessment of mixtures of the drugs. As only partial concentration-response curves were available for most of the drugs, assessment was limited to a simple mixtures experimental design. Two mixtures of five drugs were created. The simple mixture contained each drug at a ⅕ of their respective EC_{50} values and the 'something from nothing' mixture at ⅕ of ½ of their LOEC values (½LOEC) (data in Table 8). The former allowed comparison against a known toxicity value for each chemical. As there were not NOEC values for every chemical, and the resolution of the individual dose response curves were relatively course, LOEC divided by 2 was used to produce a 'something from nothing' mixture (Silva *et al.*, 2002). The hypothesis behind this design is that, although below the concentration at which single chemicals will illicit a response in a target organism, as a mixture they will.

Table 8: Pharmaceutical concentrations (µg/l) used to make the mixtures for the algae mixtures experiments.

Drug	EC_{50}	⅕ EC_{50}	LOEC	⅕ ½LOEC
5-fluorouracil	435	88	1	0.20
imatinib	6 430	1 286	50	10
cisplatin	7 400	1 480	1 000	200
hydroxycarbamide	97 200	19 440	5 000	1 000
cyclophosphamide	3 179 000	635 800	50 000	10 000

Concentrated dosing stocks were made up for each chemical and diluted to a concentration equal to the relevant EC_{50}/½LOEC values. Aliquots were taken from each of these and diluted further to one fifth of the relevant values. These were produced to allow comparison between the mixtures effects and those of the single chemical at their constituent concentrations. Equal amounts of the remaining EC_{50}/½LOEC stocks were then combined to make the mixtures. Algae were added at an initial density of 1 million cells per

ml, and the solutions transferred to long path cells. Exposure conditions and data collection methods were the same as those for the single chemical exposure tests in section 2.2.1.

The optical density readings were entered into excel spreadsheets provided with the kit. These converted optical density into cell number, which allowed the calculation of a percentage growth inhibition for the exposed concentration of the drug. However, it was not possible to calculate the EC_x values in weight per volume, as a range of concentration values were required. Instead, growth inhibition as a percentage compared to the controls, caused by the concentration of the single drug/mixture, was reported.

2.3 Results

2.3.1 Exposures to single cytotoxic drugs

Growth inhibition was observed for all drugs apart from carboplatin, for which no growth inhibition was observed at the highest concentration tested, which was 100 mg/l. Algae exposed to cyclophosphamide were the least inhibited, with an EC_{50} value of 2.18×10^6 µg/l. 5-fluorouracil elicited the greatest amount of growth inhibition, with the lowest EC_{50} value (435 µg/l), and it produced a response over the widest concentration range. All other drugs produced a steep concentration-response curve (Figure 9). It should be noted that the hydroxycarbamide exposure was only run once. In addition, although the cisplatin exposure was repeated, there was a large amount of variation, particularly in the highest concentration.

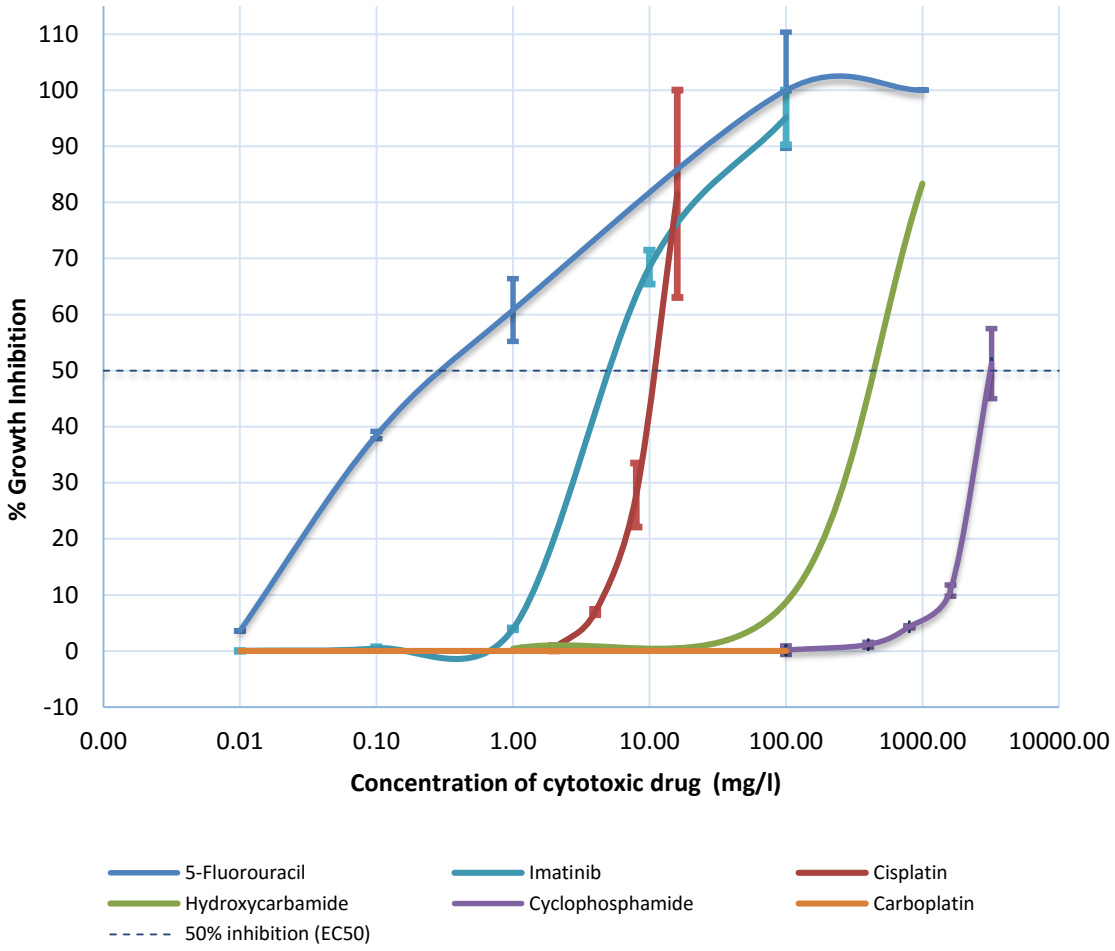


Figure 9: Concentration-response curves for the single chemical algae growth inhibition tests. The error bars are standard deviation around the mean.

Table 9: $EC_{10, 20}$ and $_{50}$ values obtained from the single chemical algae growth inhibition tests in µg/l , ordered by EC_{50} toxicity, highest to lowest. a Straub (2010), b Martín et al. (2011), c Mahnik et al. (2004), d Besse et al. (2012) * Conservative PEC as refined PEC not available, e Rowney et. al. (2009) † Combined PEC with 4 other cytotoxic drugs, f Kümmerer & Helmers (1997), g Vyas et al. (2014), h Buerge et al. (2006), i Mark-Kappeler et al. (2011) j Booker et al. (2014)

				MEC range	
Drug	EC_{10}	EC_{50}	PEC	Hospital effluent (untreated)	Surface Waters
5-fluorouracil	10.00	435.00	0.00265a	2.0b – 122c	-
imatinib	1 430.00	6 430.00	0.00499d*	-	-
cisplatin	5 885.00	10 920.00	0.00697 – 0.01072†e	0.021f – 100g	-
hydroxycarbamide	93 550.00	97 200.00	0.078d	-	-
cyclophosphamide	1 520 000.00	3 179 055.00	0.0041 – 0.040j	0.0045f	0.00005h – 0.0648i
carboplatin	>100 000.00	>100 000.00	0.002 – 0.022j	0.096f – 0.165f	-

None of the drugs tested elicited a response at predicted environmental concentrations or at those measured in surface waters. The EC_{10} for 5-fluorouracil (10 µg/l) was the only value that fell within those measured in untreated hospital effluent, which would be expected to contain much higher concentrations of cytotoxic drugs than in surface waters.

2.3.2 Exposure to mixtures of cytotoxic drugs

All the single chemicals at one fifth of their respective EC_{50} values inhibited growth, apart from cisplatin. The algae exposed to 5-fluorouracil and hydroxyurea experienced the greatest amount of growth inhibition, both having 26.36% less cells at the end of the exposure period, when compared to the controls. Cyclophosphamide had the lowest measured response with 1.46% less cells. The sum of the growth inhibition for the single chemicals was 68.71%, around one and half times greater than the 44.78% inhibition observed in the algae exposed to the mixture. Growth inhibition was only detected in algae

exposed to 5-fluorouracil, cyclophosphamide and hydroxyurea at one fifth of their half LOEC, all at less than 1%. The sum of the single chemical inhibitions was 2.09%, nearly double that of the mixture at 1.07% (Figure 10).

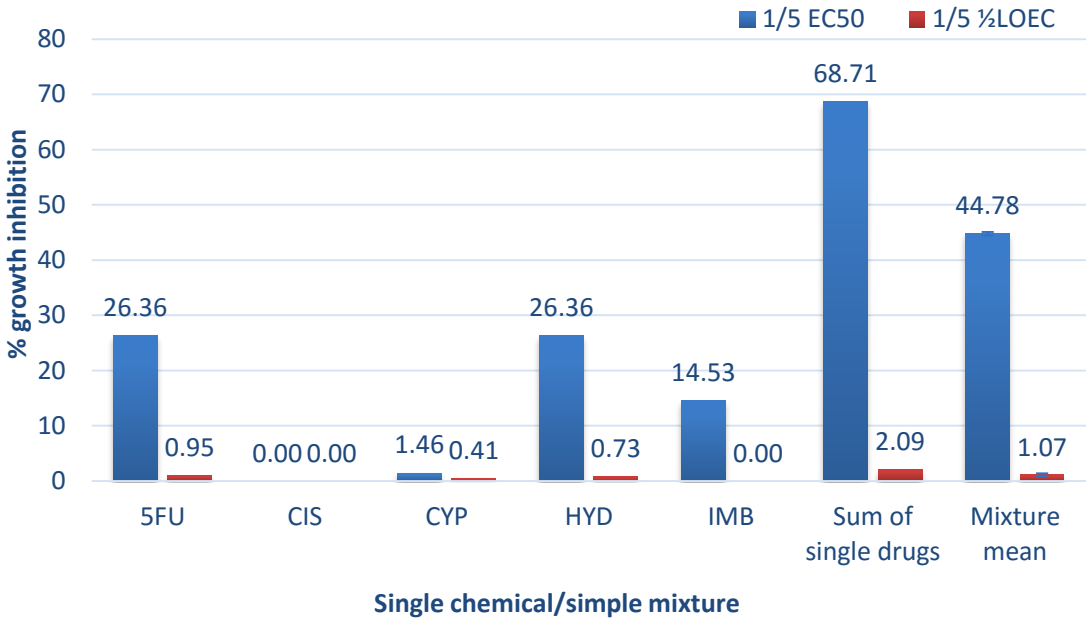


Figure 10: Percentage growth inhibition (defined as the number of algae cells in exposed cf. controls) for algae exposed to single chemicals at 1/5 their EC50 and ½LOEC, and also mixtures of these chemicals at these concentrations. The percentage inhibition for each of the single chemicals has been summed (Total (single chemicals)), to provide a comparison to the mixtures growth inhibition. The mixtures exposures had two replicates each, with a standard error of 0.33 and 0.29.

2.4 Discussion

2.4.1 Single drug exposures

All drugs inhibited alga growth at concentrations orders of magnitude higher than concentrations measured and predicted in surface waters. However, the results for hydroxycarbamide and cisplatin should be treated with caution. The former drug was only tested once, although it was run in triplicate in that experiment. In addition, these were only range finder results run with a concentration series of 0.01-100 mg/l, with a tenfold change between concentrations. Only the top two concentrations elicited growth inhibition of approximately 9% and 83%, respectively. It would be good practice to repeat these exposures at least twice more with a concentration series that covered the entire growth

inhibition curve. Cisplatin was repeated twice, each time, in triplicate but there were relatively large variations in the results between the two tests. Another repeat of this exposure may help to assess the variation between them, although the elapsed time period would mean that it would not be possible to do this from the same batch of cisplatin or algae. This risks introducing further variation in the results, due to chemical and/or biological variability between batches of algae.

5-fluorouracil inhibited algae growth by 10% at a concentration of 10 µg/l, which is a concentration within the range measured in untreated hospital effluents (Table 9). However, these values do not represent a continuous presence of these drugs at these concentrations, but rather a snap shot of the input at the time the measurements were taken. Concentrations will vary due to factors such as time of day and what day of the week it is. This is mainly influenced by the treatment regime. Oral and bolus administration are likely to occur during working hours on a week day, with initial excretion occurring some hours later. Continuous intravenous infusion may occur for a period of one or more hours up to several days, resulting in continued excretion during and after administration. In addition, chemotherapy often employs a cocktail of drugs, with different drugs administered at different time points during treatment (Vyas *et al.*, 2014). Elimination of the parent compound and metabolites from the patient varies greatly between the drugs, with complete elimination of cisplatin taking several weeks (Monjanel-Mouterde *et al.*, 2003). If a drug is administered to day or out-patients, then the majority of excretion (or removal by washing in the case of topically applied 5-fluorouracil) is likely to occur outside the hospital, once the patient has left (Kümmerer, 2001). In developed nations, hospital effluent would either be treated on site or at the local sewage treatment works. In either case the concentrations measured in the hospital effluent will not persist, either being reduced by treatment or diluted during transport to the treatment works, where it is further reduced by treatment. The concentration-response of 5-fluorouracil was unusual, in that it spanned four orders of magnitude, rather than the two to three orders of magnitude observed for the other drugs. This relatively shallow curve indicates that toxicity increases at a slower rate with increasing concentration of 5-fluorouracil, when compared to the other drugs. However, why this should be the case is not known.

The design of the experiment could have been improved with the addition of a positive control. This would have been a substance that caused DNA damage, in the same manner as the anticancer drugs tested. The concentration-response curve for the substance would be well documented, so that an observer could be confident that *x* DNA damage would occur if the algae were exposed at *y* concentration. If used as a positive control in this experiment, this damage would need to be translated into growth inhibition. It would then have provided an indication that the alga had been exposed correctly, if the expected amount of growth inhibition was observed in the algae exposed to the positive control. Unfortunately, it was not possible to identify a chemical that could reliably reproduce growth inhibition in a concentration dependent manner.

One other potential issue was identified in the literature. This concerned the fate of effluent from drug manufacturing in some low and middle-income countries. Several studies have detected concentrations of pharmaceuticals in manufacturing effluents in China and India in the tens of milligrams per litre range (Larsson *et al.*, 2007; Li *et al.*, 2008; Fick *et al.*, 2009). Li *et al.* (2008) went on to measure Oxytetracycline (an antibiotic) at concentrations in the hundreds of micrograms per litre range, 20 km downstream from the wastewater treatment plant (WWTP) discharge site. Fick *et al.* (2009) detected antibiotics in the milligram per litre range in two lakes near a major production area for pharmaceuticals. These lakes were not part of the catchment in which the WWTP responsible for treating the manufacturing waste discharged. It was believed that illegal dumping of manufacturing waste was the source of this pollution. Furthermore, the same study also detected antibiotics in at concentrations > 1 µg/l in several village wells.

No evidence was found in the literature that cytotoxic drugs were detected in manufacturing effluent. Larsson et al. (2007) did analyse samples for cyclophosphamide, but it was not detected above the LOD of 0.1 µg/l. However, with increasing amounts of drug manufacturing moving to these countries (Larsson and Fick, 2009; Pruden *et al.*, 2013) and with the majority of the target drugs of this study available generically (all but imatinib according to www.drugs.com/availability), it is possible that they could be produced in these countries. Unlike therapeutic sources, the drugs have not passed through patients, where some retention/degradation would occur. Instead, a greater percentage of the parent compounds at much higher concentrations is received by the WWTP, which acts as

a point source (Larsson *et al.*, 2007). It is also foreseeable that illegal dumping of waste containing cytotoxic drugs could occur, in which case there is little opportunity for degradation before entering surface waters. These factors could result in the presence of cytotoxic drugs in surface waters at concentrations that could inhibit algal growth. Without knowing production volumes for these cytotoxics it is not possible to say which of the tested drugs would pose a risk in these circumstances. If one was to compare the ECx values with the measured concentrations in the previous paragraph, then 5-fluorouracil, and potentially cisplatin and imatinib in cases of illegal dumping, would be capable of inhibiting algal growth.

However, the two scenarios described above only represent a small percentage of the environment. In the majority of locations where these drugs are likely to be present, for example in UK rivers downstream from sewage treatment works. In these situations they will be at much lower concentrations, in the nanograms per litre range.

2.4.2 Results from the literature

Several studies on the effects of some of these drugs on *R. subcapitata* or similar species were found in the literature. The findings of these studies are summarised in Table 10 on the next page, and compared with the results generated in this project.

Table 10: Comparison of EC_{10} and EC_{50} values from the literature with those calculated in this study, for R. subcapitata and other species of freshwater green algae.

			Literature (µg/l)		Thesis (µg/l)	
Drug	Species	Author	EC_{10}	EC_{50}	EC_{10}	EC_{50}
5-fluorouracil	*D. subspicatus*	Cleuvers 2001 in (Straub, 2010)	11 000	21 000	10	435
	R. subcapitata	(Zounková *et al.*, 2007)	-	110		
	D. subspicatus	(Zounkova *et al.*, 2010)	-	48 000		
	R. subcapitata	(Brezovšek *et al.*, 2014)	20	130		
imatinib	*R. subcapitata*	(Brezovšek *et al.*, 2014)	790	2 290	1 430	6 430
cisplatin	*R. subcapitata*	(Zounková *et al.*, 2007)	-	2 300	5 885	10 920
	R. subcapitata	(Brezovšek *et al.*, 2014)	610	1 520		
hydroxycarbamide	No results found in the literature					
cyclophosphamide	*R. subcapitata*	(Sanderson *et al.*, 2003)	-	(ECOSAR) 11 000	1 520 000	3 179 055
	R. subcapitata	(Zounková *et al.*, 2007)	-	930 000		
	R. subcapitata	(Grung *et al.*, 2008)	-	>100 000		
	R. subcapitata	(Mater *et al.*, 2014)	-	>10		
carboplatin	No results found in the literature					

2.4.2.1 5-fluorouracil

The literature results found for 5-fluorouracil were highly variable, with EC_{50} values ranging from 110 µg/l to 48 000 µg/l. The former result from Zounková *et al.* (2007), was four times lower than the EC_{50} calculated in this study. The same species of algae were used and the experimental design was based on the EN ISO 8692:1989 protocol, which is analogous to OECD 201. However, it had been altered to use 96 well microplates for the exposure. The

well volume of the plates used is not stated but typically they are around 350 µl, hence were much smaller volumes than those of the 25 ml long path cells employed by the AlgalToxkit F kits. It may be that the difference in replicate volumes impacted on EC_{50} values, perhaps by reducing the transfer of CO_2 from the atmosphere due to the much smaller surface area. This could retard algae growth rates, leading to the calculation of a lower EC_{50} value. However, Daniel (2004) tested the microplate exposure method against the AlgalToxkit F kits, using industrial effluents from a range of sources. The study concluded that the AlgalToxkit results correlated well (using the Pearson correlation coefficient on rank analysis, $p < 0.01$) with the microplate method. A comparison of the EC_{50} values for the respective methods showed differences ranging from a couple of decimal points to nearly 18 times. Daniel (2004) also noted that the microplate method had been approved by the UK Environment Agency and was widely used for effluent assessment.

Millington *et al.* (1988) found growth medium composition affected the toxicity of chemicals. It had been noted in a previous study (Adams and Dobbs, 1984) that aminotriazole (a herbicide) was less toxic to *R. subcapitata* when the concentrations of nitrogen and phosphorous in the medium were reduced. This was contrary to the author's expectations that nutrient stress would increase toxicity. In the Millington *et al.* (1988) study, three medium recipes (OECD, EPA & Bold's Basal Medium (BBM)) were tested with three species of green algae, including *R. subcapitata*, against a four different chemicals, widely used in agricultural and industrial processes.

It would appear that the growth rate-limiting threshold concentrations of the basic nutrients is somewhere between the OECD and EPA recipes (Figure 11, top). Millington *et al.* (1988) found that medium composition did impact toxicity (Figure 11, bottom). However, the effect was not consistent or predictable, varying in different directions between the chemicals. Neither did water solubility of the test chemicals appear to be a factor. Although there is the potential for interactions between the toxicants and the chemical components in the medium, this could not be assessed in the results of this study. Millington *et al.* (1988) also found that growth rates in the different mediums varied between species (Figure 11, top) and also in the presence of the different chemicals (data not shown).

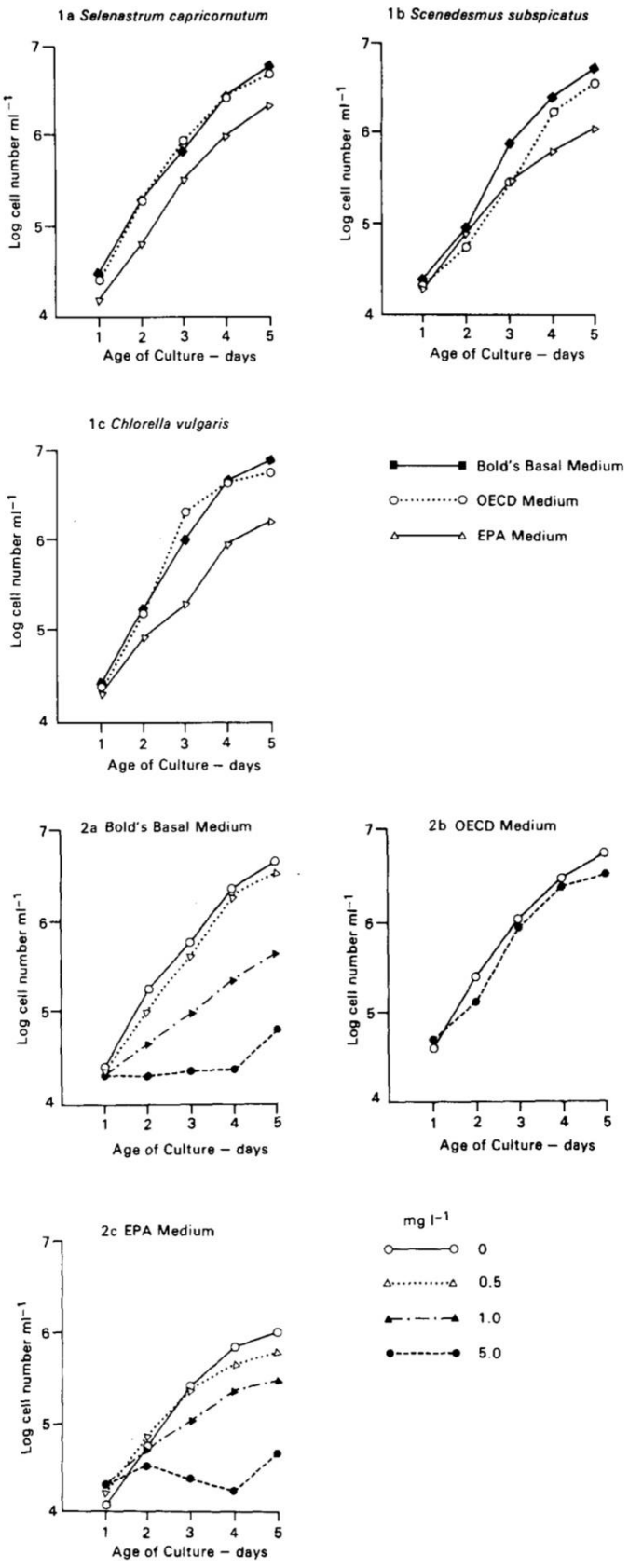


Figure 11: Top set of three graphs, growth rates of three species of freshwater green algae in three different growth mediums. Bottom set of three graphs, growth rate of R. subcapitata in three different growth mediums containing a concentration range of Triphenyl Phosphate. Only concentrations that cause a change in growth that differs significantly from the control are shown (Millington et al., 1988).

Zounková *et al.* (2007) used 50% medium, mixing Zehnder Z-Medium, BBM and distilled water in the ratio 1:1:2. The AlgalToxkit F medium contains the components at the concentrations defined in the OECD 201 protocol. In addition, stock solutions for this study were made up in algae medium, whereas Zounková *et al.* (2007) used buffered saline solution. It is not known how the Zounková *et al.* (2007) recipe varies from the OECD recipe used in this study, as the greater concentration of basic nutrients in BBM has been altered by the Zehnder medium and diluted by the distilled water. However, whether it is the availability of nutrients, interactions with the toxicants and nutrient components or a combination of the two, it cannot be stated that the use of different growth mediums is an issue. It is also not known whether the use of buffered saline for stock solutions would affect toxicity. Nevertheless, these factors could have contributed to the difference in EC_{50} values, although the direction of change exerted is not known.

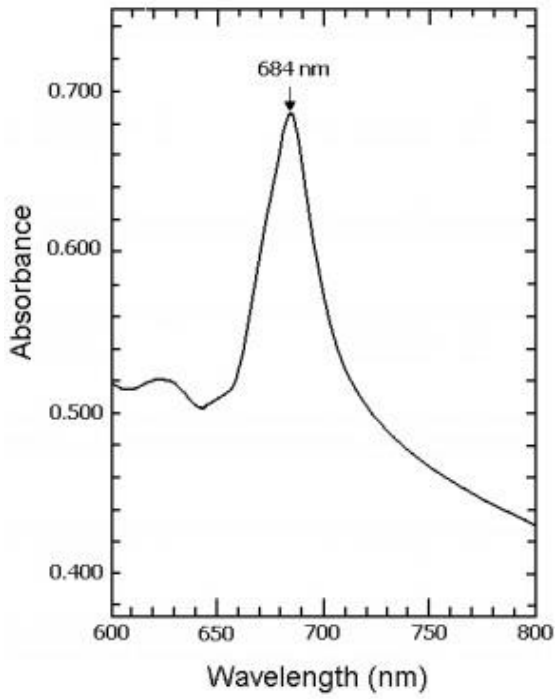


Figure 12: Part of the total absorption spectra of light for R. subcapitata, with a peak in absorption at 684 nm (Rodrigues et al., 2011)

The same method for calculating algal growth was used, extrapolating cell number by measuring light absorbance at a wavelength that is strongly absorbed by chlorophyll. In this study absorbance was measured at 670 nm, whereas Zounková *et al.* (2007) used 680 nm. A peak in light absorption [2] has been calculated for *R. subcapitata* at 684 nm (Figure 12). As can be seen, it is a sharp peak with 670 nm sitting further down the left hand slope than 680 nm. Nevertheless, as growth rate is calculated relative to the control

[2] This peak is at a similar wavelength to absorption peaks for chlorophyll a (662 nm) and chlorophyll b (642 nm), although other pigments in the alga cell contribute towards the total absorption spectra. Larger peaks exist at shorter wavelengths, as can be seen in Figure 13. However, these are broader which, perhaps make them less useful as a diagnostic of algal cell numbers.

replicates in the experiment, and providing the same wavelength is used throughout, this should not lead to erroneous EC_{50} values.

No chemistry data for concentrations in the test media before or after the exposure were reported in Zounková *et al.* (2007), so it is not possible to confirm if the nominal concentrations were achieved. Nevertheless, no chemistry was conducted in the algae exposures for this study either, so it was possible that actual concentrations were lower than nominal, resulting in the calculation of a higher EC_{50} value. In this project, chemical analysis of the 5-fluorouracil zebrafish exposures was commissioned, the results of which showed that the actual concentrations were within an acceptable range to the nominal (section 4.3.3). The same protocol for making up the dosing stocks was used, giving some confidence that the algae were exposed at the desired concentrations.

The results of Zounková *et al.* (2010) are 100 times higher than either this or the 2007 study at 48 000 µg/l (Table 10). The same protocol as Zounková *et al.* (2007) was used, but with the following modifications. A different species of algae was used; it was *D. subspicatus*, another species of green algae approved in the OECD 201 guidelines. 96 well microplates were substituted for 24 well plates. Again well volume is not stated but the average is approximately 3 500 µl, ten times that of a 96 well plate. This would give a larger surface area, increasing CO_2 transfer from atmosphere. All other criteria remaining equal between the 2007 and 2010 experiments, the most probable affect would be to increase algal growth.

No media recipe was given, but instead of buffered saline, stocks were made up in H_2O, before dilution in media. Although this would have slightly diluted the medium, it is highly doubtful that this would impact the growth rate, resulting in the much higher EC_{50} value observed. In Zounková *et al.* (2010) the microplates were shaken discontinuously throughout the exposure for 30 minutes at 400 rpm, with 15-minute rest periods, unlike this study where samples were only shaken before the optical density readings were taken. As algae settle if there is no motion, discontinuous shaking keeps them suspended. This provides the population of algae in the sample more equal exposure to light and nutrients. This is likely to increase the growth rate of the population.

The same method for calculating growth rate was used, but in Zounková *et al.* (2010) absorbance was measured at 650 nm. Absorption spectra for *D. subspicatus* was not found in the literature. Absorption spectra for other species of green algae (Figure 13) showed similar peaks to that of *R. subcapitata*, although a smaller peak at 650 nm was also present. However, as noted above, if the algal growth rates are calculated relative to the control replicates, then the wavelength used is not critical, providing it is significantly absorbed by the species of algae used.

In the Zounková *et al.* (2010) study, chemical analysis of the exposure medium was conducted. The authors noted that a minor decline was observed in 5-fluorouracil concentrations during the exposure. However, it was not possible to assess this claim as numerical values were not given.

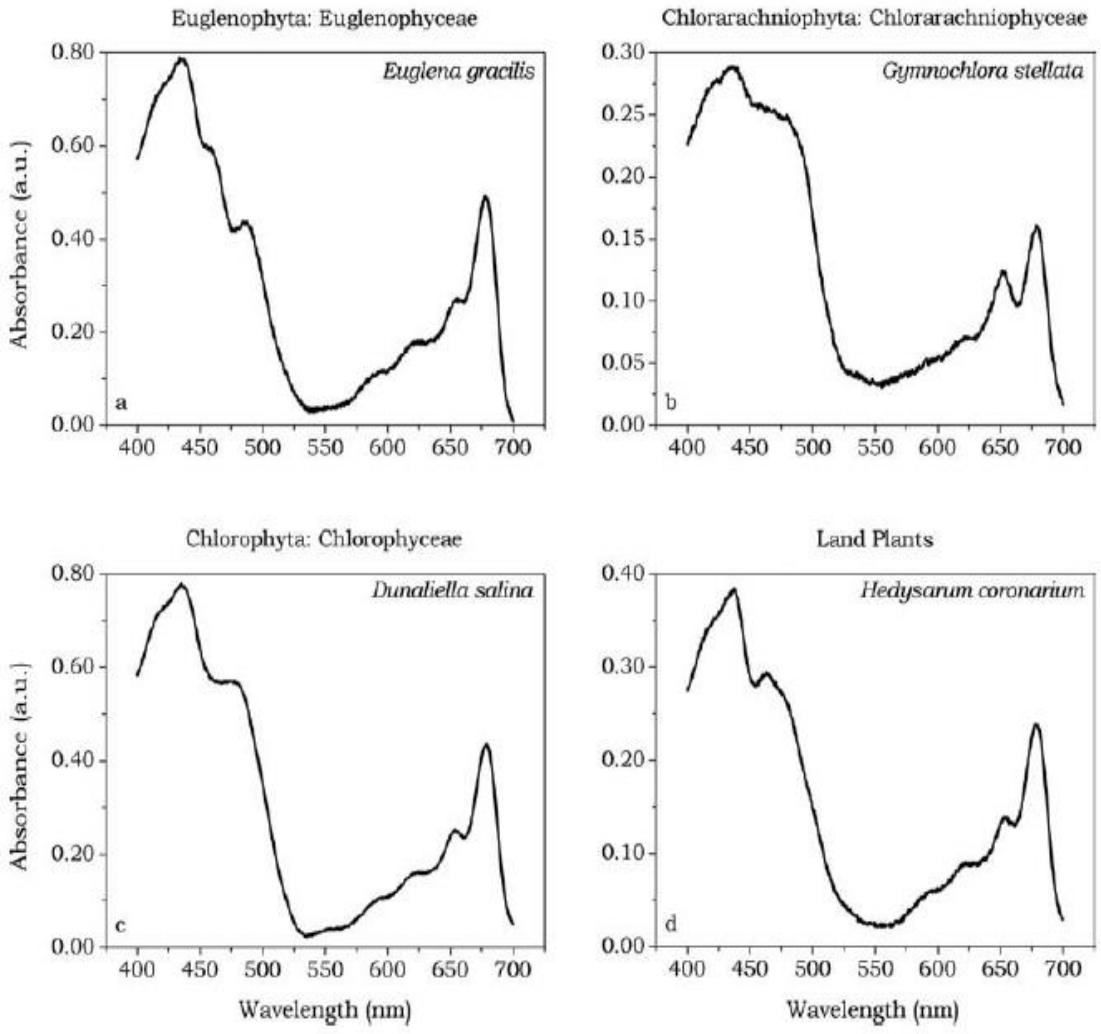


Figure 13: Absorption spectra for a selection of green algae and land plants. Both R. subcapitata and D. subspicatus belong to the Class Chlorophyta, represented here by D. salina (bottom left) (Barsanti and Gualtieri, 2006).

Notably the large discrepancy in EC_{50} values between the 2007 and 2010 experiments was not discussed by the authors. However, the 2010 results are comparable to those obtained by Cleuvers (2001) in Straub (2010), which also produced EC_{10} and $_{50}$ values in the tens of milligrams per litre range. Unfortunately, the Cleuvers paper was cited in German so it was not possible to assess these results. However, in both cases *D. subspicatus* was used, suggesting that the test organism used may have had a strong influence on the toxicity of 5-fluorouracil. This is supported by the findings of Millington *et al.* (1988), as outlined above.

Most recently, Brezovšek *et al.* (2014) generated an EC_{10} of 20 µg/l and an EC_{50} of 130 µg/l. Following the OECD guidelines, *R. subcapitata* was exposed in OECD growth medium in Erlenmeyer flasks (no volume was given, but it is likely to be c. 50 ml) and was shaken constantly throughout the exposure period. Stock solutions were made up in DMSO but solvent controls up to 5000 µl/l showed no statistical difference in growth rates. Chemical analysis was undertaken and no significant change in concentration was observed, although no data was shown. Cell numbers were counted using a coulter counter and growth rates calculated relative to the controls. Exposures were repeated three times in triplicate. The EC_{50} value is comparable to Zounková *et al.* (2007) but 4 times lower than that calculated for this study. However, the EC_{10} value is twice that of this study (no EC_{10} was given in Zounková *et al.* (2007)).

In all cases in the literature, algae cells were taken from continuous laboratory cultures maintained on site. This study used algae cells supplied fixed in a matrix, which were re-suspended before exposure. Daniel *et al.* (2004) saw this as a benefit, as this meant the algae were from a single source and were not subject to the variability that would occur between laboratories. This highlights that biological variability could also be a factor in the different EC_x values between studies.

One other point of interest was that Zounková *et al.* (2010) also tested the 5-fluorouracil metabolite, fluoro-β-alanine (FBAL). An EC_{50} of 80 000 µg/l was calculated, nearly double the EC_{50} concentration of the parent drug. FBAL is not an active metabolite of the cytotoxic mechanisms of action of 5-fluorouracil. However, it has been implicated as a causative agent of neuro and cardiotoxicity (section 1.4.1). Why this metabolite and not one of the

ones in the cytotoxic pathways was tested was not discussed in this paper. Xenobiotic metabolism pathways have been identified in green algae. A search of a green algae genome database identified some of the enzymes involved in 5-fluorouracil metabolism in species related to *R. subcapitata* (Kanehisa Laboratories, 2015). Therefore, the possibility that algae can metabolise 5-Fluoruracil to its active components exists. In addition, 5-Fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), the active metabolite that inhibits thymine synthesis, is available for purchase.

2.4.2.2 Imatinib

Brezovšek *et al.* (2014) was the only study found in the literature to have assessed imatinib under the conditions described above. Stock solutions were prepared in OECD medium, as for this study and chemical analysis showed no significant reduction in concentration. EC_{10} and EC_{50} values were respectively around two and three times lower than this study. In addition, whereas this study found imatinib to be more toxic than cisplatin, the opposite was found in Brezovšek *et al.* (2014). However, the difference in EC_x values was relatively small (Table 10).

2.4.2.3 Cisplatin & carboplatin

Zounková *et al.* (2007) also assessed the effect of cisplatin on *R. subcapitata* growth. The EC_{50} value was 2 300 µg/l, nearly 5 times lower than the 10 920 µg/l calculated by this study. As with the 5-fluorouracil and cyclophosphamide results, which were both around 4 times lower than this study, the difference could be due to one or more of the factors described in section 2.4.2.1.

Cisplatin also formed part of the Brezovšek *et al.* (2014) study. The EC_{10} and EC_{50} values were much lower than in this study by around ten and seven times, respectively (Table 9). In comparison to the Zounková *et al.* (2007) results, the Brezovšek *et al.* (2014) EC_{50} result was one and half times lower. For both of these studies the tests were repeated three times in triplicate, whereas in this study tests were run twice in triplicate. As noted in the results for the cisplatin experiment in this project, there was a relatively large variation in the results between the two tests, particularly at the highest concentration tested. This may have contributed to the large differences in the EC_x values. Although no chemical analysis

was conducted on the algae test medium, chemical analysis of the aquarium water in the zebrafish cisplatin experiments was carried out. Although there was some variation between the actual and nominal concentrations, this variation was within acceptable levels. As the same protocol for making up the algae exposure medium was the same, this gives some confidence that the algae were exposed to concentrations of cisplatin close to the nominal values.

The effects of carboplatin on algae did not appear to be represented in the literature. Although it has the same mechanism of action as cisplatin, no growth inhibition was observed in this study, up to the maximum tested concentration of 100 000 µg/l. This may be due to the additional metabolism step required for carboplatin, which is beneficial in a therapeutic context as it reduces or even eliminates some of the severe side effects associated with cisplatin (section 1.4.3)

2.4.2.4 Hydroxycarbamide

No results for the effects of hydroxycarbamide on algae were found in the literature. Research on other species was present, with studies in rats have shown neurotoxicity in the offspring of rats whose mothers were exposed to 550 000 µg/kg during gestation (Vorhees *et al.*, 1979). Foetal abnormalities were also observed in the offspring of rabbits injected with 650 000 µg/kg of the drug during gestation (Desesso *et al.*, 1994). The highest concentration tested in algae was 100 000 µg/l, resulting in 83 % growth inhibition, a volume of drug 5-6 times lower than that used in the rat and rabbit experiments. It could be likely that some algal growth inhibition would be detected at comparable concentrations. However, for both mammals and plants, the concentration of hydroxycarbamide required to produce adverse effects is far higher than those found in the environment.

2.4.2.5 Cyclophosphamide

Zounková *et al.* (2007) was the only paper found to have experimentally calculated an EC_{50} value for the effect of cyclophosphamide on algae. At 930 000 µg/l, this was around three times less than the value calculated in this study. It is possible that this difference may be due to one or more of the issues outlined above, e.g. differences in the capacities of the

test vessels and/or medium composition. Chemical analysis of the aquarium water from the zebrafish cyclophosphamide experiments showed a large decrease in concentration between the nominal and actual concentrations, with a greater decrease in actual concentration at higher nominal concentrations. This is discussed in more detail in section 4.3.3, but it is possible that this is due to chemical degradation via hydrolysis, once cyclophosphamide is put into solution in ddH_2O. As the same protocol was used for making up the algae exposure medium, it is likely that the algae were exposed at lower actual concentrations, which could explain the difference between the EC_{50} value from this study and that of Zounková *et al.* (2007).

Grung *et al.* (2008) followed OECD Guideline 201, selecting *R. subcapitata* and exposing them in 100 ml vessels containing 50 ml of medium. Test vessels were continuously agitated throughout the exposure period on a reciprocating table. The number of algae cells was counted at 24 hour intervals using a Coulter counter and growth calculated relative to the controls. However, the highest concentration of cyclophosphamide tested was 100 000 µg/l. All test concentrations (3 200, 5 600, 10 000, 18 000, 32 000 and 56 000 µg/l) produced growth 1-3% higher than the control, apart from the highest concentration, where the percentage growth was the same as the control. This finding agreed with this study in that no difference in growth was observed at 100 000 µg/l.

Mater *et al.* (2014) used the same test kits as were used in this thesis project, so the conditions of the experiments should have been highly comparable. However, Mater *et al.* (2014) tested a much lower concentration range. They claimed a non-monotonic concentration-response curve, with 0.01 µg/l causing 5-10% growth inhibition ($p > 0.05$), no significant response at 0.1, 0.5 and 1.0 µg/l and 5-10% growth proliferation ($p > 0.001$) at 10.0 µg/l. No higher concentrations were tested, so no EC_{50} value was reported. It could be argued that the 1-3% increase in growth compared to the controls, observed by Grung (2008), gives some support to this claim, although there was no overlap in the concentration series between these two experiments. However, small changes in growth could be a consequence of other factors rather than an effect of the drug. For example, biological variation between replicates and treatments could be responsible for the results reported.

The only other relevant data found in the literature was a value of 11 000 µg/l predicted for algae using ECOSAR (Sanderson *et al.*, 2003). This value is ten times lower than Zounková *et al.* (2007) reported, and 30 times lower than the value obtained in this project. Given that these calculations make assumptions about the toxicity and environmental concentrations of the target chemical, and are conservative by design, this discrepancy is unsurprising.

One other point of interest is that Vannini et al. (2011) exposed *R. subcapitata* to a mixture of thirteen pharmaceuticals that included cyclophosphamide at 10 ng/l. They then analysed the algal cells (using HPLC-MS-MS) for the thirteen chemicals and could find all but cyclophosphamide and one other drug. This may be evidence that *R. subcapitata* is not able to take up this drug, which would be supported by the high EC_x values. However, to detect cyclophosphamide at or below 10 ng/l would require a highly sensitive technique. For comparison, the LOD for cyclophosphamide in this project was 0.13 µg/l.

2.4.2.6 Single drug conclusions

The null hypothesis states that: "The selected cytotoxic pharmaceuticals, at the tested concentrations, will not inhibit alga growth, with the effects not being additive".

For 5-fluorouracil, in the specific environmental scenarios discussed in section 2.4.1, it was possible to reject the null hypothesis. For all other drugs, it was not possible to reject the null hypothesis.

None of the drugs tested appear to cause algae growth inhibition at concentrations found in surface waters in developed countries. 5-fluorouracil did inhibit growth at concentrations observed in hospital effluents. However, these effluents are likely to be greatly diluted by other effluents within the sewerage system, before the drug is further degraded and diluted within the sewage treatment works.

Waste from drug manufacturing in low and middle income countries has the potential to contain cytotoxic drugs. If these were to occur at the same orders of magnitude as the drugs measured in the literature, 5-fluorouracil, and potentially imatinib and cisplatin could be at concentrations shown to inhibit algal growth in this study.

Although there was some variation between the results of this study and those found in the literature, they supported the conclusions above.

2.4.3 Exposure to simple mixtures of cytotoxic drugs

Both the one fifth EC_{50} and half LOEC mixtures produced lower amounts of algal growth inhibition, than the sum of the single chemicals. This difference was about 40% for the EC_{50} mixture and 50% for the half LOEC. This could be described as antagonistic behaviour, with interactions between the drugs reducing the toxicity of the mixture below the sum of their individual actions. This is contrary to expectations for at least some of the components of the mixture. Antineoplastic drugs are generally administered in combination, as their synergistic effects can enhance their tumour supressing abilities. For example, an in-vitro study pre-exposed cancer cells to 5-fluorouracil before exposing them to cisplatin. The study found that pre-exposure increased the cells retention of cisplatin and led to an enhancement of the latter's cytotoxicity and a reduction in the cells resistance to cisplatin. The research team postulated that this was due to 5-fluorouracil inhibiting the cell's DNA repair capability (Tanaka *et al.*, 2001). These two drugs form a drug regimen termed FP, which is used as a treatment for advanced gastric cancer. To provide some comparison between therapeutic and environmental concentrations, this regimen is administered intravenously, with cisplatin at 80 mg/m^2 on day 1 and 5-fluorouracil at 800 mg/m^2 on days 1-5. This regimen is repeated every 3 weeks until halted by a range of factors, such as toxicity, disease progression or withdrawal of patient consent (Kang *et al.*, 2009). Other drug regimens that include the tested drugs are described in section 1.5.

When compared with the predicted and measured environmental concentrations (Table 11), as for the single chemical exposures, only 5-fluorouracil was present in the mixture at concentrations that fell within the measured range for hospital effluents. Even the lower concentration used in the 'something from nothing' mixture was three orders of magnitude higher than that predicted in surface waters. The only other drug that was close to environmental values was cisplatin, whose one fifth the half LOEC value was twice that found in hospital effluents. As mentioned above, these two drugs are used together therapeutically, due to their synergistic behaviour. Given that the results of the 'something from nothing' mixture suggests that the five drugs together might have an overall

antagonistic effect, one way to assess this further would be to conduct a binary mixture exposure. However, given the complex nature of mixtures experiments outlined in the previous paragraph, it could be more prudent to design a more robust mixtures experiment.

Table 11: A comparison of the individual drug concentrations (µg/l) that made up the simple mixture (⅕ EC_{50}) and 'something from nothing' mixture (⅕ ½LOEC) with the Predicted Environmental Concentrations (PEC) and Measured Environmental Concentrations (MEC). a Straub (2010), b Martín et al. (2011), c Mahnik et al. (2004), d Besse et al.(2012) * Conservative PEC as refined PEC not available, e Rowney et. al. (2009) † Combined PEC with 4 other cytotoxic drugs, e f Kümmerer & Helmers (1997), g Vyas et al. (2014), h Buerge et al. (2006), i Mark-Kappeler et al. (2011)

				MEC range	
Drug	⅕ EC_{50}	⅕ ½LOEC	PEC	Hospital effluent (untreated)	Surface Waters
5-fluorouracil	88.00	0.20	0.00265a	2.0b – 122c	-
imatinib	1 286.00	10.00	0.00000499d*	-	-
cisplatin	1 480.00	200.00	0.00697 – 0.01072†e	0.021f – 100g	-
hydroxycarbamide	19 440.00	1 000.00	0.000078d	-	-
cyclophosphamide	635 800.00	10 000.00	0.00697 – 0.01072b	0.0045f	0.00005h - 0.0648i

2.4.3.1 Experimental design of the simple mixture experiment

If designed ideally, the components of the mixture should cause a similar level of inhibition to each other when exposed singly. However, when exposed by itself, cisplatin elicited no measurable algal growth inhibition at one fifth its EC_{50}. This may have been due to the variability observed between the two repeats of the single chemical cisplatin exposures, resulting in an inaccurate EC_{50} calculation. This is possibly supported by the EC_{50} values from the literature, which were five to seven times higher. It is therefore possible that the concentrations of cisplatin used in the mixtures exposure were too low to have inhibited algal growth. The same could be said of the cyclophosphamide results, in which only a 1.46% inhibition in response to the one fifth EC_{50} concentration was observed. However, more confidence can be had in the single chemical exposure values, as there was much less variability between the repeats. Imatinib was closest to the expected value of 10%

inhibition with an actual result of 14.53%. Both 5-fluorouracil and hydroxycarbamide inhibited growth at more than twice the expected value of 26.36%. It should be noted that due to the high cost, the single drug exposures in this mixtures experiment were not repeated, although they were tested in triplicate. It is therefore possible that these results could have been compromised by an error, for example a mistake made in weighing the drug to make up the dosing stocks.

Another possible explanation can be seen if the EC_{10} and one fifth EC_{50} values are compared to each other (Table 12). For cisplatin, where no response was seen, the EC_{10} is nearly four times larger than the one fifth EC_{50} concentration. For cyclophosphamide the EC_{10} is nearly 2.5 times greater. In the opposite direction the 5-fluorouracil EC_{10} is nearly nine times smaller. The two values for imatinib, however, are nearly the same, with the EC_{10} only slightly greater than the one fifth EC_{50}. With hindsight, simply dividing the EC_{50} by the number of chemicals could be seen as a naïve design. It may have been more appropriate to use the EC_{10} values instead. This would have taken into account not only the non-linear concentration-response curves, but also the differences the slopes of the concentration-response curves between the drugs. This may have achieved the equipotent mixture that was the aim of the original design. It should be noted that the hydroxycarbamide values do not support this argument. However, the lack of data available to assess the accuracy of the EC_x values due to the single chemical exposure not being repeated is a more likely explanation.

Table 12: Comparison of the EC_{10} and EC_{50} calculated for the algae single chemical exposures and the ⅕ EC_{50} concentrations (µg/l) used to make the simple mixture exposure.

Drug	EC_{10}	EC_{50}	⅕ EC_{50}
5-fluorouracil	10.00	435.00	88.00
imatinib	1 430.00	6 430.00	1 286.00
cisplatin	5 885.00	7 400.00	1 480.00
hydroxycarbamide	93 550.00	97 200.00	19 440.00
cyclophosphamide	1 520 000.00	3 179 000.00	635 800.00

Another possible factor in the disparity observed between results is the steepness of the concentration-response curves, particularly in the central region covering the EC_{50} values. This steepness can make the values calculated more sensitive to slight changes in concentration or biological variability and result in greater variability in EC_x values between experiments.

2.4.3.2 'Something from nothing' mixture experimental design

As stated previously, the concept of a 'something from nothing' design is that drugs at individual concentrations that elicit no response in an endpoint, when combined in a mixture will produce a response. However, in this design, three of the five drugs, namely 5-fluorouracil, cyclophosphamide and hydroxycarbamide, all produced small but measurable amounts of growth inhibition when tested individually (Figure 10). The sum of this inhibition was approximately two percent; one percent greater than the effect of the mixture. The most likely reason for this is the use of half the LOEC values as the single drug concentration. Ideally a NOEC value should be used in this design. However, these values were not available for all drugs, so the arbitrary value of half the LOEC was used as a proxy. It would appear that this still did not fall below the NOEC for these three chemicals. This could be rectified by repeating the single chemical exposures with greater resolution at the bottom of the concentration-response curve, in order to detect the NOEC values.

2.4.3.3 Mixtures of cytotoxic drugs in the literature

Very few mixtures studies containing the target drugs were found in the literature. Vannini *et al.* (2011) included cyclophosphamide, at an environmentally relevant concentration of 10 ng/l, in a mixture with 12 other pharmaceuticals of other classes and exposed *R. subcapitata* to a 1 x and 10x concentration. No growth inhibition was observed. The study also assessed DNA damage and changes in the proteome. No DNA damage was observed but some changes in protein expression were observed, mainly in proteins involved in photosynthesis. However, it is not possible to assess what role, if any, cyclophosphamide played.

Mater *et al.* (2014) also exposed *R. subcapitata* to cyclophosphamide in a mixture with ciprofloxacin (an antibiotic that inhibits repair and replication of DNA in bacteria) and

tamoxifen (an oestrogen receptor antagonist used in breast cancer treatment). As noted above, little if any effect was observed when the algae were exposed singly to cyclophosphamide at a concentration range of 0.01 - 100 µg/l with a 10-fold change between each step. However, when combined with tamoxifen at 0.01, 0.1 0.5, 1.0 & 10 µg/l (1 – 5 in Figure 14) and ciprofloxacin at 0.01 - 100 µg/l, significant growth inhibition was observed at all concentrations (Figure 14).

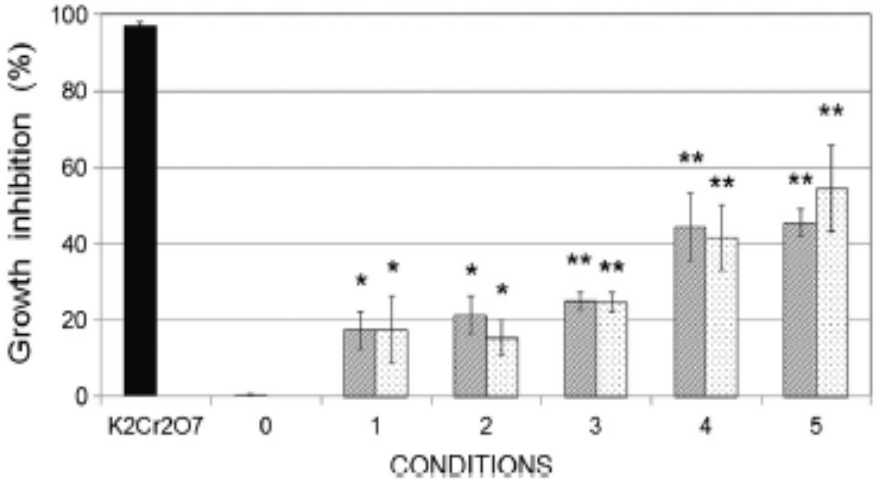


Figure 14: Comparison of growth inhibition of R. subcapitata exposed to ciprofloxacin + tamoxifen (left: dark stippled bar) and ciprofloxacin + tamoxifen + cyclophosphamide (right: dark stippled bar), at the concentrations (1 – 5) described in the text. 0 is the algae medium control and potassium dichromate the positive control (far left: black bar). Data are median values and error bars standard deviation (Mater et al., 2014).

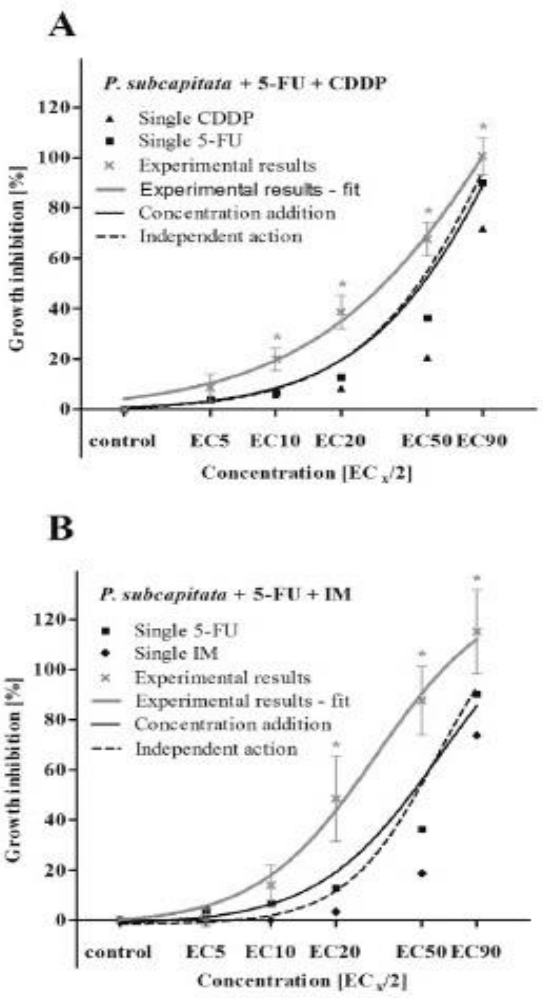


Figure 15: Predictions of the CA and IA models for the growth inhibition of R. subcapitata, exposed to two binary mixtures of cytotoxic drugs, at a concentration series of their ECx values and compared to actual exposure results (Brezovšek et al., 2014)

The only paper found that assessed more than one of the target drugs, looked at binary mixtures of 5-fluorouracil + cisplatin and 5-fluorouracil + imatinib (Brezovšek *et al.*, 2014). The two binary mixtures were chosen as they form part of chemotherapy regimens. Each mixture was tested as a concentration series of their individual EC_5, $_{10}$, $_{20}$, $_{50}$ and $_{90}$ concentrations divided by 2, using the results of the individual exposure series critiqued in sections 2.4.2.1 - 2.4.2.3 above.

The experimental results of the binary mixtures were assessed against the predictions of the Concentration Addition (CA) and Independent Action (IA) models. Both models significantly underestimated the effect of the 5-fluorouracil + cisplatin (Figure 15A) and the 5-fluorouracil + imatinib (Figure 15B) binary mixtures at all but the EC_5 concentration. Unfortunately, the study did not include a 5-fluorouracil + imatinib mixture, which would have aided assessment of the mixtures in this project. Nevertheless, two combinations of three of the five drugs in the project mixtures show an effect opposite to that observed by Brezovšek et al. (2014). It could be suggested that cyclophosphamide and/or imatinib and/or hydroxycarbamide had an antagonistic effect that overrode the potentially synergistic effects observed by Brezovšek *et al.* (2014). However, this would require 10 binary mixtures to explicitly test this.

2.4.3.4 Mixtures conclusions

The null hypothesis states that: “The selected cytotoxic pharmaceuticals, as mixtures at environmentally relevant concentrations, will not inhibit alga growth”.

For either mixture design, it was not possible to reject the null hypothesis.

Although the results would suggest the five chemicals might be displaying antagonistic behaviour, when combined in a mixture, issues with the experimental designs would require refinement and retesting to better assess this. Based on the current data, it is not possible to draw any further conclusions on the results of the effects of mixtures of these drugs at environmentally-relevant concentrations. However, the literature findings, particularly the apparently strongly synergistic effect of ciprofloxacin with cyclophosphamide, underline that single chemical testing alone is not capable of fully providing all the environmental implications of that, or any other chemical.

3 Impact of cytotoxics on Daphnia mobility

3.1 Introduction

Commonly known as water fleas, Daphnia species are freshwater planktonic crustaceans, belonging to the Phylum Arthropoda, in the Kingdom Animalia. They have a broad geographical range, having been identified in Europe, North and South America and Africa.

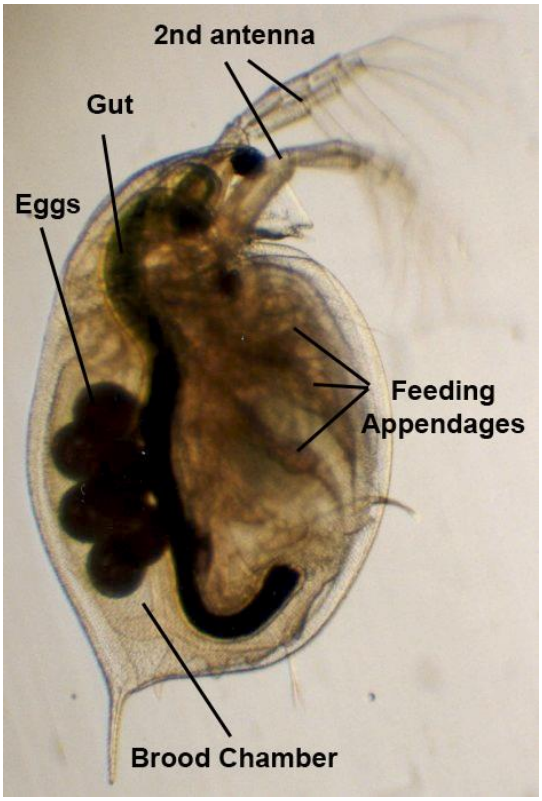


Figure 16: Photograph of a female adult D. magna with a clutch of parthenogenic eggs in her brood chamber. The greenish tinge in the gut indicates that she has been feeding on algae. The 2nd antenna are used for locomotion. An adult D. magna is 1-5 mm long. Image modified from Ebert (2005)

They are filter feeders, subsisting mainly on green algae, but are capable of filtering smaller organisms such as bacteria. Their lifecycle is one of cyclical parthenogenesis, producing asexually through diploid amictic eggs during the growing season (Spring and Summer months, periods of drought or low productivity), from a population that mostly comprises of females. Towards the end of the growing season, triggered by ecological factors (such as carrying capacity) and environmental factors (such as decreased temperature), females produce resting eggs that are haploid and fertilisable by the males. A protective shell layer, called an ephippium, forms around one to two resting eggs whilst in the brood chamber (Figure 16) and is cast off when the daphnia next moults. The ephippia lay dormant until external stimuli such as increased temperature or rising water levels prompt hatching (Ebert, 2005).

Daphnia species are often the dominant zooplankton in a wide range of still freshwater bodies (Ebert, 2005). As such they are keystone species in freshwater food webs. As primary grazers of phytoplankton and bacteria they can have a substantial impact on the abundance of these species (Lampert *et al.*, 1986; Jürgens, 1994). In turn they are a key

food source for planktivorous fish and some invertebrates (Ebert, 2005), so their abundance can have significant impact on their higher trophic levels (Luecke *et al.*, 1990). As such they are also an important ecotoxicology test species, with *Daphnia magna* being the preferred species in the OECD guidelines.

Ideally, due to the genotoxic potential of the target drugs, a chronic multiple generation assay would be conducted, as was the case for the algae exposures. The OECD has defined an assay with Daphnia, based on offspring inhibition as the end point. However, due to time and cost constraints, an acute assay that used adult immobilisation as the end point was chosen.

3.1.1 Aims

- Calculate effects concentrations for 10% and 50% daphnia immobilisation (EC_{10} & EC_{50}), when the daphnia are exposed to the individual cytotoxic drugs, which are listed in Table 13.
- Expose algae to the two simple mixtures defined in section 3.2.2 and compare results with each other and with the results of the single chemical exposures.

3.1.2 Hypothesis

"The selected cytotoxic pharmaceuticals, at the tested concentrations, will immobilise daphnia, with the effects being additive".

3.2 Methods & materials

OECD 202 "Daphnia species acute immobilisation test" (OECD, 2004) defines the testing procedure for determining the effects of a substance on the immobilisation of juvenile Daphnia, a freshwater crustacean. Batches of Daphnia are exposed to a concentration range of the target substance for a period of 48 hours. At the end of this period the number of immobilised Daphnia is counted and a percentage immobilised is calculated. From this EC_x values can be determined, where $_X$ is the percentage daphnia killed or immobilised.

For the reasons outlined for the algae tests in section 2.2.1, DaphToxkit F™ kits were purchased from MicroBioTests Inc., Belgium. This kit was also independently tested by

AstraZeneca (Daniel *et al.*, 2004) and the results also found comparable to self-cultured daphnia. The kit contains the test organism (*Daphnia magna*) and the OECD defined medium (to be made up in deionised water). The kit also contains 10 ml 36 well plates, in which the daphnia are exposed in a standard incubator.

3.2.1 Single chemical exposures

The tests were designed and run by the project second supervisor, Dr. Catherine Harris, following the supplied protocol, with the modifications detailed below. The standard freshwater is supplied in four vials, containing the required minerals to make up standard freshwater. The contents of these vials are added to 2 litres of deionised water and aerated with a filtered aquarium pump for 15 minutes prior to use. *D. magna* are supplied as ephippia, which require hatching prior to exposure. These are hatched in 5cm petri dishes containing 15ml of pre-aerated standard freshwater. The petri dishes are placed in a standard incubator at 21° C (± 2° C), under 10,000 lux of light at the top of the petri dish for 72 hours. At the 72-hour time point, 600 µl of standard freshwater is added to the supplied vial of *Spirulina* micro algae. The vial is shaken to homogenise the solution and poured into the petri dish. The daphnia are then left for a 2-hour pre-feeding period.

The standard kit allows for a control and five concentrations to be tested, with four replicates per treatment for statistical robustness. For range finder tests, the desired concentration ranges were either 0.001 to 100 or 0.01 to 1000 mg/l in ten-fold steps. For definitive tests the fold change and range were defined based on the toxicity of the chemical. The pharmaceuticals and their definitive concentration ranges are listed in Table 13. All definitive concentration series were run twice, apart from carboplatin, which was only run once and 5-fluorouracil, which was repeated three times. The definitive tests for 5-fluorouracil and cyclophosphamide were run at two different concentration series.

Table 13: Pharmaceuticals information and definitive exposed concentration ranges (mg/l) for the daphnia single chemical exposures. *numbers in brackets are the fold change, five concentrations in a range.

Pharmaceutical	CAS number	Supplier	Supplier name	Definitive range (fold change)*
5-fluorouracil	51-21-8	Sigma-Aldrich	5-fluorouracil	50, 125-1000 (2) 8-5000 (5)
cyclophosphamide	6055-19-2	Sigma-Aldrich	cyclophosphamide monohydrate	625-10,000 (2) 1,000, 2,000, 3,000, 8,000, 10,000
cisplatin	15663-27-1	Sigma-Aldrich	cis-Diammineplatinum(II) dichloride	0.1-10 (3)
hydroxycarbamide	127-07-1	Sigma-Aldrich	hydroxyurea	50-800 (2)
imatinib	220127-57-1	Cayman Chemical	imatinib (mesylate)	6-270 (3)
carboplatin	41575-94-4	Sigma-Aldrich	carboplatin	50, 125-1000 (2)

Instead of following the dilution series method detailed in the protocol, a concentrated stock solution was made up in standard freshwater and diluted to the desired concentration (also in standard freshwater) to a total of 60 ml per concentration in the series, with a further 60 ml of standard freshwater only for the control. 10 ml aliquots were transferred to the 30 well plate; four replicates and one rinsing well per concentration (Figure 17). Approximately 25 daphnia are placed into each rinsing well (Figure 17). The purpose of these wells is to reduce the amount of dilution that would occur if daphnia were added directly from the petri dish. Daphnia are then distributed to the concentrations, 5 per well. The 30 well plate is placed in the incubator, at the same temperature but with the light extinguished, for a period of 48 hours, with the number of immobilised daphnia counted every 24 hours. Each well is gently swirled with a pipette and the number of dead and immobilised daphnia counted after 15 seconds. A daphnia is counted as immobilised if it cannot propel itself through the standard freshwater. There may still be movement from its antennae.

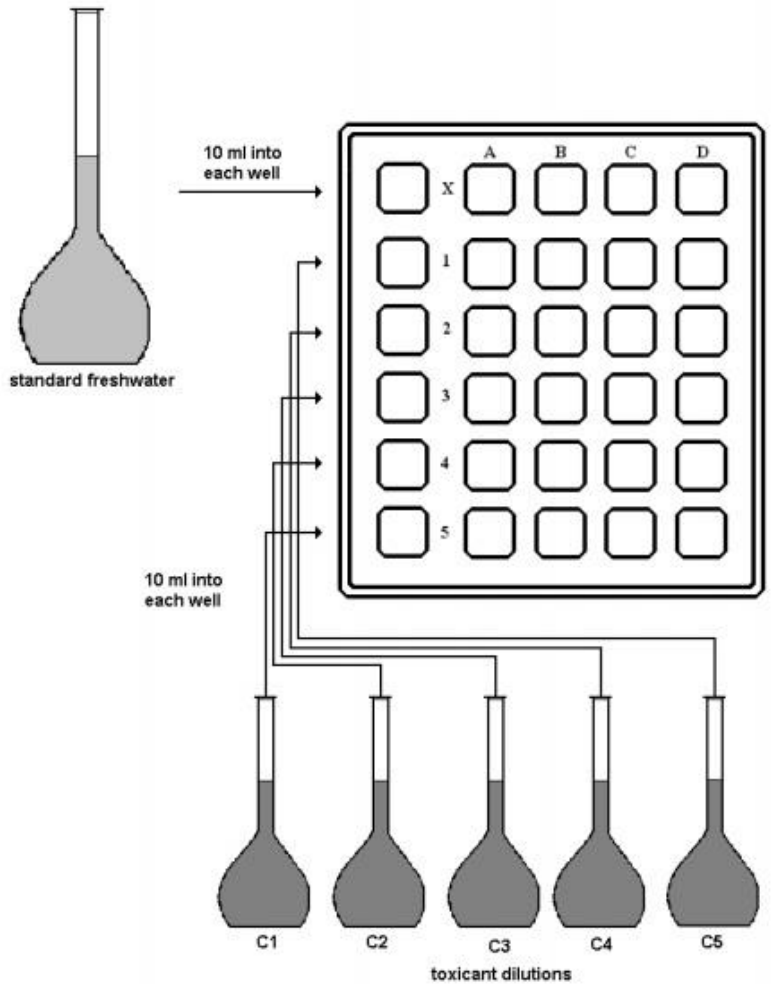


Figure 17: The loading process for the 30 well plate used in the daphnia exposure experiments. The left hand column is the rinsing wells where the daphnia are initially placed before distributing them to the exposure wells.

An excel spreadsheet was prepared that plotted dead/immobilised daphnia on a semi-log graph and allowed calculation of any given EC_X value between 1 and 100 percent. Although the OECD guidelines for this species only require the EC_{50}, the EC_{10} and EC_{20} vales were also reported, for comparison with the algae results.

3.2.2 Mixtures exposure

The protocol defined in section 3.2.1 was followed but further adapted to allow assessment of mixtures of the target drugs. The design was the same as for the algae mixtures experiments (section 2.2.2). Daphnia exposed to carboplatin had been immobilised (EC_{50} 266.92 mg/l). However, there was only sufficient room in the daphnia kit to run five chemicals. This was to allow a comparison with the algae mixtures experiment, which comprised of the same five drugs as in Table 14.

Concentrated dosing stocks were made up for each chemical and diluted to a concentration equal to the relevant EC_{50}/½LOEC values (rounded up to the nearest whole number apart from the cisplatin ½LOEC value). Aliquots were taken from each of these and diluted further to one fifth of the relevant values (Table 14). These were produced to allow comparison between the mixtures effects and those of the single chemical at their constituent concentrations. Equal amounts of the remaining EC_{50}/½LOEC stocks were then combined to make the mixtures.

Table 14: Pharmaceutical concentrations (µg/l) used to make the simple mixture and 'something from nothing' mixture for the daphnia mixtures experiments. All one fifth values were rounded to the nearest hundred/thousand to simplify the calculations. *A mistake was made when calculating the cyclophosphamide ½LOEC and the mixture was run with this drug at 50 000 µg/l instead of 500 000 µg/l

	cisplatin	imatinib	hydroxycarbamide	5-fluorouracil	cyclophosphamide
EC_{50}	1 650	65 780	213 450	319 240	1 025 110
Simple mixture (⅕ EC_{50})	300	13 000	43 000	64 000	205 000
LOEC	300	54 000	50 000	36 000	1 000 000
½LOEC	150	27 000	25 000	18 000	50 000*
'Something from Nothing' (⅕ ½LOEC)	30	5 400	5 000	3 600	10 000

These were transferred to the 36 well plates and the daphnia added as described in section 3.2.1. Exposure conditions and data gathering were also the same as for the single chemical exposures. However, as for the algae mixtures exposure, it was not possible to calculate EC_X values, as a range of concentrations would be required. Instead, the percentages immobilised at the exposed concentration of single drugs/mixture were reported.

3.3 Results

3.3.1 Single chemical exposures

Immobilisation was observed for all drugs. The concentration-response curves are shown in Figure 18, and the EC_{10} and EC_{50} values in comparison to the available predicted and measured environmental concentrations, are shown in Table 15.

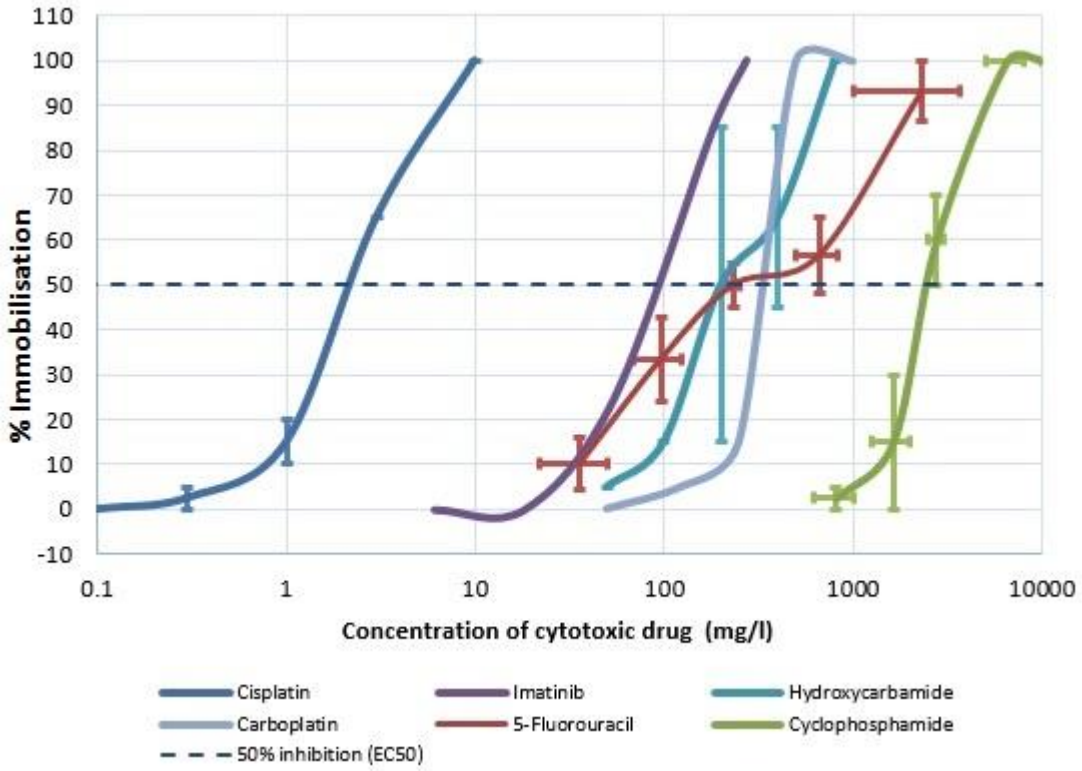


Figure 18: Concentration-response curves from the single drug daphnia immobilisation experiments. Due to the variation in concentration series used for 5-fluorouracil and cyclophosphamide, the mean concentration has been plotted with horizontal error bars. All error bars are standard error.

Daphnia appeared to be most sensitive to cisplatin (EC_{50} = 1.65 mg/l). Cyclophosphamide was observed to be the least toxic (EC_{50} = 2318.22 mg/l). Three drugs produced relatively similar EC_{50} values, from 213.45 mg/l for hydroxycarbamide, 266.92 mg/l for carboplatin to 319.24 mg/l for 5-fluorouracil. It should be noted that the carboplatin exposure was not repeated, so its EC_{50} value is generated from only one experiment. Hydroxycarbamide was repeated at the same concentration series, but the third and fourth concentrations (200 mg/l and 400 mg/l) in the series produced a wide range of immobilisation. 15% immobilisation was observed in the 200 mg/l concentration and 45% in the 400 mg/l in the first run. In the second run, 85% immobilisation was observed for both concentrations.

Table 15: EC_{10} and EC_{50} values for the single chemical daphnia immobilisation exposures in µg/l, ordered by EC_{50} toxicity, highest to lowest. a Straub (2010), b Martín et al. (2011), c Mahnik et al. (2004), d Besse et al.(2012) * Conservative PEC as refined PEC not available, e Rowney et. al. (2009) † Combined PEC with 4 other cytotoxic drugs, e f Kümmerer & Helmers (1997), g Vyas et al. (2014), h Buerge et al. (2006), i Mark-Kappeler et al. (2011)

				MEC range	
Drug	EC_{10}	EC_{50}	PEC	Hospital effluent (untreated)	Surface Waters
cisplatin	350	1 650	0.00697 – 0.01072†e	0.021f – 100g	-
imatinib	17 930	65 780	0.00499d*	-	-
hydroxycarbamide	88 540	213 450	0.078d	-	-
carboplatin	110 270	266 920	-	0.096f – 0.165f	-
5-fluorouracil	44 430	319 240	0.00265a	2.0b – 122c	-
cyclophosphamide	1 025 110	2 318 220	0.00697 – 0.01072e	0.0045f	0.00005h - 0.0648i

The experiment with 5-fluorouracil was repeated three times, the first two times at the same concentration series with a two-fold change and the third using a wider concentration series (five-fold change). Each run produced a different range of results, with no apparent concentration-dependent response. For example, the highest concentration (1,000 mg/l) in the first two runs produced 100% and 40% inhibition, respectively. In the third run the highest concentration of 5,000 mg/l resulted in 80% inhibition of the exposed daphnia. The experiment using cyclophosphamide was also repeated with two different concentration series, although they both covered a similar range (Table 13). The experiment with imatinib was not repeated, as bacterial growth was observed in the highest two concentrations of the range-finding and first definitive exposure, impeding daphnia movement and producing unreliable data.

To counter the use of different concentration series for some of the drugs, the results for all experiments for a given drug were plotted onto semi-log XY scatter plot (Figure 19). The EC_x values were then calculated from the equation of the slope of the linear best-fit trend lines (Table 15).

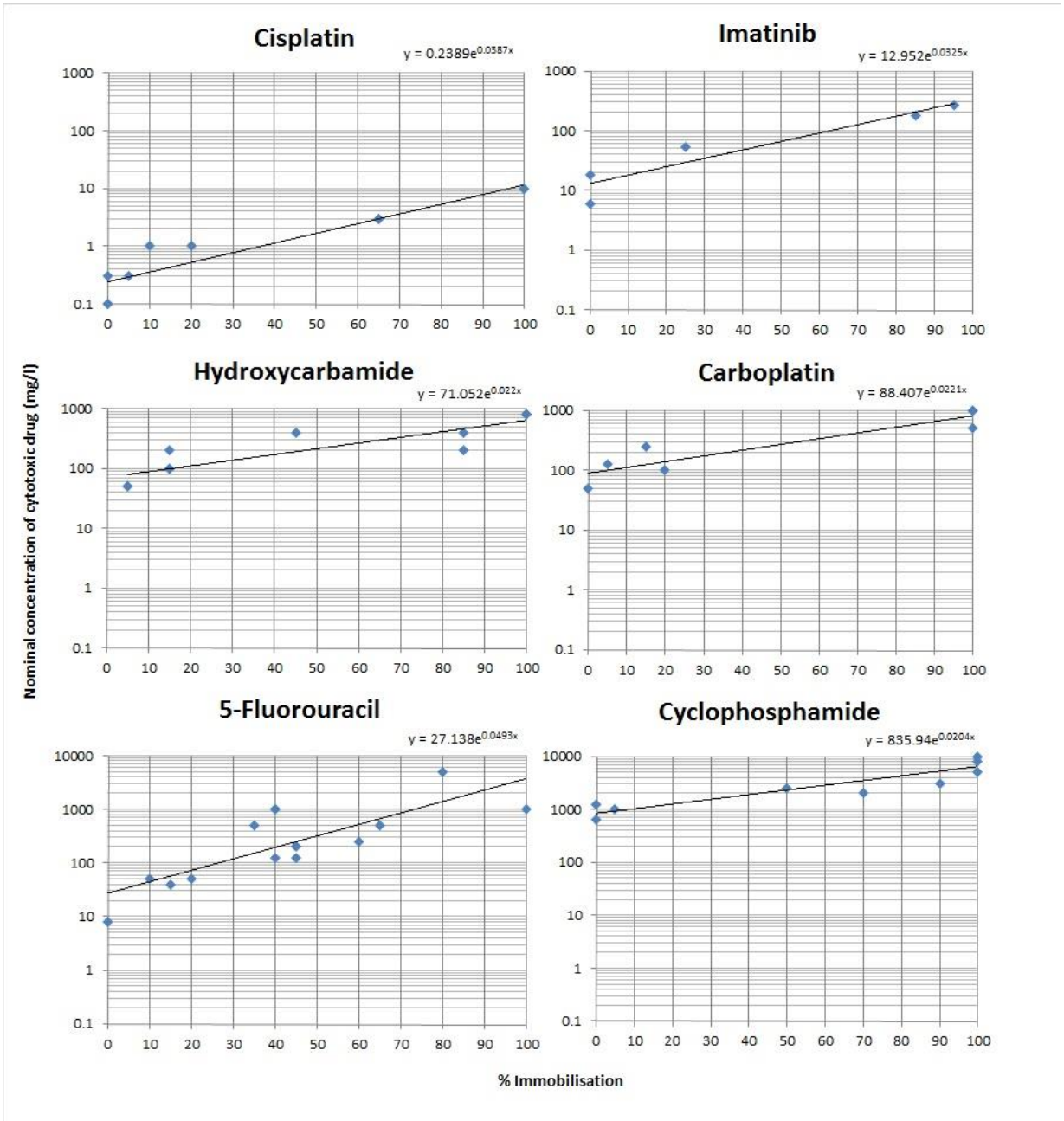


Figure 19: Results of the single drug daphnia immobilisation experiments graphed as XY scatter plots with linear best fit trend lines and their trend line equations. The slope equations were used to accurately calculate the $EC_{10, 20}$ and $_{50}$ values reported in Table 15, using the equation $y \times e^{(ECx \times x)}$ where e (a mathematical constant), the base of the natural logarithm, is raised to the value of the required EC value multiplied by the x value of the trend line equation.

None of the drugs tested elicited a response at predicted environmental concentrations or at those measured in surface waters. The EC_{10} for cisplatin (350 µg/l), the most potent

cytotoxic drug was still three times the concentration measured in untreated hospital effluent (Table 15).

3.3.2 Mixtures experiments

All the single chemicals in the simple mixture experiment (one fifth of their respective EC_{50} values) caused immobilisation, apart from imatinib. 5-Fluorourail experienced the greatest amount of immobilisation at 15%. The sum of the responses to the single chemicals in the simple mixture experiment was 35%. In comparison, the mean of the simple mixture (the mixture was run in duplicate) of these chemicals was two and a half times greater at 87.5% (Figure 20). The results of the 'something from nothing' mixture are not shown as no immobilisation was observed, either in the single chemicals or the mixture.

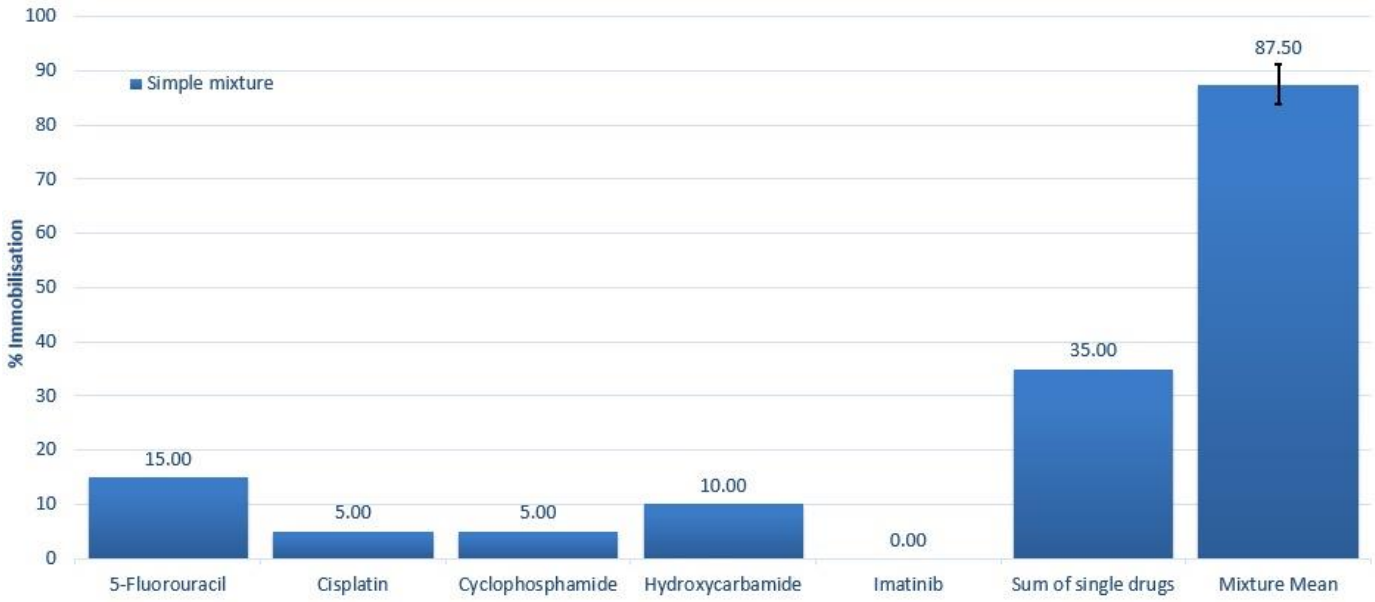


Figure 20: Percentage immobilisation* for daphnia exposed to single chemicals at 1/5 their EC_{50} values, and also a simple mixture of these chemicals at these concentrations. The percentage immobilisation for each of the single chemicals has been summed (Column: Sum of single drugs), to provide a comparison to the mixture results (Column: Mixture Mean). The mixture exposure was repeated twice; error bars are standard error around the mean. *defined as the number of immobilised daphnia as a percentage of the daphnia in that treatment

3.4 Discussion

3.4.1 Single drug experiments

All drugs inhibited daphnia mobility, albeit at concentrations orders of magnitude higher than measured and predicted surface water concentrations (Table 15). In addition, all drugs had EC_x values higher than those measured in hospital effluents, although the cisplatin EC_{10}

of 350 µg/l was only around three times greater, so the possibility for some overlap exists. As discussed in section 2.4.1, drug manufacturing in low and middle income countries has resulted in levels of pharmaceuticals in the hundreds of micrograms per litre in some surface waters. However, there is no evidence to show that this is the case for the target drugs in this study. As noted previously, this may be because chemical analysis for these drugs has not been conducted (with the exception of cyclophosphamide). Further research is required into the possibility that production of this class of pharmaceutical in low to middle income countries could lead to high concentrations in the environment.

The results for several of the drugs should be treated with caution. The experiment with carboplatin was not repeated, although it was run with quadruplicate replicates. Ideally this experiment would have been run at least once more, to give more confidence in the EC_x values calculated.

In both the range finder and first definitive tests conducted with imatinib, filamentous bacterial growth was observed in the highest two concentrations. The growth appeared to be concentration-dependent, with larger amounts of the bacteria in the higher concentration. This could be seen to impede daphnia motility. This posed the risk of producing a false positive for daphnia immobilisation. Due to this problem, the second definitive exposure was not run. However, the data generated from the first experiment was reported, as the decision was made to include imatinib in the simple mixture. This was to allow comparison with the algae mixture results.

The source of the bacteria was not discovered. The design of the kit does not call for autoclaving of vessels or the deionised water used to make up the daphnia Standard Freshwater. In any case, the flexible plastic multiwell plates would not be robust enough to be autoclaved. In addition, the ephippia would not be sterile and although the outer membrane is protective, it is unlikely it would protect the eggs from standard sterilisation techniques.

The experiment with hydroxycarbamide was repeated twice, but there were large variations observed between the repeats, in the percentage of immobilised daphnia in the third and fourth concentrations (200 mg/l and 400 mg/l). To help assess the variability, the exposure should have been repeated at least once more. The observed variation may have

been due to an error in making up the concentrated or dosing stocks during one of the runs of the experiment. A third run could have had the possibility of highlighting any inconsistencies between the first two. The raw data were checked to see if immobilisation was skewed to certain wells, possibly signifying an error in loading the medium. However, there was no significant difference between wells. In addition, analytical chemistry of the exposure medium would have identified any deviation from the nominal concentrations. Unfortunately, this was not conducted for any of the daphnia exposures, due to cost constraints. Biological variability is another possible causative factor, although the same batch of daphnia ephippia were used for all exposures.

The 5-fluorouracil experiment was repeated three times. The first two experiments were run with the same concentration series, but resulted in large variations in the amount of immobilisation observed. The highest concentration in the first experiment produced 100% immobilisation. However, in the second experiment, this concentration (1000 mg/l) only produced 40% immobilisation. The experiment was repeated with a series spanning a broader range of concentrations. The highest concentration was 5000 mg/l, but the percentage inhibition observed was 80%. A better design for the third experiment would have been to repeat it at the same concentration range as the first two. As with the hydroxycarbamide results, the observed differences may have been due to errors in making up the dosing or exposure stocks and/or in loading the wells and/or due to biological variability. In other studies, 5-fluorouracil is often dissolved in Dimethyl sulfoxide (DMSO). However other sources, including manufacturers' material safety data sheets (MSDS), state that it is dissolvable in water at the concentrations used. It was the experience of this author that vortexing for 15-30 minutes fully dissolved 5-fluorouracil, and that the drug stayed in solution for at least four days (the length of time between changing stock bottles in the zebrafish experiments). Careful observation of the concentrated stock is required during vortexing, to ensure the drug is fully dissolved before making up the dosing stock. As was seen in the algae experiments, the concentration-response of 5-fluorouracil was unusual, in that it spanned four orders of magnitude, rather than the two to three orders of magnitude observed for the other drugs. This relatively shallow curve indicates that toxicity increases at a slower rate with increasing concentration of 5-fluorouracil, when compared to the other drugs. However, why this should be the case is not known.

The experiment with cyclophosphamide was repeated, but with different concentration series. As before, a more robust design would have been to repeat the experiment using the same concentrations. However, broadly the same range was covered. There was some variation in the effects of the lower concentrations, for which a third repeat of this experiment would have been useful, to aid assessment.

Although chemical analysis of the daphnia exposure medium, to compare actual concentrations of the drugs to the nominal values was not conducted, this was carried out for 5-fluorouracil, cyclophosphamide and cisplatin in the zebrafish experiments (section 4.3.3). Variations in 5-fluorouracil concentration was within the range stipulated by the OECD protocol (OECD, 1984), cisplatin was outside but within an acceptable difference and cyclophosphamide saw a large reduction in actual concentration, that increased with increasing nominal concentrations. As the same protocol for making up the dosing stocks of the drugs was the same between the two species, this gives some indication of the actual concentrations the daphnia were exposed to.

In addition to the possible causes for variation described above, daphnia immobilisation is a relatively imprecise endpoint. Each daphnia represents five percent, whereas the same value in the algae assay is comprised of many more individual organisms. Due to factors such as biological variability (e.g. an individual's osmoregulation ability) or disease, some individuals can be more susceptible to a toxicant than others. This would have a larger impact in the daphnia results, than in the algae experiments. Nevertheless, this protocol has been strenuously tested and ratified by the OECD, so the results can be considered reliable in this context.

With regards to the accuracy of the EC_x calculations, given that the concentration-response curves calculated in Figure 18 are non-linear, it may be that another method would be more accurate. For example, instead of fitting a linear equation to semi-logged data it could be more accurate to fit a non-linear model to linear data. However, due to time constraints, it has not been possible to follow this line of reasoning further.

3.4.2 Daphnia immobilisation and the effects of the target cytotoxics in the literature

Several studies on the effects of some of these drugs on *D. magna* were found in the literature. The findings of these studies are summarised in Table 16, in comparison with the results generated for this thesis.

Table 16: Comparison of EC_{10} and EC_{50} values from the literature with those calculated in this study for immobilisation of D.magna. The Sanderson et al. (2003) results were calculated by the ECOSAR model, rather than calculated experimentally. Those results marked OECD 211 are from the Daphnia 21 day reproduction assay.

			Literature values (µg/l)		Thesis values (µg/l)	
Drug	Species	Author	EC_{10}	EC_{50}	EC_{10}	EC_{50}
cisplatin	*D. magna*	(Zounková *et al.*, 2007)	-	640	350	1 650
		(Parrella *et al.*, 2014)	-	940		
imatinib	*D. magna*	(Parrella *et al.*, 2014)	-	11 970	17 930	65 780
hydroxycarbamide	*D. magna*	(Bristol-Myers Squibb Company, 2010)	>100 000 (NOEC)	>100 000	88 540	213 450
carboplatin	No results found in the literature				110 270	266 920
5-fluorouracil	*D. magna*	Cleuvers 2001 in (Straub, 2010)	1 300	25 000	44 430	319 240
		(Zounková *et al.*, 2007)	-	36 000		
		(Zounkova *et al.*, 2010)	-	15 000.00 (5 200 – 45 000)		
		(Parrella *et al.*, 2014)	-	20 840		
cyclophosphamide	*D. magna*	(Sanderson *et al.*, 2003)	-	1 795 000 (ECOSAR)	1 025 110	2 318 220
		(Grung *et al.*, 2008)	-	>56 000 (OECD 211)		

		(Zounková *et al.*, 2007)	-	>1 000 000		

3.4.2.1 Cisplatin

Two studies considered the effects of cisplatin on *D. magna*. As part of the same set of experiments that examined *R. subcapitata*, Zounková *et al.* (2007) calculated an EC_{50} for *D. magna* of 640 µg/l, more than two and half times lower than in this study. The study used a Czech protocol, claimed to be identical to European Standard EN ISO 6341:1996. From the details (length of study, number of organisms used) given in the paper, the European Standard protocol would appear to be analogous to the OECD 202 Daphnia protocol. However, it has since been revised, the 1996 version is no longer available and there is a charge to download the latest version. It is therefore not possible to assess factors that may impact toxicity, such as the medium recipe, although it is likely the same medium was used. Parthenogenic clones cultured in the laboratory were used for the exposure. EC_{50} values were estimated using the US Environmental Protection Agency Probit software. No chemistry results were reported, so it was not possible to assess the actual vs nominal cisplatin concentrations. However, Zounková *et al.* (2007) also tested 5-fluorouracil and cyclophosphamide and found cisplatin to be the most toxic to *D. magna*, as did this study.

The Parrella *et al.* (2014a) experiment generated an EC_{50} value of 940 µg/l, nearly two times less than the one calculated in this project. The exposures were run with both parthenogenic laboratory cultured daphnia and sexually reproduced daphnia hatched from ephippia. No significant difference was observed between these two groups, giving some confidence that the use of ephippia in this study would not have compromised the comparability of results between experiments. OECD 202 protocol was used with the same standard freshwater medium as this study. Non-linear regression was used to calculate the EC_{50}, which may account for the difference. Parrella *et al.* (2014a) also conducted the chronic OECD 211 *D. magna* 21-day reproduction test, which produced an EC_{50} of 1.63 µg/l and an EC_{10} of 0.25 µg/l, which are within the concentration range measured in hospital effluents, but still 1-2 orders of magnitude higher than the PEC for surface waters (Table 15).

Notably, chemical stability analysis of cisplatin was conducted using a UV-Vis spectrophotometer. Readings of an aliquot of daphnia freshwater containing 1 mg/ml cisplatin were taken every ten minutes for eight hours. Parrella *et al.* (2014a) claimed the results showed a decrease in cisplatin concentration, until a stable concentration was observed after eight hours. However, there was insufficient data in the paper to assess this claim. In addition, the aliquot did not contain daphnia, so the impact of the organism, if any, was not assessed. Finally, no chemical analysis was conducted of actual vs nominal cisplatin concentrations in the experiment.

Despite the difference in concentration between the EC_x values found in the literature and the values calculated in this project, all three values are around 1 mg/l, far higher than measured or predicted environmental concentrations.

3.4.2.2 Imatinib

Parrella *et al.* (2014a) was the only study found in the literature that assessed the effect of imatinib on *D. magna*. The EC_{50} was 11 970 µg/l, five and half times lower than in this study; lower even than the EC_{10} by 6 000 µg/l. However, as described previously, the imatinib results from this study were included for comparison purposes only, as the bacterial growth was believed to have compromised the exposure. No mention was made in the Parrella paper with regards to bacterial contamination. Any sterilisation procedures undertaken for the equipment, reagents and/or organisms were also not described.

The Parrella *et al.* (2014a) 21-day reproduction exposures produced an EC_{50} of 308 µg/l and an EC_{10} of 8.34 µg/l, both many orders of magnitude higher than the PEC for this drug (Table 15).

3.4.2.3 Hydroxycarbamide

No independent studies into the effects of hydroxycarbamide on *D. magna* were discovered within the literature. EC_{50} and NOEC values were found in a Material Safety Data Sheet (MSDS) (Bristol-Myers Squibb Company, 2010). However, these were both higher than the highest concentration tested of 100 000 µg/l. The EC_{10} value calculated in this study was lower, at 88 540 µg/l. The MSDS contained no details of how the exposure was

conducted, so it is not possible to conduct further comparison and assessment of these results. Nevertheless, both studies are in agreement that very high concentrations of hydroxycarbamide are required to immobilise daphnia.

3.4.2.4 Carboplatin

No *D. magna* experiments using carboplatin were found in the literature. Although it has the same mechanism of action as cisplatin, it was observed to be far less toxic to daphnia in this study. This may be due to the additional metabolism step required for carboplatin, which is beneficial in its therapeutic context as it reduces or even eliminates some of the severe side effects associated with cisplatin (side effects described in section 1.4.3). It is not known if daphnia have the required enzymes to carry out the additional metabolism, but this ability, or lack of, could be a factor in the reduced toxicity observed in this study.

3.4.2.5 5-fluorouracil

5-fluorouracil was the most represented drug in the literature. All results found were an order of magnitude lower than those calculated in this project. Four studies were found with results ranging from 15 000 µg/l to 36 000 µg/l. As with the algae exposures, it was not possible to assess the Cleuvers (2001) experiment (EC_{50} = 25 000 µg/l), as the original paper was in German. The Zounková *et al.* (2007) results (EC_{50} = 36 000 µg/l) were obtained under the same conditions as cisplatin (3.4.2.1), so the same arguments apply to 5-fluorouracil.

The Zounková *et al.* (2010) experiments used the same protocol as Parrella *et al.* (2014a), although a different medium was selected (Aachener Daphnien Medium). This medium was also designed to represent natural freshwater. Whether this had the potential to impact toxicity, as was found for algae medium (Millington *et al.*, 1988), is unknown. Concentrated stocks of 1 g/l were made up in water. Whether this was the standard freshwater is unclear. However, the use of distilled or deionised water was unlikely to be an issue, considering how dilute the exposed concentrations would be. Chemical analysis was conducted and minor declines of < 15% were reported during the exposure period. EC_{50} values were estimated using the US Environmental Protection Agency Probit software. Notably all the drugs exposed to *D. magna* in the Zounková *et al.* (2010) paper generated EC_{50} values with

large amounts of variation. For 5-fluorouracil it was 15 000 µg/l ± 5 200 µg/l – 45 000 µg/. In comparison the algae exposure in the same study generated an EC_{50} of 48 000 µg/l ± 44 000 µg/l – 51 000 µg/l. The large variation was not discussed in the paper. However, this is in common with the variation in this study, as discussed in section 3.4.1. Parthenogenic clones cultured in the laboratory were used for the exposure. As with the algae exposures, Zounková *et al.* (2010) also exposed daphnia to the 5-fluorouracil metabolite α-fluoro-ß-alanine, but no response was observed at the highest concentration of 100 000 µg/l.

Parrella *et al.* (2014a) also exposed daphnia to 5-fluorouracil (EC_{50} = 20 840 µg/l), dissolving it in DMSO to make the concentrated stock solution. The paper stated that a DMSO control was included, but no further information or results were provided as to how this fared in comparison to the standard freshwater control. Chemical analysis showed that 5-fluorouracil was very stable under test conditions, with actual concentrations varying from nominal values by < 10%, although the direction of variation was not reported. As with the other drugs, no significant differences were observed between the asexual and sexually reproduced daphnia. In contrast to this project and the results of the Zounková *et al.* (2010) paper, less variation was seen in the results of the Parrella *et al.* (2014a) study at 18 070 µg/l – 24 040 µg/l around the EC_{50} value.

Both Zounková *et al.* (2010) and Parrella *et al.* (2014a) conducted the *D. magna* reproduction assay. The former generated an EC_{50} of 100 µg/l and the latter an EC_{50} of 26.40 µg/l and an EC_{10} of 4.60 µg/l. All of these results are within the MEC range found in hospital effluents; however, they are all many orders of magnitude higher than the more broadly environmentally relevant PEC for surface waters of 0.00265 µg/l (Table 15).

3.4.2.6 Cyclophosphamide

Only Zounková (2007) experimentally calculated an EC_{50} value using the daphnia acute immobilisation test. However, no immobilisation was observed at the highest concentration tested of 1 000 000 µg/l, just below the EC_{50} of this project of 1 025 110 µg/l. This seems to be a large difference, but no reasons for this could be identified in the paper. As with their 5-fluorouracil experiment, no chemical analysis was reported. Parthenogenic clones, cultured in the laboratory, were used, compared with sexually reproduced eggs

hatched from ephippia in this project. However, Parrella *et al.* (2014a) found no difference between these two life stages, for the cytotoxics they tested. Although it is possible that a different medium recipe was used, it seems unlikely that this would generate such a large difference in the results.

Grung *et al.* (2008) carried out the OECD 211 daphnia reproduction test, but found no immobilisation at the highest concentration tested of 56 000 µg/l. As can be seen from the other reproduction test results reported above, this protocol tends to be more sensitive to cytotoxic drugs than the acute assay. It can be expected that the EC_{50} for the reproduction assay would be lower than for the immobilisation test. However, further testing would be required to assess this possibility for cyclophosphamide.

The only other relevant data found in the literature were an EC_{50} value generated by the ECOSAR software model of 1 795 000 µg/l (Grung *et al.*, 2008). Given that this model makes assumptions about the toxicity and environmental concentrations of the target chemical, and are conservative by design, a larger value than the EC_{50} value calculated in this study is not unexpected.

3.4.2.7 Conclusions from the experiments with the single drugs

The null hypothesis states that: “The selected cytotoxic pharmaceuticals, at the tested concentrations, will not immobilise daphnia”.

For all the drugs tested, it was not possible to reject the null hypothesis.

None of the drugs tested appear to cause daphnia immobilisation at concentrations above those found in surface waters in developed countries.

Waste from drug manufacturing in low and middle income countries has the potential to contain cytotoxic drugs. If these were to occur at the same orders of magnitude as the drugs measured in the literature, cisplatin, and potentially also imatinib could be present at concentrations shown to immobilise daphnia in this study. 5-fluorouracil may also be an issue, due to the reasons outlined below.

Although there was some variation between the results of this project and those found in the literature, they all supported the conclusions above; apart from 5-fluorouracil. Given the issues outlined with the 5-fluorouracil experiments in this study, they could not be considered robust. The EC_{50} values for this chemical in the literature were an order of magnitude less than those obtained from this project, and were very similar to each other. The balance of probabilities would suggest that the literature values are more accurate.

3.4.3 Mixtures experiments

The simple mixture, made up of five drugs at one fifth their respective EC_{50} concentrations, caused immobilisation one and a half times greater than the sum of the single chemicals (87.5% vs 35%). This could be described as synergistic behaviour, meaning that interactions between the drugs increasing the toxicity of the mixture above the sum of their individual actions. As described in section 1.5, when administered to patients, anti-cancer drugs are often given in combination, as their combination actions can enhance their tumour supressing abilities. The simple mixture was run in quadruplicate and repeated, although the repeats were run at the same time, due to time constraints and dosing stocks being made up from the same concentrated stock. Ideally the second experiment would have been run separately, in case any errors were made in the setup of the first. However, there was little variation between the two simple mixture repeats. This suggested that if the experiment was run correctly, the simple mixture results were representative of the mixtures effects on daphnia mobility.

None of the simple mixture component drugs were at concentrations within the range of the PEC/MEC values found in the literature (Table 11). The cisplatin value is similar to the EC_{10} concentration calculated in the single drug experiment and the arguments made regarding environmental relevance are therefore the same (section 3.4.1). All other drugs were used at concentrations many orders of magnitude higher than any of the environmental scenarios discussed.

Table 17: A comparison of the ⅕ EC_{50} and ⅕ ½LOEC used in the simple and 'something from nothing' mixtures with the PEC and MEC concentrations. All values are µg/l. a Straub (2010), b Martín et al. (2011), c Mahnik et al. (2004), d Besse et al.(2012) * Conservative PEC as refined PEC not available, e Rowney et. al. (2009) † Combined PEC with 4 other cytotoxic drugs, e f Kümmerer & Helmers (1997), g Vyas et al. (2014), h Buerge et al. (2006), i Mark-Kappeler et al. (2011)

				MEC range	
Drug	⅕ EC_{50}	⅕ ½LOEC	PEC	Hospital effluent (untreated)	Surface Waters
cisplatin	300	30	0.00697 – 0.01072†e	0.021f – 100g	-
imatinib	13 000	5 400	0.00499d*	-	-
hydroxycarbamide	43 000	5 000	0.078d	-	-
5-fluorouracil	64 000	3 600	0.00265a	2.0b – 122c	-
cyclophosphamide	205 000	10 000	0.00697 – 0.01072e	0.0045f	0.00005h - 0.0648i

Based on the results of the simple mixture experiment, this mixture displayed apparent combination effects, with the drugs in combination appearing to enhance their ability to immobilise daphnia. However, without proper modelling and a more robust mixture experiment design, it is not possible to discuss this further.

The 'something from nothing' mixture caused no immobilisation, and neither did the drugs at their respective half LOEC concentrations, when exposed singly. The intention of the design was to use single drug concentrations that on their own produced no measurable response in the endpoint, but in combination could, thus displaying a 'something from nothing' outcome. The fact that no observable response occurred could be due to the single chemical concentrations being too low. However, other factors may be more influential on the observed outcome. These are discussed in more detail below.

3.4.3.1 Simple mixture experimental design

As described in section 2.2.2, the aim of this mixtures design was to choose concentrations of the individual drugs that are equipotent to each other. The suggestion made there was that the EC_{10} value may have been more suitable, rather than one fifth of the EC_{50} value. The full argument for this thinking is laid out in that section, but it was based in part on a

comparison of the EC_{10} values with the one fifth EC_{50}s. A trend was noted between the level of growth inhibition of the algae and the differences between these two values. This trend was not repeated in the daphnia immobilisation simple mixture experiment. To follow the proceeding discussion, the data it is based on is contained in Table 12.

The EC_{10} and one fifth EC_{50} values for cisplatin were roughly the same. However, the percentage of daphnia immobilised was only five percent, against an expected ten percent. These two values were also broadly similar for imatinib, but no immobilisation was observed in the single chemical component of the simple mixture experiment. Whilst ten percent immobilisation was observed for hydroxycarbamide, the EC_{10} value was twice the one fifth EC_{50}, a scenario in which the expected immobilisation would be nearer five percent. The cyclophosphamide one fifth EC_{50} concentration was five times smaller than the EC_{10}, yet five percent immobilisation was recorded, much higher than would have been expected if the trend seen in the algae had been observed in the daphnia. Only the 5-fluorouracil values and percentage immobilisation followed the trend seen in the algae growth inhibition simple mixture experiment.

Table 18: Comparison of the EC10 and EC50 calculated for the daphnia single chemical exposures and the ⅕ EC50 concentrations (µg/l) used to make the simple mixture exposure.

		Simple Mixture	
Drug	EC_{10}	⅕ EC_{50}	% immobilised
cisplatin	350.00	300.00	5
imatinib	17 930.00	13 000.00	0
hydroxycarbamide	88 540.00	43 000.00	10
5-fluorouracil	44 430.00	64 000.00	15
cyclophosphamide	1 025 110.00	205 000.00	5

As was discussed in section 3.4.1, there were several issues with the running of the single drug exposures, which cast some doubt on the accuracy of the EC_x values calculated. In addition, it was noted that due to the small number of organisms in this assay, percentage

immobilisation was a relatively inaccurate endpoint. It is possible that the inconsistencies described above could be attributed to these factors, to a lesser or greater extent.

3.4.3.2 'Something from nothing' mixture experimental design

As with the algae experiments, not all the daphnia single chemical experiments generated NOEC concentrations. In order to design a 'something from nothing' mixture the decision was made to use a concentration at half the LOEC, in order to estimate a concentration that singly might elicit no response in the endpoint tested. The results would suggest that this has been achieved. Unfortunately, it was not possible to conduct chemical analysis, so this cannot be empirically tested. No immobilisation was seen in the 'something from nothing' mixture which raises some questions, given that the simple mixture experiment appeared to show that the drugs acted in combination to increase toxicity.

Firstly, were the LOEC values used too low? Ideally single drug experiments would be conducted that provide a higher resolution of the LOEC/NOEC boundary. However, this was not possible. Examining the percentage immobilisation caused by the available LOECs, showed that for cisplatin, hydroxycarbamide and cyclophosphamide these values were as low as possible for this end point, as five percent immobilisation corresponds to one daphnia (Table 19). Imatinib and 5-fluorouracil caused higher percentages of immobilisation, at twenty-five and ten percent respectively. For these two drugs there was still the potential for their half LOEC values to cause immobilisation, particularly for imatinib. For a sigmoidal curve, which the concentration-response curves for the daphnia experiments broadly resemble, a change on the x axis at the bottom of the curve represents a much smaller change on the Y-axis. Given both these factors, it is possible that at least some of the LOECs were not low enough and that some immobilisation could be expected. However, this was not observed.

Table 19: LOEC and the percentage immobilisation they caused in the daphnia single chemical exposures.

Drug	LOEC	% immobilisation
cisplatin	300	5
imatinib	54 000	25
hydroxycarbamide	50 000	5
5-fluorouracil	36 000	10
cyclophosphamide	1 000 000	5

Another area of concern is the accuracy of the LOEC values. Given the issues described for the single drug exposures in section 3.4.1, there was a good possibility that they were not accurate for some, if not all of the drugs. As for the single drug exposures, with the existing data it was not possible to assess this further. A further issue discussed in section 3.4.1, was the relative inaccuracy of the daphnia immobilisation endpoint and its susceptibility to chemical and/or biological variability. This potential problem could have made it difficult to design a robust 'something from nothing' mixture experiment.

Finally, all calculations were based on nominal concentrations. Given the results of the aquarium water analysis from the zebrafish experiments (Chapter 4), it is unlikely that the actual concentrations were always the same as the nominal. This would have generated inaccurate EC50s and LOECs.

3.4.3.3 The effects of cytotoxic mixtures reported in the literature

Only one study containing the target drugs and using *D. magna* was found in the literature, although it used the OECD 211 reproduction protocol. Parrella *et al.* (2014b) created binary mixtures of cisplatin, imatinib and 5-fluorouracil, using the results of the Parrella *et al.* (2014a) study. In contrast to this study and the binary mixtures study conducted on *R. subcapitata* by Brezovšek *et al.* (2014), where the mixtures are made up of EC_x values divided by the number of drugs in the mixture, a more complex design was used. Due to the different mechanisms of action of the drugs, the Independent Action (IA) model was selected as the basis for the design. The intention was to have equal effects of the drugs

both singly and in combination. Parrella et al (2014b) then goes on to describe the use of isobolograms to determine these concentrations based on the work of Tallarida (2006). It is assumed that this is for the drugs singly and in binary mixtures, as different concentrations are used in the two scenarios (see x axes in Figure 21). Finally, based on the concentration-response data from the individual drugs, two "optimally discriminating" endpoints of 31.6 and 63.3 percent were chosen, using a log logistic function $R(x) = A(x/\mu)^{\beta}/(1+(x/\mu)^{\beta}$. It is not clear from the paper how this equation was formulated; whether it was based on the data produced from the isobolograms or from the data in Parrella *et al.* (2014a). It is also not clear why these two percentages are optimally discriminating. This lack of clarity may be due to the inexperience of this thesis author in the design of mixtures experiments. However, with this inexperience in mind, this design would appear to be more robust than the one used in this study. It would appear to take into account the differences in the slopes of the individual concentration-response curves. It also provides two endpoints (31.6 and 63.3 percent), rather than the single endpoint of one fifth the EC_{50} used in the simple mixture experiment in this project, thus providing greater resolution of the organism's response to the individual drugs and binary mixtures.

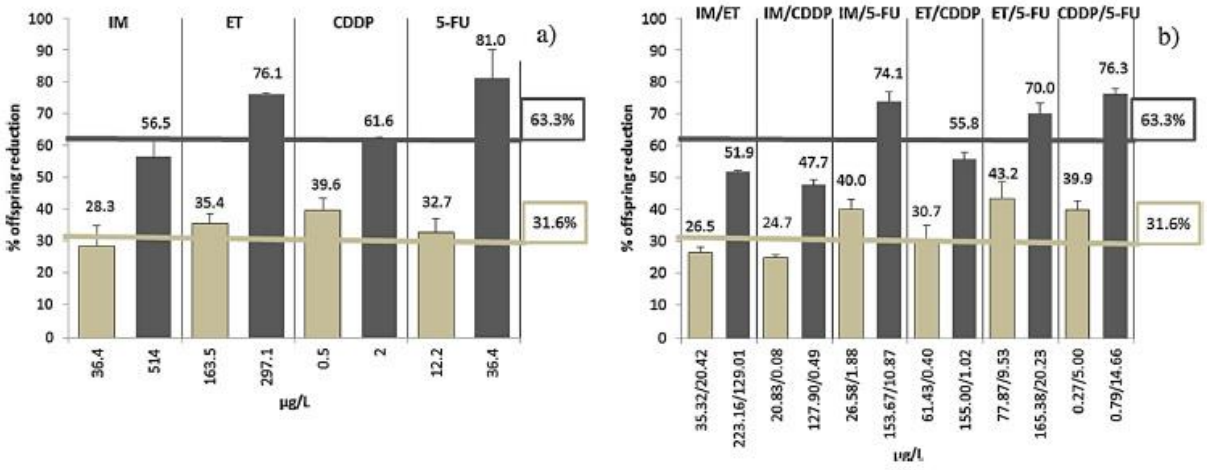


Figure 21: Results of a 21-day D. magna reproduction assay from Parrella (2014b). Left: Percentage of offspring produced compared to the controls for daphnia exposed to imatinib (IM), Etoposide (ET), cisplatin (CDDP) & 5-fluorouracil (5-FU) singly at concentrations predicted to cause 31.6 and 63.3 percent reproduction inhibition. Right: Percentage of offspring produced compared to the controls for daphnia exposed to different binary mixtures of the four cytotoxic drugs, at concentrations predicted to cause 31.6 and 63.3 percent reproduction inhibition.

The concentrations calculated for the single chemicals corresponded relatively well with their target reproduction inhibition percentages. Parrella *et al.* (2014b) conducted statistical analysis of the single chemicals vs the binary mixtures in which they were

components. They found that for all but imatinib/Etoposide and imatinib/cisplatin, their interactions were best represented by Independent Action. These two binary mixtures were considered to have antagonistic interactions. The higher than predicted results for the mixtures containing 5-fluorouracil were attributed to the higher than predicted results for the single 5-fluorouracil exposure. The authors reported variability in the 5-fluorouracil results between this study and the one reported in Parrella *et al.* (2014a). However, as noted previously, their intra-study variability was far less than in this study. The antagonism or independent action described in these papers does not support the strong combination actions observed in the simple mixture experiment in this project. However, this is a mixture of five drugs in an acute exposure so it is uncertain how comparable these findings could be.

3.4.3.4 Mixtures conclusions

The null hypothesis states that: "The selected cytotoxic pharmaceuticals, as mixtures tested, will not immobilise daphnia".

For either mixture design, it was not possible to reject the null hypothesis.

Although the results would suggest the five chemicals display either additive or a combination action when combined in a mixture at their one fifth EC_{50} values, issues with the experimental design would require refinement and retesting to confirm this. It is not possible to say whether the results of the 'something from nothing' mixture accurately reflect their behaviour at the nominal concentrations, as there were issues with the single chemical experiments from which these nominal concentrations were derived.

Based on the current data, it would appear that the mixtures effects of these drugs at environmentally relevant levels is not of concern. However, the strong combination action observed in the simple mixture, underlines that single chemical testing alone is not capable of understanding the environmental implications of a given drug.

4 Impact of cytotoxic drugs on zebrafish morphology & blood

4.1 Introduction

The third group of aquatic species to be assessed were the vertebrates. This group was represented by the freshwater teleost *Danio rerio*, commonly known as the zebrafish. This Cyprinid species is a native of the southeast Himalayas but, due to its small size, hardy nature and ease of breeding, is a popular aquarium species (Spence *et al.*, 2008). These features also lend it to scientific research, and as such it is one of the approved freshwater fish species defined in OECD 203 "Guideline for testing chemicals" (OECD, 1992), cross referenced from OECD 204 204 "Fish, Prolonged Toxicity Test 14-Day Study" (OECD, 1984). In addition, this species' genome has been mapped, allowing the identification and use of genes involved in DNA damage repair, as potential end points for molecular analysis (See Chapter 5).

Given the impact of anticancer drugs on rapidly replicating cells, it could be argued that testing zebrafish at the larval rather than adult life stage would be preferable. This is due to the greater rate of cell replication during the rapid growth that is experienced by the larval life stage. However, adult zebrafish were used as one of the partners on the PHARMAS project were carrying out experiments on larvae. The intention was to compare the results between the adults and the larvae. However, this was not possible due to time constraints.

4.1.1 Aims

- Assess non-lethal effects of 5-fluorouracil, cisplatin and cyclophosphamide on zebrafish morphology and blood, at concentrations analogous to those found in the environment and with reference to the results of the algae and daphnia exposures.
    - Fish to be exposed to individual drugs in a concentration series and to a simple mixture of the cytotoxic drugs, the concentrations of the latter being the median concentrations from the single chemical concentration series.

4.1.2 Hypothesis

This hypothesis has been condensed for conciseness, morphology and blood will be considered separately in the following sections.

“The selected cytotoxic pharmaceuticals, at the tested concentrations, will cause significant effects on adult zebrafish morphology/blood”.

4.2 Methods & materials

OECD 204 “Fish, Prolonged Toxicity Test 14-Day Study” (OECD, 1984) defines the testing procedure for determining the effects of a substance on adult freshwater fish. Batches of freshwater fish are exposed to a concentration range of a test substance for a period of 14 days. The fish are housed in aquaria with a flow through system that constantly refreshes the tank water. Light, temperature, pH and water hardness are maintained within defined parameters. The test substances are fed into the aquaria at a constant rate, to ensure the fish are continuously exposed to the required concentrations. At the end of this period the fish are killed and, depending on the desired end points, tissue samples are collected and morphological measurements such as weight and length recorded. Values such as EC_x, LOEC and NOEC are then calculated using the results of the tissue and/or morphological data analysis. This protocol is described in more detail in the proceeding sections.

4.2.1 Exposure of zebrafish to cytotoxic drugs

The tests were run in accordance with the requirements of OECD 204 (OECD, 1984). Brunel has a dedicated aquatic environmental toxicology facility that enables the test environment to be configured and maintained as described below.

The fish were sourced as defined in Table 20. Prior to exposure the fish were housed in mixed sex holding tanks, within specified per litre carrying capacities for this species, for at least 14 days. The tank conditions were maintained within the criteria defined in Table 21.

Table 20: Strains, ages, sources and sex ratio per tank for the zebrafish used in the single chemical and mixture experiments. The TL strain had to be used for the mixture experiment as no WIK strain could be sourced within the relevant timeframe. The 5:3 ratios for the cyclophosphamide series were due to a shortage of female fish. The concentrations of the drugs used in each experiment can be found in Table 22.

Exposure & date (MM/YY)	Fish supplier	Strain	Age at Exposure	Planned sex ratio per tank (M:F)	Actual sex ratio per tank (M:F)	# tank replicates
1st Pilot 03/13	University College London	WIK	~12 months	6:6	6:6	1
5-fluorouracil series 06/13	In house – bred from UCL stock	WIK	~10 months	6:6	6:6	2
5-fluorouracil repeat 08/13	University College London	WIK	~12 months	6:6	6:6	1
cyclophosphamide series 12/13	AZ Brixton Laboratory	WIK	~12 months	5:5	5:3	3 control, 2 per conc.
cisplatin series 08/14	University College London	WIK	~18 months	5:5	5:3	3 control, 2 per conc.
Mixtures 09/14	University College London	TL	~12 months	5:5	Single 7:3 Mix 5:5	3 control, 2 per conc.

The WIK strain was used as it was the wild type strain that was available at the time from the supplier. An alternative wild type strain, TL, was used for the last exposure as the supplier did not have sufficient WIK fish available. Although the two strains exhibit different phenotypes (Figure 22) and the literature suggests caution in using different strains (Guryev *et al.*, 2006), they were expected to be comparable for the purposes of this study. This was due to the Mechanisms of action (MOA) of the drugs tested, which in some way directly damage DNA, a molecule that will not differ between strains. In addition, the use of different strains should not affect the molecular analysis reported in Chapter 5, as the DNA repair enzymes have been shown to be highly conserved between different species of fish and therefore were thought not likely to differ between stains of zebrafish.

Figure 22: The two zebrafish strains used in this study. On the left hand side are the female (top) and male (bottom) of the WIK strain. On the right hand side is a TU strain fish, possibly female.

Power analysis was conducted (using software package GPower v3.1.7) to calculate the minimum number of fish required to be statistically significant (Table 20). A priori calculations specified a sample size of six fish per sex. Subsequently, using the data from the first pilot study and altering the input criteria on the advice of a biostatistician (Power reduced from 95% to 80%), sample size was reduced to five fish of each sex. An uneven sex ratio was used for the cyclophosphamide series exposure due to an accident in the holding tank, which led to the death of a large portion of the female fish. An even sex ratio was planned for the cisplatin exposure (Table 20). However, their condition at the time of the experiment made sexing difficult, as the females were thinner than typically observed, possibly due to the age of the fish. Although it was thought that the fish were assigned evenly, when it came to sampling at the end of the experiment there were a greater number of females in the controls and each concentration. In addition, there was a shortage of male stock fish that led to their only being four male fish available for two of the control tanks and one of the 100 µg/l tanks. An even sex ratio was also planned for the

mixtures exposure, but the TL strain fish that were received from the supplier proved very difficult to sex based on physical appearance (Table 20). Although it was thought that the fish were assigned evenly, when it came to sampling at the end of the experiment there were a greater number of females in the controls and each concentration, apart from the mixtures tanks. The difficulty in sexing the fish may have been an issue with this particular batch of fish as, although sufficient fish of each sex were ordered; the final sex ratio observed meant that more female fish had been delivered than male. It would therefore appear that the suppliers also found the fish difficult to sex accurately.

The specific criteria for the tank parameters and their values can be found in Table 21. Seven days before exposure to the drugs began, the fish were moved into holding tanks that mirrored exposure conditions (Figure 23). These were twelve litre glass tanks, sealed with silicon and with lids of polycarbonate plastic, to reduce evaporation and prevent the fish from jumping out. Oxygenation was provided by a central sponge filtered air pump feeding to each tank via silicon tubing terminated with a steel weight and a porous stone air stone. Water was gravity fed from header tanks, which were themselves fed by carbon filtered mains water and heated to the required temperature range (Table 21 and Figure 23). Where possible each aquarium was fed via a flow meter (Figure 25), which for all studies was set to a rate of 12 litres per hour. Due to there being a fixed number of flow meters in the exposure room, for the cyclophosphamide and cisplatin studies, one control tank had to be fed directly from the header tank, with the flow rate manually monitored daily. As the mixtures study had two additional aquaria, all three control aquaria were fed directly from the header tank. Only minor variations in flow rate were observed for these aquaria.

Table 21: The environmental requirements for the zebrafish aquaria during the holding, acclimatisation and exposure phases of the studies.

Experiment environment criteria	Required Values
Water quality	Carbon filtered tap water
Water temperature	26-28 °C
pH	6.0 – 8.5
Dissolved O_2 concentration	60 – 80 % air saturation value
Hardness	50 – 250 mg $CaCO_3$ per litre
Light	12-hour daily photoperiod

Temperature, pH and dissolved O_2 concentration were measured and recorded daily using a handheld electronic probe (Hache, HQ40d). Hardness and ammonia levels were measured weekly using dip sticks (API, 5 in 1 Aquarium Test Strips and API, Test Strips Ammonia).

Figure 23: Adult zebrafish 14-day exposure room setup. (a) Header tanks containing carbon filtered mains tap water feeding to (b) individual flow meters and tank feeds. (c) Temperature controls for header tanks. (d) Air pump leading to (e) individual tank air feeds. (f) Pre-study 7-day acclimatisation tanks. (g) 14-day exposure tanks. (h) Individual tank out flows, draining to mains sewerage system.

Due to the duration and cost of the adult fish experiments, only three drugs were tested both singly and as a simple mixture. These were 5-fluorouracil, cyclophosphamide and cisplatin, which were selected due to their usage (section 1.3.1), cost to purchase and the results of the algae (section 2.3) and daphnia (section 3.3) tests. The United Kingdom Home Office Project Licence (70/7023) and Personal Licence (30/9555) under which these tests were conducted limits concentrations of test chemicals to a couple of orders of magnitude above environmentally relevant levels. The environmental concentrations and literature sources for these are listed in Table 22. The concentration range for the main cyclophosphamide study was supposed to be 10, 100 and 1000 µg/l, but a mistake was made in the dosing calculations, leading to a doubling of the concentrations. This was discovered half way through the exposure but, as there were no mortalities or signs of acute effects in the exposed fish, the study was completed at these concentrations.

Table 22: Test chemicals, the test concentration ranges and measured environmental concentrations and their literature sources for the adult zebrafish exposures. CAS number, supplier and supplier name for the test chemicals are as for algae section 2.2. The measured environmental concentrations in the µg range were taken in hospital effluents, those in the ng range, were measured in surface waters. A more detailed description of these measurements can be found in Table 4.

Drug	Exposure	Concentration range µg/l (fold change)	Measured environmental concentrations	Source
5-fluorouracil	1st Pilot	100	20 – 122 µg/l	(Mahnik *et al.*, 2004)
	Series	4, 20, 100, 500 (5)		
	Repeat	100, 500 (5)		
cyclophosphamide	1st Pilot	100	0.05 – 0.17 ng/l 46.1 – 64.8 ng/l 20 – 4500 ng/l	(Buerge *et al.*, 2006; Zaharie, 2006; Steger-Hartmann *et al.*, 1997)
	Series	20, 200, 2000 (10)		
cisplatin	1st Pilot	10	125 ng/l	(Kümmerer and Helmers, 1997)
	Series	1, 10, 100 (10)	100 µg/l	(Vyas *et al.*, 2014)
Simple Mixture		5-fluorouracil 100µg/l cyclophosphamide 200µg/l cisplatin 100 µg/l	As above	As above

A NOTE ABOUT THE SIMPLE MIXTURE DESIGN

As there were no concentration-response effects observed in the morphology and blood endpoints in the single drug exposures, it was not possible to design a robust mixtures experiment. Instead a simple mixture design was used, where either a concentration that had been measured/predicted in the environment (5-fluorouracil and cisplatin) or the median concentration from the single drug experiment (cyclophosphamide) was selected. Fish were then exposed to both the single drugs at these concentrations and a mixture of these drugs at these concentrations.

Stock solutions of the test chemicals were made up every four days. The drugs were dissolved in ddH_20, either by hand shaking or stirring on a magnetic stirrer, at a concentration that will achieve the desired concentration once diluted in the exposure tanks. These were fed by a peristaltic pump (Figure 24) into mixing vessels, along with the filtered mains water supplied by the flow meters. The mixing vessels then fed into the aquaria (Figure 25). The rate of flow from the peristaltic pump and flow meters were monitored daily and adjusted so that the required concentrations of chemical were continually fed into the aquaria.

Figure 24: Delivery system for drug dosing. (a) 2.5L brown glass (to reduce photolysis of drug) bottles with foam stoppers to reduce evaporation and hold tubing in place. This photograph was taken during the mixtures experiment, so the stock bottle on the right contains the three drugs at the same concentration as their individual stocks. In each experiment the control tanks were dosed with ddH_20 (stock bottle back left). All replicates at a given concentration and control are fed from one dosing stock bottle. (b) Each stock bottle sits on a magnetic stirrer and the stock solution is kept in motion with a magnetic stirrer bar, to prevent the drug from coming out of solution. (c) The stock solutions are fed to the mixing vessels via a peristaltic pump. This works by massaging narrower gauge tubing with rollers rotating in the direction of flow. This keeps the stock solution within a "closed" circuit, reducing the chance of both cross contamination of dosing stocks and contamination of pumping equipment. (d) Control panel for setting rotation rate for approximate dosing rate. (e) Individual adjustment knobs to fine tune flow rate to each exposure tank. Further tubing (not visible above) continues to individual mixing vessels (Figure 25).

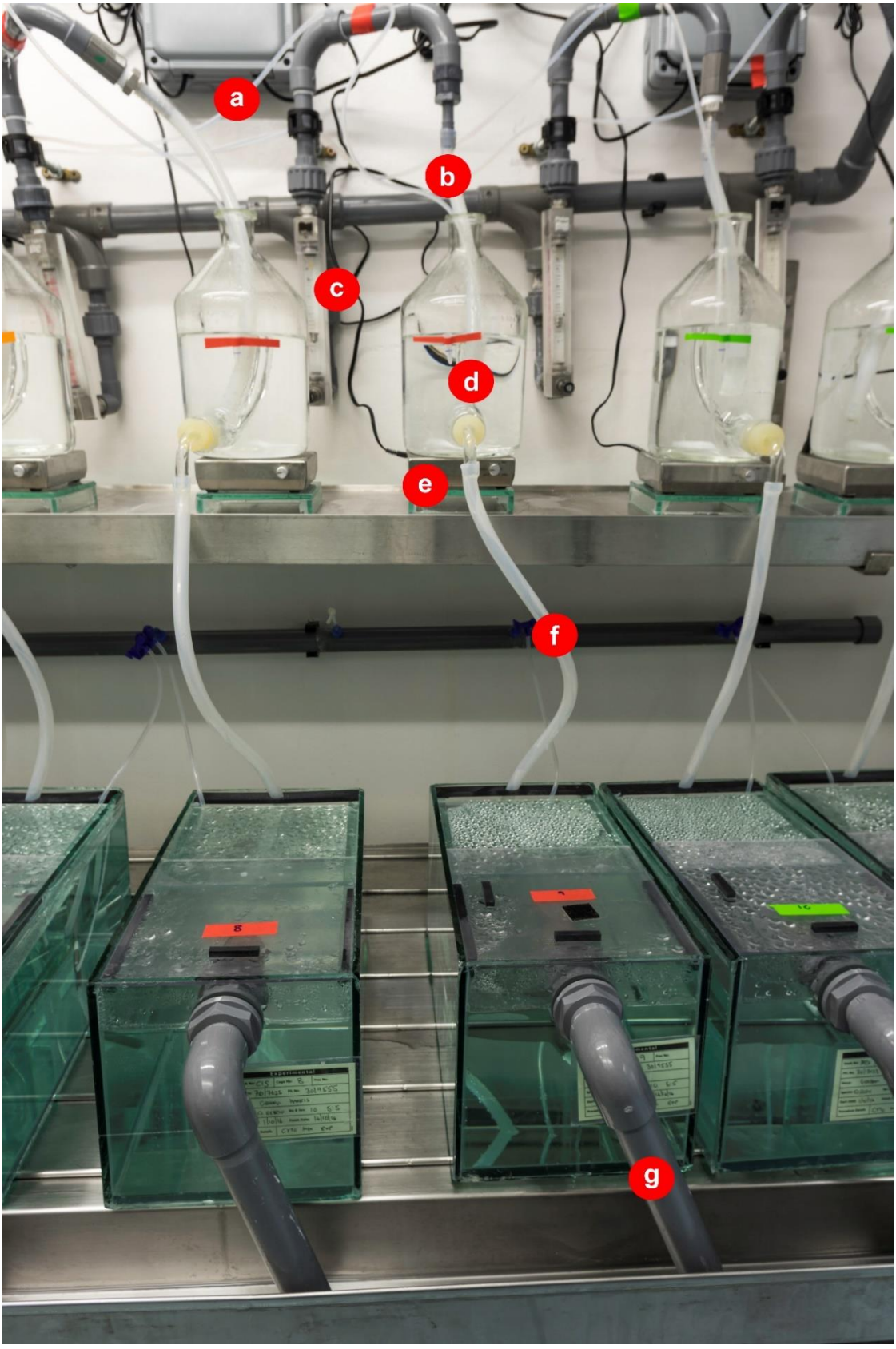


Figure 25: Exposure tank dosing apparatus. (a) Silicon tubing fed by the peristaltic pump from the dosing stock bottles along with (b) aquarium water feed regulated by (c) individual flow gauge leads into (d) mixing vessel sat on a (e) magnetic stirrer that ensures homogenisation of the individual tank inputs. Each tank is gravity fed (f) and drained (g), ensuring a constant flow of aquarium water containing the desired concentration of drug.

4.2.2 Chemical analysis of aquarium water

To confirm the fish were exposed to the intended drug and at the intended concentration, ~250 ml water samples were taken, in 500 ml amber polypropylene bottles, one from each aquarium at both the beginning and end of each exposure period. These were stored at -20 °C until they were tested at the institutes in Table 23.

Table 23: Institutes employed to conduct chemical analysis of water samples from 14 day exposure experiments.

Drug	Test Institute	Method	Limit of Detection (µg/l)
5-fluorouracil	Institute for Environmental Studies (IVM), VU University, Amsterdam, Netherlands	HPLC-ESI-MS	2.0
cyclophosphamide	Norwegian Institute for Water Research (NIVA), Oslo, Norway	UPLC-QTOF	0.15
CIS	School of Geography, Earth and Environmental Sciences, Plymouth University, UK	ICP-MS	0.13

Cisplatin concentrations were analysed by measuring the platinum concentration and from this calculating the corresponding cisplatin concentration. It was noted that the silicone used to for the tubing that transported the dosing stock to the mixing vessels had been vulcanised (a curing process that hardens a material) with platinum. As the study had already been conducted, it was not possible to procure another type of tubing (other curing processes are available). To assess whether the platinum-cured tubing contributed to platinum concentrations, aquarium water was passed several times through a length of new, unused tubing and then the tubing immersed in this water for 48 hours before storing it at -20 °C prior to analysis.

4.2.3 Single drug and simple mixture morphological endpoints

After the 14-day exposure period, the fish were killed in aquarium water containing a pH-buffered (7.4 ± 0.4) solution of Tricaine mesylate (MS222) at a concentration of 500 mg/l. This is an approved method for killing fish in accordance with Schedule 1 of the Animals (Scientific Procedures) Act (1996). MS222 is an anaesthetic and muscle relaxant, which meant the fish lost sensation and then the ability for non-voluntary muscle contractions,

stopping the heart and killing the fish. Fish were placed, one at a time, into the solution and were considered dead when no movement of the operculum (gill cover) was observed.

Blood was collected by removing the tail, placing a heparinised (to reduce clotting) capillary tube against the exposed caudal vein and letting the blood flow into the tube via capillary action. To maximise the amount of blood collected, the heart was massaged by gentle pressure from the thumb and forefinger against the body. The blood was placed into 0.5 ml micro-centrifuge tubes and kept on ice until processed. For the mixtures experiment only, 10% aprotinin was added to the micro-centrifuge tubes, to further reduce clotting. The specific blood endpoints analysed for each of the experiments are described in section 4.2.5.

The fish, including the detached tail, were then weighed and measured from nose to tail fork. The gills, gonad and liver were dissected and the latter two organs weighed. The livers were halved, with one half placed in formaldehyde to be fixed for histology. The gonad was used to confirm the sex of the fish. The other half of the livers, gonads, gills and bodies were placed individually in cryovial tubes and snap frozen in liquid nitrogen. These tissues were stored at -80 °C. The liver halves were then used for gene expression analysis (see chapter 5). The other tissues were stored in case of future analysis requirements. The specific morphology endpoints analysed for each of the experiments are described in section 4.2.4.

4.2.4 Single drug and simple mixture morphology endpoints

A range of morphological endpoint analyses were initially considered for the tissue samples, however only the ones summarised in Table 24 were conducted. Although tissues were also collected for histological and molecular analysis, only expression analysis of mRNA associated with DNA damage proteins and Vitellogenin production was actually conducted (Chapter 5).

Table 24: Morphology endpoints conducted for each 14 day exposure. *Gill/†Gonadal somatic index were not calculated for the pilot/mixture exposure as the gill/gonad was not collected and weighed in that experiment.

End point	Technique	Tissue
Condition factor	Ruler & balance	Whole body
Somatic index	Balance	Liver, Gill*, Gonad†

The Condition Factor of a fish is calculated by dividing the weight of the fish in milligrams by the cube of its length in millimetres. The outcome of this is then multiplied by 100, acting as a scaling factor to bring the value close to 1. This can be known as Fulton's condition factor (Nash *et al.*, 2006) and can be expressed as:

$$K = \frac{W}{L^3} \times 100$$

The somatic index of an organ is calculated by dividing the weight of the organ in grams by the weight of the fish in grams. A scaling factor of 100 is again used to bring the value close to one.

4.2.5 Single drug and simple mixture blood endpoints

A variety of blood endpoint analyses were attempted with the blood samples and these are summarised in Table 25. The methods are described in detail in the following sections. Not all endpoints were conducted or completed for all experiments, the reasons for which are described in the relevant sections in the discussion (section 4.4).

Table 25: The blood endpoints planned for the zebrafish 14 day exposure experiments. *Not all were conducted and these are marked with an asterix, with an explanation as to why in the discussion.

Exposure	End point	Technique	Blood constituent
Pilot	Blood cell viability	Automated Haemocytometry	Whole Blood
	DNA damage*	Micronucleus	Nucleated Cells
5-fluorouracil series	Blood cell viability	Imaging Flow Cytometry	Nucleated Cells
	DNA damage*	Micronucleus	Nucleated Cells
5-fluorouracil repeat	Blood cell viability	Imaging Flow Cytometry	Nucleated Cells
	DNA damage*	Micronucleus	Nucleated Cells
cyclophosphamide series	Blood cell viability*	Imaging Flow Cytometry	Nucleated Cells
	DNA damage*	Micronucleus	Nucleated Cells
cisplatin series	Vitellogenin*	ELISA	Plasma
Mixture	Vitellogenin*	ELISA	Plasma

4.2.5.1 Red blood cell viability using Automated haemocytometry

This assay was conducted using a Countess Automated Cell Counter (Invitrogen). This instrument uses digital analysis of optical images of highly diluted aliquots of whole blood. The cells are stained with a dye that is only taken up by cells with permeable membranes, in effect dead or dying cells. This allows a calculation of the proportion of viable and non-viable cells in a sample. The majority of the cells in a sample of whole blood are red blood cells, so it is predominantly these cells that are counted. As fish red blood cells are nucleated, it was considered possible that the drugs could damage the nuclear DNA, causing the presence of non-viable cells in exposed fish.

2 µl of whole blood per fish were diluted 1:500 in Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich) and gently mixed by pipetting. 10 µl of the diluted blood was added to 10 µl of trypan blue stain and also gently mixed by pipetting. 10 µl of the sample mixture was loaded into each of the two chambers on the cell counting chamber slide. The slide was inserted into the cell counter and the device focused to ensure accurate counting. Live cells have bright centres and dark edges. Dead cells have permeable membranes, allowing the

take up of the trypan blue stain. The cell counter gives the total number of cells and the number of live and dead cells. These numbers are derived by the machine by taking images of the counting chamber and tagging cells that match the criteria for live and dead. This information was saved and viewed later using the supplied software. Any missed or mis-tagged cells were identified and the total re-calculated. The two chambers in each slide were read independently, providing two replicates per sample.

4.2.5.2 Red blood cell viability using Imaging flow cytometry

This assay was conducted using an ImageStreamX Imaging Flow Cytometer (Amnis Corporation). This allowed high throughput optical analysis of a sample containing hundreds of thousands of cells. Multiple images of each cell are taken, in the visible spectrum and with one or more wavelengths of light corresponding to the multiple lasers installed in the equipment. Cells can then be stained or tagged with one or more markers, which are then excited by the lasers and imaged. For this study the cells are stained with a dye that is only taken up by cells with permeable membranes due to them being dead, damaged or apoptotic. This dye stains the nuclear material of these cells, which are then imaged by the flow cytometry. Using the supplied software, the cell populations of the samples are gated into viable and non-viable, based on the staining intensity.

0.5 µl of whole blood per fish was added to 0.25ml Draq7 far-red fluorescent DNA dye (Biostatus Limited) diluted 1:500 in Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich). The solution was gently mixed by pipetting and then placed on ice and covered until processing through the imaging flow cytometer (maximum delay 60 minutes). Each sample was pipetted lightly to re-suspend the cells, 50 µl was transferred to a 250 µl micro-centrifuge tube and the tube loaded into the ImageStreamX. Each sample was run using the settings in Table 26. The data files generated were analysed using the manufacturer's analysis software IDEAS. The mean numbers of total cells and non-viable cells per treatment were recorded.

Table 26: Imaging flow cytometer settings. Cell classifiers defined the smallest and largest object that is imaged. The two laser settings defined the laser's power. The number of events defined the number of objects counted.

Settings	Value
Cell Classifiers	15-300
Main laser	20.0
Side scatter laser	2.0
# events	10,000

4.2.5.3 Sample preparation for micronucleus assessment

2 µl of whole blood was pipetted onto a glass polylysine coated (to aid cell adhesion) microscope slide. Using the edge of the end of another microscope slide, held at 45 degrees and dragged gently along the length of the slide, the blood drop was smeared evenly over the slide surface. The smear was left to air dry before fixing by immersing the slide in methanol for five seconds and rinsing it under running water.

Wright-Giemsa stain was selected to aid visualisation of the cell nucleus. This stain also aids identification of cell type, due to the differential staining of cell organelles. Slides were placed in a steel staining rack and submerged in 350 ml undiluted Wright-Giemsa stain (Sigma-Aldrich) in a 500 ml glass staining bath for 30 seconds. The staining rack is then removed from the stain and submerged in 350 ml of deionised water (pH 5.8-7.2) for ten minutes. After air drying, cover slips were mounted using DPX mountant (Sigma-Aldrich).

The goal was to conduct the micronucleus assay on the fixed cells. This assay is an established technique for the detection of DNA damage. Double strand breaks in the DNA caused by a cytotoxic agent are not always repaired before the cell goes through replication. This can lead to the formation of micronuclei from the DNA fragments that become separated from the cell nucleus during replication. These micronuclei are counted visually, using a light microscope. Experience is required in order to differentiate between micronuclei and similar objects within the cells.

4.2.6 Fish blood plasma concentration calculations

Experiments conducted on the elimination of several polychlorinated biphenyls (PCBs) via the gills of rainbow trout, produced observations that showed that the concentration of PCBs in the fish's blood reached near equilibrium with the water it expelled during respiration (Fitzsimmons *et al.*, 2001). The blood:water partition values ($P_{blood:water}$) calculated from these measurements were averaged for each PCB, log-transformed (base 10) and plotted against the corresponding log-transformed K_{ow} values for each PCB. The equations of the linear fit and the linear regression of these values were shown to be statistically insignificant, so the two equations were combined to form the following fitted relationship.

$$LogP_{blood:water} = (0.73 \times LogK_{ow} - 0.88) \quad (1)$$

Building on this work, Huggett *et al.* (2003) produced an equation that allowed the calculation of the steady state plasma concentration ($F_{SS}PC$) of a chemical in a fish, when the fish is exposed to the chemical at a given environmental concentration (EC).[3]

$$F_{SS}PC = EC \times (P_{blood:water}) \quad (2)$$

This was then used to estimate the risk the chemical posed at this environmental concentration, by comparing $F_{SS}PC$ to the blood plasma concentration required in a human to have a therapeutic effect (H_TPC). This risk is presented as an effect ratio (ER).

$$ER = H_TPC \div F_{SS}PC \quad (3)$$

[3] In this equation the inverse of $LogP_{blood:water}$ is required so the value must first be exponentially raised by ten.

In order to take into account the effect of pH on partitioning, equation 1 was further modified by replacing LogKow with $LogD_{7.4}$. This value best represents freshwater, which has an average pH of 7.4.

$$LogP_{blood:water} = (0.73 \times LogD_{7.4} - 0.88) \quad (4)$$

These equations were used in this project to calculate the $F_{SS}PC$ of the three drugs in the zebrafish, at the highest experimental concentrations they were exposed to. The $F_{SS}PC$ of the drugs in the zebrafish, at the drugs measured and predicted environmental concentrations, were also calculated. The ERs were then calculated for each of the drugs in these two scenarios.

4.2.7 Statistical analysis techniques

Statistical analysis was conducted using the R programming language and environment. To aid understanding of the test selection process described below, refer to the flow chart in Figure 26. Before testing, the data were displayed graphically as boxplots to identify outliers. These were then checked in the raw data to see if their outlier status was due to data entry or calculation mistakes, and if necessary corrected. These boxplots have been included in the results section 4.3.1.

The replicates in each group were tested for homogeneity of variance using Levene's test on the median of each endpoint, grouped by sex. The advantage of using Levene's test is that it does not assume that the data are from a Gaussian (normal) distribution. Using the median instead of the mean is considered to be more robust, as it is not affected by a skewed distribution (Brown and Forsythe, 1974). If the test passed (no significant difference was observed between the replicates), the replicates in each group were pooled for further analysis. If this test failed and there were only two replicates, then that group would have to be discarded. If there were three replicates (as was the case for the controls in the cyclophosphamide, cisplatin and mixtures experiments) and one replicate was different from the other two, then that replicate would have to be discarded and the other two pooled.

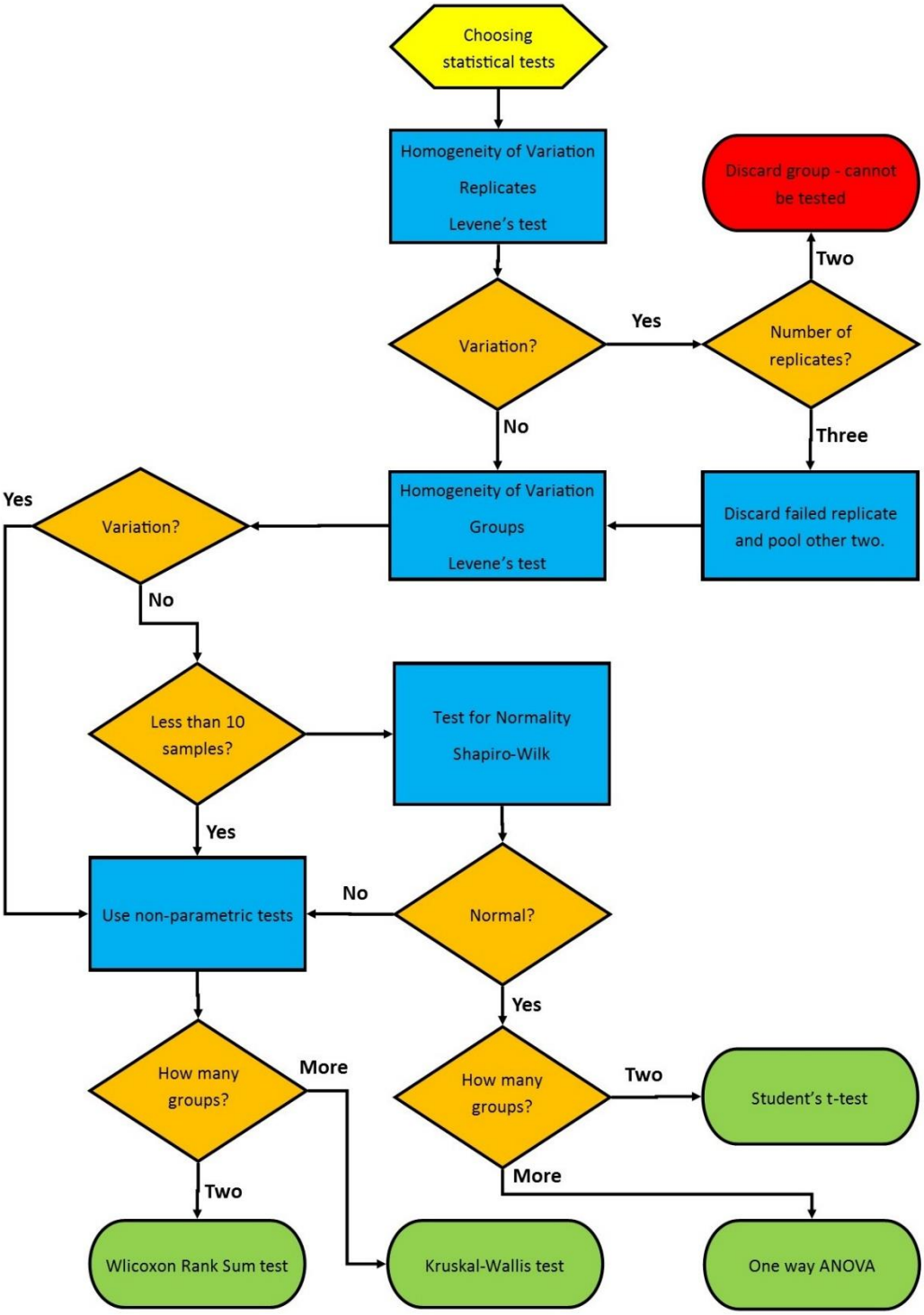


Figure 26: Flow chart describing the process for selecting the correct statistical tools for analysing the zebrafish morphology endpoints.

Homogeneity of variance between groups (a group being defined as the control or one of the concentrations of drug/mixture) was then tested with Levene's test, as described above. If no statistical difference in variance was detected and sample sizes were greater

than ten, the distribution of each group was tested for normality, using the parametric Shapiro-Wilk test.

UNEQUAL VARIANCE, NON-NORMALITY & THE USE OF TRANSFORMATION

Failure of homogeneity of variation or normality testing can sometimes be due to outliers, as these can skew the sample. As described earlier, outliers can be detected by graphically displaying the data as boxplots. If the outlier is not due to an error, then it is not good practice to change or remove it. Some statisticians suggest changing the value, using rules such as the mean plus two standard deviations. However, due to the large amount of variation in biological data, this approach is generally seen as unacceptable. Another method is transformation, which can also deal with other caused of unequal variance and non-normality. Various methods are possible such as log or square root transformation of the sample if it fails variance or normality tests. If this method is used, then the same transformation method must be applied to all data sets.

There is a great deal of dispute over the use of transformation, with some statisticians claiming there is little or no benefit, or even adverse effects from using it (Field, 2009). However, failure of variance or normality testing requires the use of non-parametric testing due to the inherent assumptions in parametric tests. As the former are less robust and are therefore more conservative in their output, there will always be compromises to be made in choosing the correct path through the statistical analysis process.

If both these tests were passed (no significant difference was observed in either variance or normality), the groups were then tested for differences between the controls and exposed. For the pilot and mixture experiment a Student's t-test would be used as there was only one concentration of each drug to be tested against the control group. For the 5-fluorouracil, cyclophosphamide and cisplatin exposures a one-way ANOVA would be used, as there were several groups to be tested against each other (a control and multiple concentrations per drug). If a statistically significant difference was found by the ANOVA, the post-hoc Tukey test was used, to identify between which groups the difference lay. Bonferroni correction was applied to adjust for multiple comparisons.

If the tests failed or if the sample size was less than ten, then non-parametric tests were used. Log transformation was tried in cases of failure, but this tended not to remove outliers or result in passing the failed variance/normality tests. In the case of the pilot and mixture experiments, the non-parametric Wilcoxon Rank Sum test was used to test for differences between the control and exposed groups. This was because there was only one concentration for each drug. For all other exposures the non-parametric Kruskal-Wallis test was conducted. If a statistically significant difference was found the post-hoc Pairwise Wilcox test was used, to identify between which groups the difference lay. Bonferroni correction was applied to adjust for multiple comparisons.

4.3 Results

This section contains the results of the morphological endpoints in the single chemical and mixture experiments. For the blood endpoint results, please refer to section 4.4.3. For the molecular endpoint results, please refer to section 5.5.

4.3.1 Single drug and simple mixture morphological endpoints results

This section contains the morphological results of the condition factor and somatic index measurements for the single drug exposure experiments. The fish have been grouped by sex, to remove any differences due to natural difference in size between the two sexes. All error bars are confidence intervals at the 95% confidence level. Confidence intervals were used to provide an indication of how representative the sample mean is of the population mean. Variability should be assessed using the associated box plots. Results that are significant at p = <0.05 are marked with a single asterisk, those significant at <0.01 are marked with a double asterisk.

4.3.1.1 Pilot study

As this was a pilot study, the fish were only exposed at one concentration of each drug, so no concentration-response could be obtained. To provide greater resolution of the variability in the data, the results were graphically described as boxplots. The following discussion relates to the set of boxplots in Figure 27.

The three drugs had relatively little, if any, effect on condition factor. The only significant difference observed in condition factor was in female zebrafish exposed to cyclophosphamide at a concentration of 100 µg/l. For this group of fish, the mean condition factor was significantly less than that of the control females. The extreme outlier for the female control in the condition factor (CF) boxplot was checked in the data and appeared to be the result of correctly recorded weight and length.

A significant difference was observed in the hepatosomatic index of both female and male zebrafish exposed to 5-fluorouracil at a concentration of 100 µg/l and of male fish exposed to cyclophosphamide, also at 100 µg/l. In all cases the hepatosomatic index of the exposed fish was lower than the controls.

The only significant difference observed in gill somatic index was observed in female zebrafish exposed to cisplatin at a concentration of 10 µg/l. For this drug, the mean gill somatic index was significantly more than that of the control females. It should be noted that although the male cisplatin Gill somatic index results were found to be statistically significant, there was a great deal of variability within the sample.

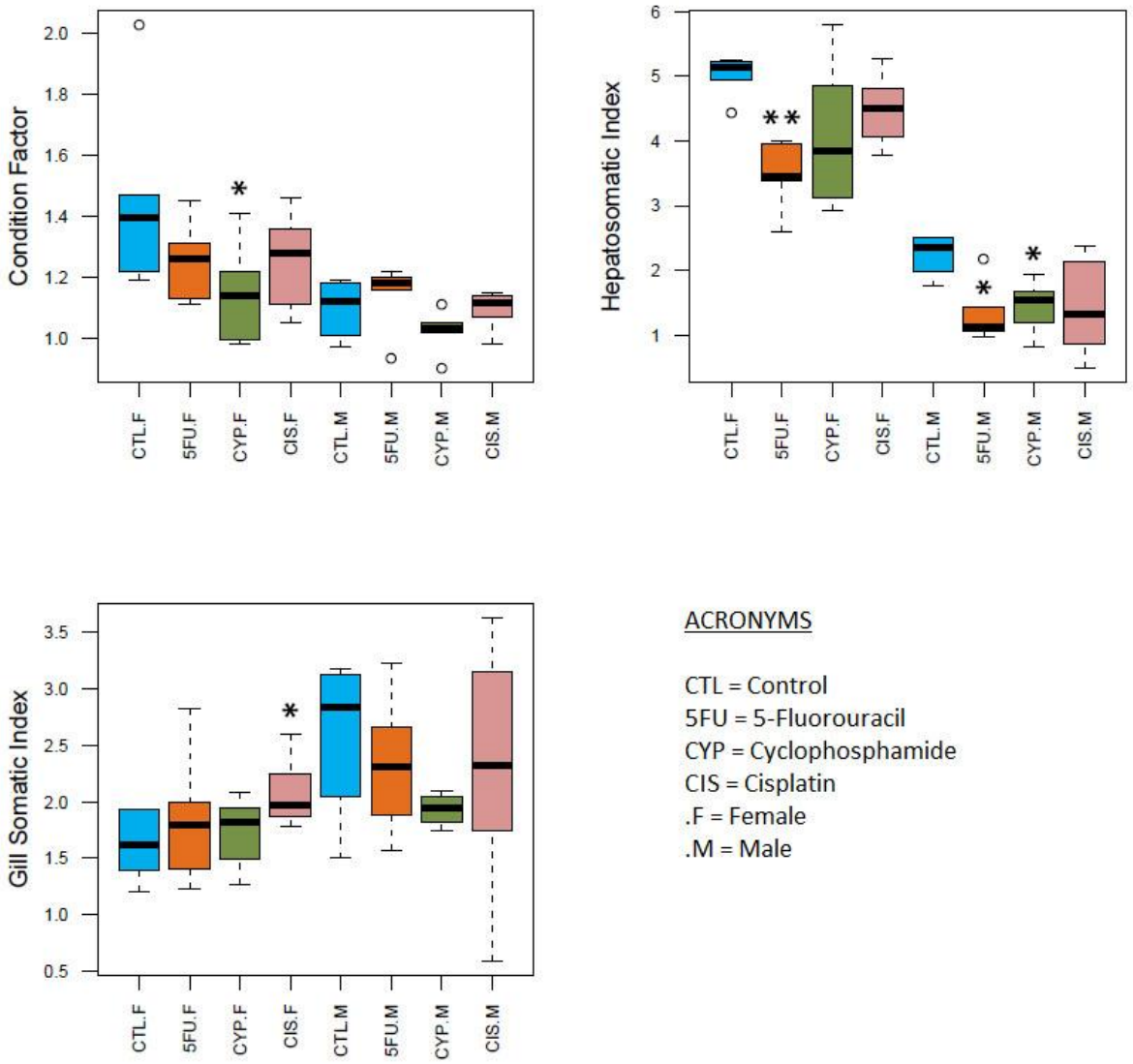


Figure 27: Boxplots of the zebrafish morphology results generated in the Pilot experiment. Open circles are outliers. Thick black horizontal lines inside the boxes are the median value. The box contains the interquartile range, which are the values between the 25th and 75th percentile. The "whiskers" (vertical dotted lines with T bar terminators) display the adjacent values, defined as the value of the largest/smallest observation that is less than or equal to the upper/lower quartile plus 1.5 times the length of the interquartile range. Any values that fall outside of the adjacent values are plotted as outliers.

4.3.1.2 5-fluorouracil concentration series

No significant difference in the condition factor, hepatosomatic, gill somatic or gonadal somatic indices of adult zebrafish was observed, when comparing the control group to any of the groups exposed to increasing concentrations of 5-fluorouracil; nor did there appear to be a concentration-dependent response (Figure 28).

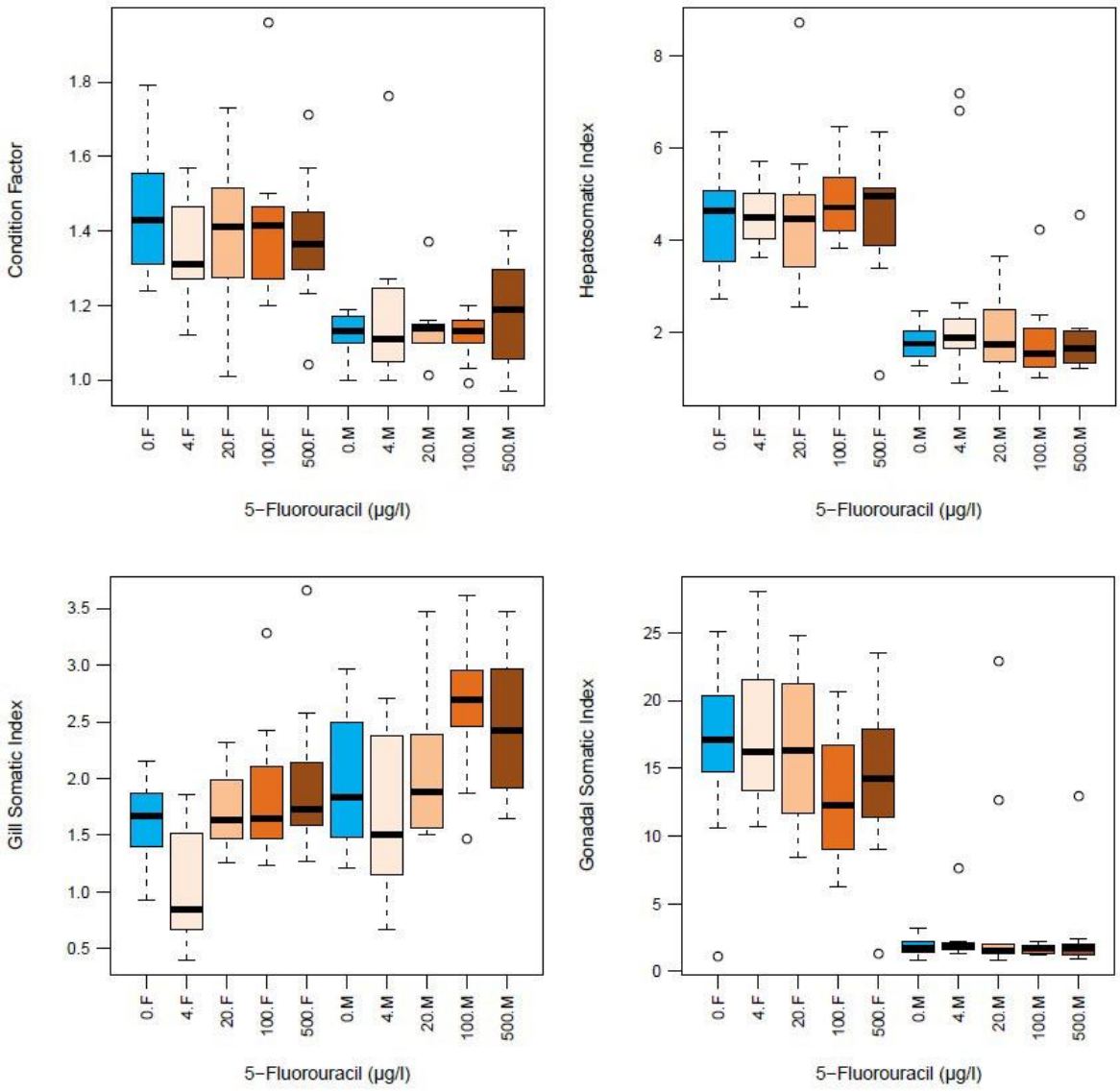


Figure 28: Boxplots of the zebrafish morphology results generated in the 5-fluorouracil concentration series experiment. Open circles are outliers. Thick black horizontal lines inside the boxes are the median value. The box contains the interquartile range, which are the values between the 25th and 75th percentile. The "whiskers" (vertical dotted lines with T bar terminators) display the adjacent values, defined as the value of the largest/smallest observation that is less than or equal to the upper/lower quartile plus 1.5 times the length of the interquartile range. Any values that fall outside of the adjacent values are plotted as outliers. The numbers in the x axis are the concentration of the drug and the letters refer to the sex of the fish.

4.3.1.3 Repeat 5-fluorouracil partial concentration series

No significant differences in the condition factor, hepatosomatic, gill somatic or gonadal somatic index of adult zebrafish were observed, when comparing the control group to any of the groups exposed to increasing concentrations of 5-fluorouracil. There appeared to be a concentration-dependent response in both the female and male mean hepatic somatic index, however this was not statistically significant (Figure 29).

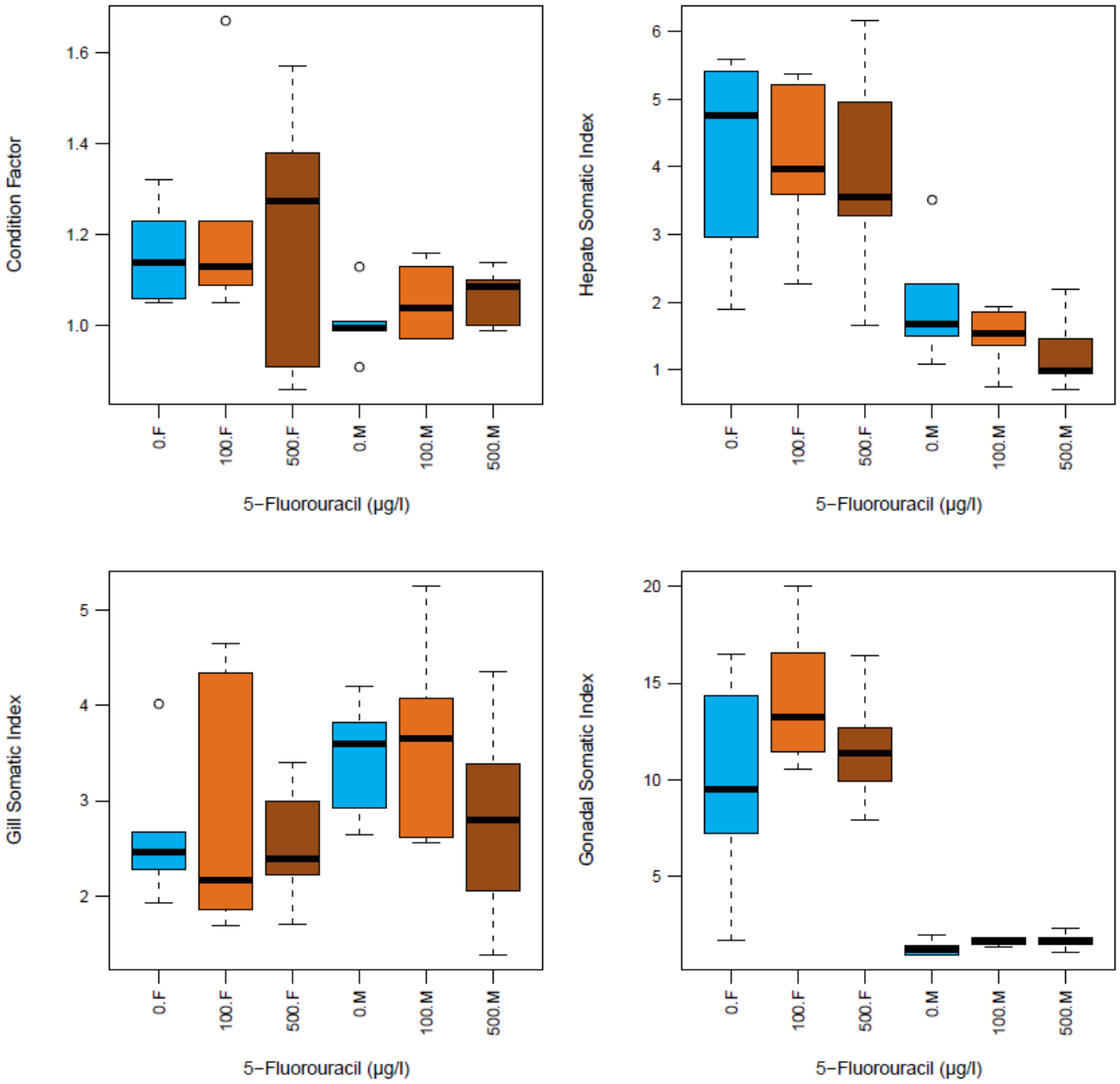


Figure 29: Boxplots of the zebrafish morphology results generated in the 5-fluorouracil partial concentration series experiment. Open circles are outliers. Thick black horizontal lines inside the boxes are the median value. The box contains the interquartile range, which are the values between the 25th and 75th percentile. The "whiskers" (vertical dotted lines with T bar terminators) display the adjacent values, defined as the value of the largest/smallest observation that is less than or equal to the upper/lower quartile plus 1.5 times the length of the interquartile range. Any values that fall outside of the adjacent values are plotted as outliers. The numbers in the x axis are the concentration of the drug and the letters refer to the sex of the fish.

4.3.1.4 Cyclophosphamide concentration series

No significant differences in the condition factor, hepatosomatic, gill somatic or gonadal somatic index of adult zebrafish were observed, when comparing the control group to any of the groups exposed to increasing concentrations of cyclophosphamide. Nor did there appear to be a concentration-dependent response (Figure 30).

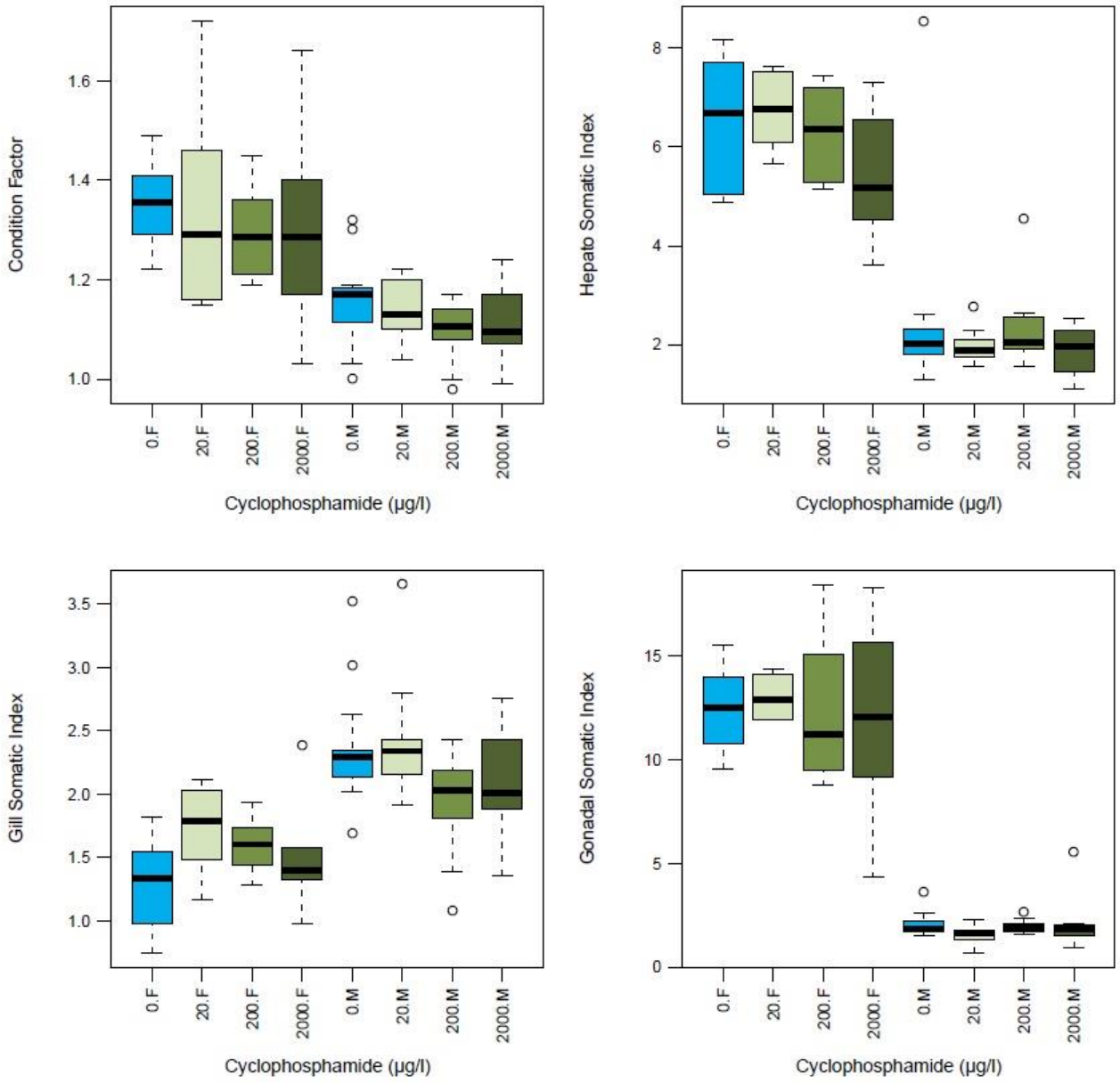


Figure 30: Boxplots of the zebrafish morphology results generated in the cyclophosphamide concentration series experiment. Open circles are outliers. Thick black horizontal lines inside the boxes are the median value. The box contains the interquartile range, which are the values between the 25th and 75th percentile. The "whiskers" (vertical dotted lines with T bar terminators) display the adjacent values, defined as the value of the largest/smallest observation that is less than or equal to the upper/lower quartile plus 1.5 times the length of the interquartile range. Any values that fall outside of the adjacent values are plotted as outliers. The numbers in the x axis are the concentration of the drug and the letters refer to the sex of the fish.

4.3.1.5 Cisplatin concentration series

No significant differences in the condition factor, hepatosomatic, gill somatic or gonadal somatic index of adult zebrafish were observed, when comparing the control group to any

of the groups exposed to increasing concentrations of cisplatin; nor did there appear to be a concentration-dependent response (Figure 31).

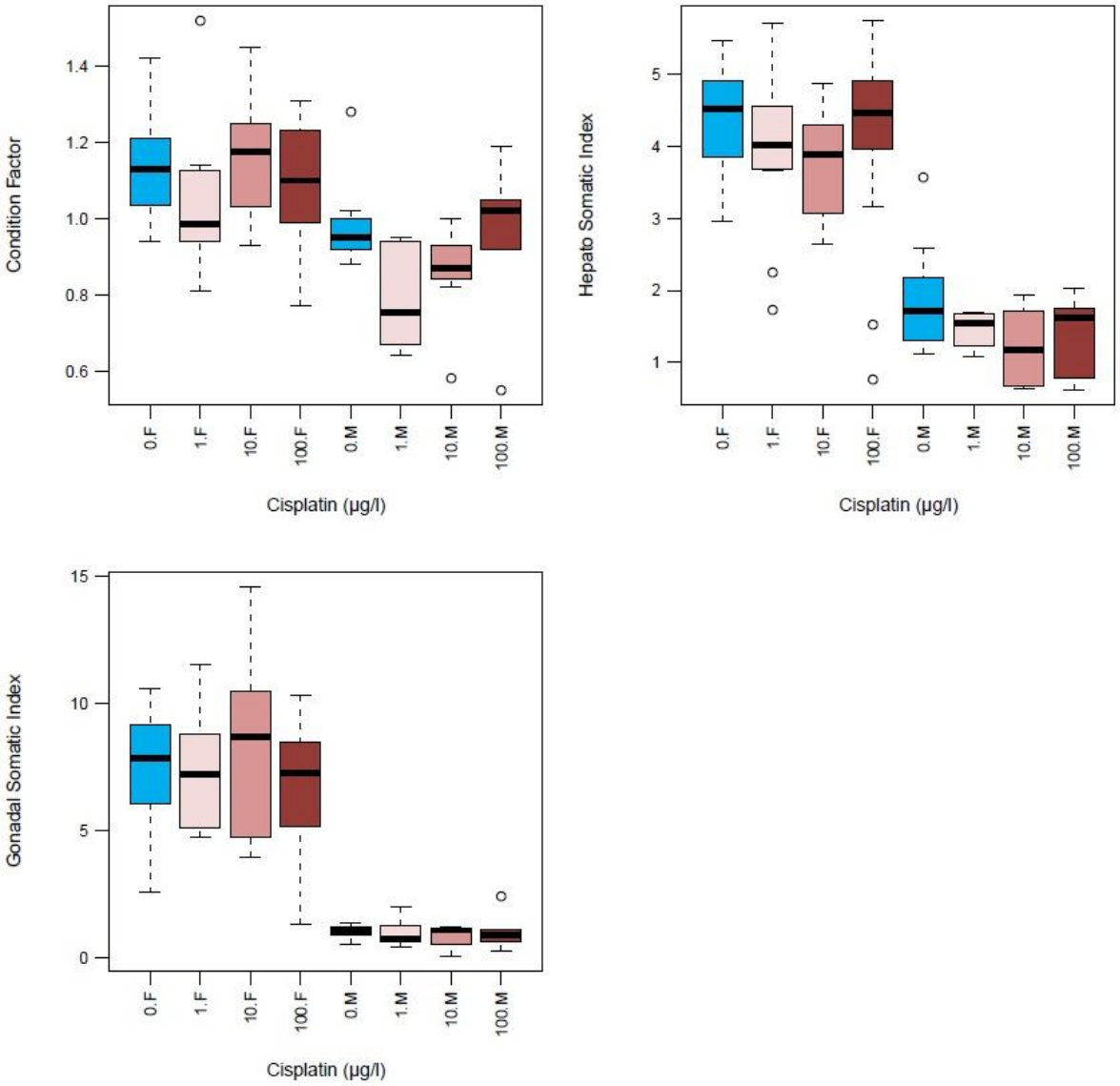


Figure 31: Boxplots of the zebrafish morphology results generated in the cisplatin concentration series experiment. Open circles are outliers. Thick black horizontal lines inside the boxes are the median value. The box contains the interquartile range, which are the values between the 25th and 75th percentile. The "whiskers" (vertical dotted lines with T bar terminators) display the adjacent values, defined as the value of the largest/smallest observation that is less than or equal to the upper/lower quartile plus 1.5 times the length of the interquartile range. Any values that fall outside of the adjacent values are plotted as outliers. The numbers in the x axis are the concentration of the drug and the letters refer to the sex of the fish.

4.3.1.6 Simple mixture

The only significant change in mean condition factor was in male fish exposed to cisplatin, where this endpoint was higher than the male control fish. The mean hepatosomatic index of female fish exposed to cyclophosphamide and cisplatin was significantly higher than that

of the female control fish. In male fish, only those exposed to 5-fluorouracil had significantly different results, with their hepatosomatic index being lower than that of the male control fish. The only significant results for the mean gonadal somatic index were observed in male fish exposed to cyclophosphamide, where this endpoint was significantly higher than that of the male control fish (Figure 32).

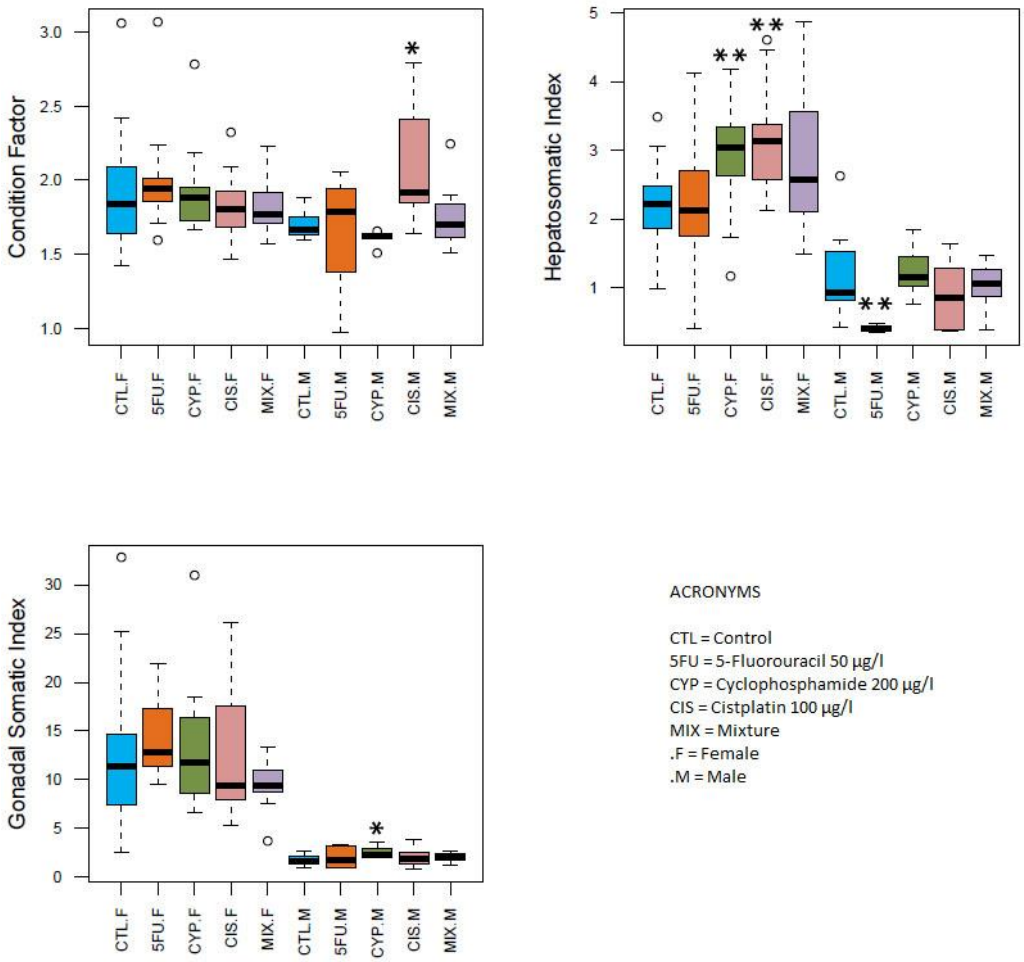


Figure 32: Boxplots of the zebrafish morphology results generated in the mixture experiment. Open circles are outliers. Thick black horizontal lines inside the boxes are the median value. The box contains the interquartile range, which are the values between the 25th and 75th percentile. The "whiskers" (vertical dotted lines with T bar terminators) display the adjacent values, defined as the value of the largest/smallest observation that is less than or equal to the upper/lower quartile plus 1.5 times the length of the interquartile range.

4.3.2 Single drug blood endpoint results

This section contains the results of the blood analysis for the single drug exposure experiments. For the blood endpoints these data have not been grouped by sex, as initial analysis showed no significant difference between the two groups (data not shown). All error bars are confidence intervals at the 95% confidence level. Results that are significant

at p = <0.05 are marked with a single asterisk, those significant at <0.01 are marked with a double asterisk.

4.3.2.1 Pilot Experiment

No significant difference in the mean number of total blood cells per ml was observed for fish exposed to any of the three drugs, when compared to the control group (Figure 33, blue bars). The mean percentage of non-viable cells in fish exposed to 5-fluorouracil was significantly higher than that of the control group. In fish exposed to cisplatin the opposite was observed, with this endpoint being significantly lower than that of the control group (Figure 33, purple bars).

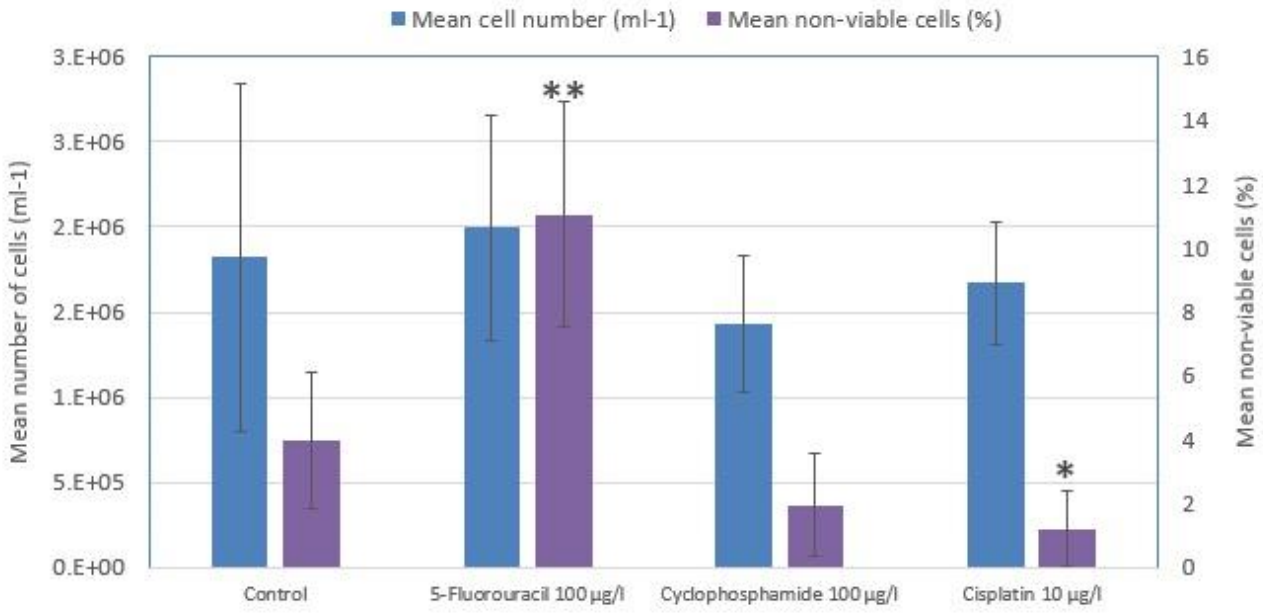


Figure 33: Mean number of total blood cells per ml and mean percentage of non-viable cells, collected from adult zebrafish exposed to three cytotoxic drugs individually at the concentrations shown in the graph.

4.3.2.2 5-fluorouracil concentration series

The only significant difference observed in the mean number of nucleated blood cells was for the group of fish exposed to 4 µg/l of 5-fluorouracil per litre, which was higher when compared to the control group (Figure 34, blue bars). All exposed groups had a significantly higher mean percentage of non-viable cells, compared to the control group. This difference

displayed a concentration-dependent response between the groups (Figure 34, purple bars).

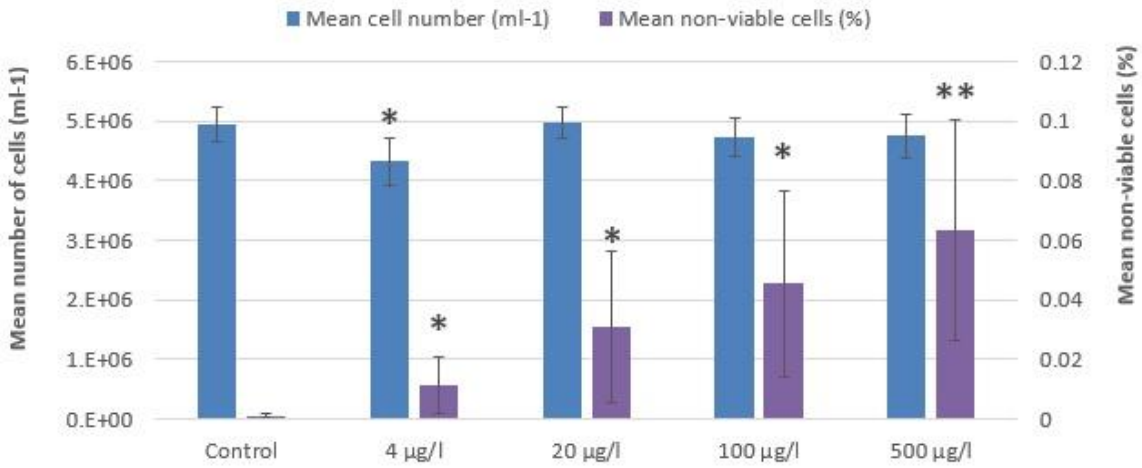


Figure 34: Mean number of nucleated blood cells and percentage of non-viable nucleated blood cells collected from adult zebrafish, exposed to a concentration series of 5-fluorouracil.

4.3.2.3 Repeat 5-fluorouracil partial concentration series

Both groups of fish exposed to 5-fluorouracil had a significantly higher mean number of cells per ml (Figure 35, blue bars) and significantly lower mean percentage of non-viable cells (Figure 35, purple bars) than those fish in the control group.

Due to the logged secondary axis, the error bar for the control – percentage non-viable cells (Figure 35, left hand side purple bar) cannot be plotted accurately. Therefore, these data have also been plotted as a box plot to illustrate the large amount of variation in this sample (Figure 36).

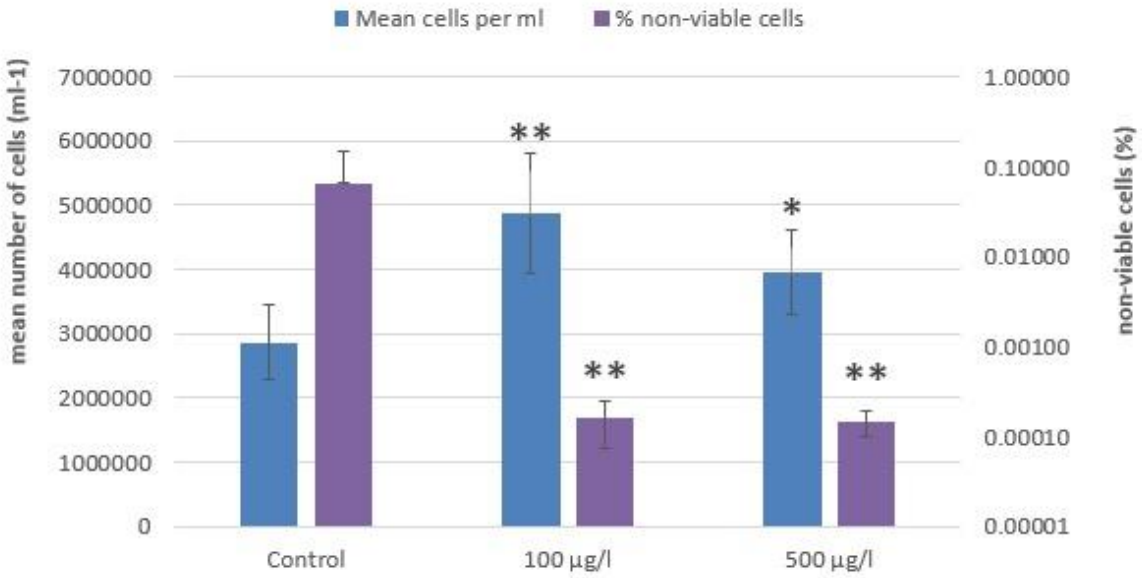


Figure 35: Mean number of nucleated blood cells and percentage of non-viable nucleated blood cells collected from adult zebrafish exposed to a partial concentration series of 5-fluorouracil. As error bars cannot be plotted accurately on a log scale, variability should be assessed using the boxplot in Figure 36.

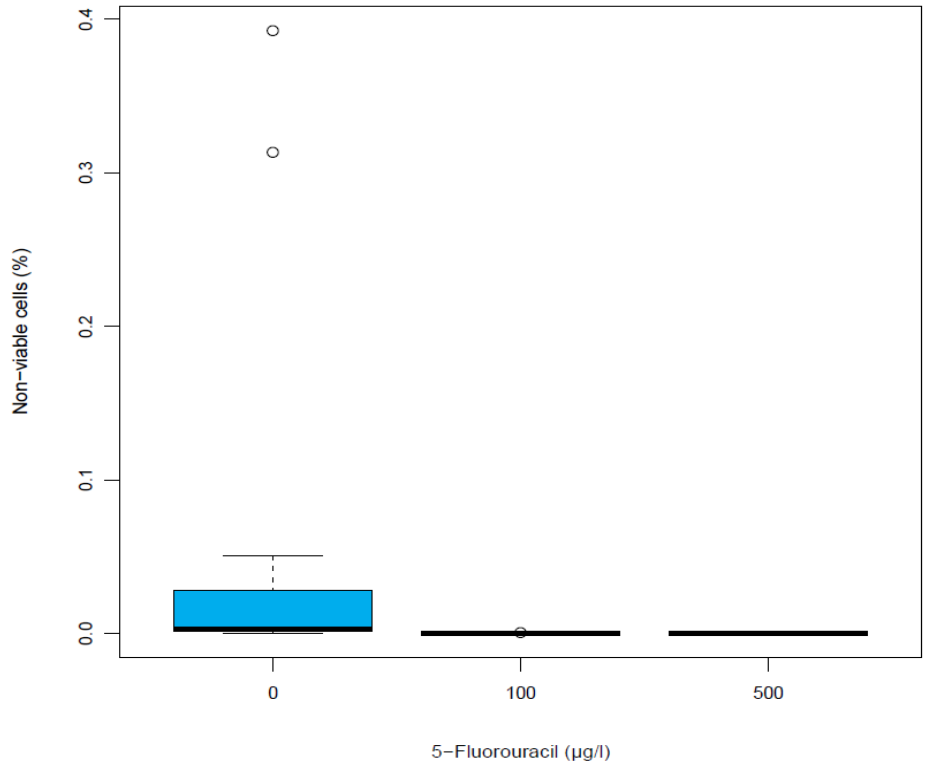


Figure 36: Mean percentage of non-viable cells in whole blood samples collected from adult zebrafish exposed to a partial concentration series of 5-fluorouracil. These data have been visualised as a boxplot in order to provide greater resolution of the variability in the control fish.

4.3.2.4 Cyclophosphamide concentration series

No data was collected for the blood endpoints from the cyclophosphamide concentration series.

4.3.2.5 Cisplatin concentration series

No data was collected for the blood endpoints from the cisplatin concentration series.

4.3.2.6 Simple mixture

No data was collected for the blood endpoints from the simple mixture.

4.3.3 Measured drug concentrations in aquarium water samples

Financial constraints meant that not all samples were analysed for all chemicals in all experiments. Table 27 contains the results of the chemical analysis that was conducted and identifies the concentrations that were not tested. The requirements for the actual chemical concentrations in the OECD 204 guidelines are that they be within twenty percent of the nominal concentrations (OECD, 1984). Due to cost, very few samples were measured in the pilot experiment and of these, only the 5-fluorouracil sample taken at the end of the study was within the defined boundary. With the exception of the nominal 20 µg/l group, all measured samples from both the 5-fluorouracil concentration series and repeat experiments were within twenty percent of the nominal concentrations. For the cyclophosphamide concentration series experiment, none of the actual concentrations measured were within the defined limits. All were consistently lower than the nominal concentrations by approximately 45-90 percent. The actual concentrations measured for the cisplatin concentration series, were all outside the twenty percent boundary apart from the nominal 100 µg/l group, at both the beginning and end of the experiment. Chemistry analysis of the dosing stocks was also conducted for this experiment, with only the dosing stock for the nominal 100 µg/l group being within twenty percent of the nominal concentration. The majority of the aquarium water and dosing stock samples taken at both the beginning and end of the mixture experiment were outside the twenty percent boundary. The exceptions were the cisplatin single chemical and mixture samples, where

all but the mixture sample taken at the beginning of the experiment were within twenty percent of the nominal concentrations.

One other noteworthy chemistry analysis result was obtained from washing an unused section of silicone tubing in which the dosing stock is delivered to the aquaria. The expected concentration for this sample was zero. However, the measured concentration was similar to that achieved in the 1 µg/l nominal concentration group.

Table 27: Chemical analysis of aquarium water samples taken at the start and/or end of the 14-day exposure period for the Pilot, 5-fluorouracil, cyclophosphamide and cisplatin exposure series and the mixtures studies. Due to financial constraints not all replicates were analysed. Results with ± values have had both replicates measured and these values are standard error of the mean. In addition, due to the cost of 5-fluorouracil analysis, only the start samples for the concentration series and end samples for the mixtures study were tested for this drug.

Exposure		Nominal concentration (µg/l)	Measured concentration (µg/l)	Deviation from nominal (%)
Pilot	5-fluorouracil start	Control 100	<LOD 72	 28
	5-fluorouracil end	Control 100	<LOD 120	 20
	cyclophosphamide start	Control 100	Not analysed	
	cyclophosphamide end	Control 100	Not analysed	
	cisplatin start	Control 10	Not analysed 7.449	 25
	cisplatin end	Control 10	<LOD (0.13) Not analysed (sample missing)	
5-fluorouracil	start	Control 4 20 100 500	<LOD 4.3 27 120 540	 7.5 35 20 8
	end	Control 4 20	 Not analysed	

		100 500		
5-fluorouracil repeat	start	Control 100 500	<LOD 100 460	 0 8
	end	Control 100 500	<LOD 100 450	 0 10
cyclophosphamide	Start	Control 20 200 2000	<LOD 9.40 ±0.1 94.75 ±3.55 207.50 ±107.6	 53 52.6 89.6
	End	Control 20 200 2000	<LOD 11.15 ±0.15 96.50 ±1.5 323.85 ±4.35	 44.3 51.75 83.8
Platinum (proxy for cisplatin)	Start	Control 1 10 100	0.168 ±0.095 0.661 ±0.069 6.357 ±1.074 104.21 ±5.01	 33.9 36.4 4.2
	End	Control 1 10 100 Dosing stocks: (1) 2,000 (10) 20,000 (100) 200,000	0.367 ±0.112 0.692 ±0.039 7.208 ±0.008 94.64 ±14.25 1,121.03 15,482.05 160,615.38	 31 28 5.36 43.9 22.6 19.7
	Tubing wash	0	0.661 ±0.095	
Mixture	start	5-fluorouracil Control Single 50 Mixture 50	 Not analysed 75 68	 50 36
		cyclophosphamide Control Single 200 Mixture 200	 25.3, <LOD, <LOD 101.35 ±10.95 323.85 ±4.35	 49.3 61.9

		cisplatin		
		Control	<LOD (0.13)	
		Single 100	107.1 ±3.6	7
		Mixture 100	125.2 ±1.6	25
	end	5-fluorouracil		
		Control	<LOD (3 µg/l)	
		Single 50	68	36
		Mixture 50	63	26
		Dosing stock single 100,000	61,000	39
		Dosing stock mixture 100,000	80,000	20
		cyclophosphamide		
		Control	<LOD	
		Single 200	109.6 ±9.9	45.2
		Mixture 200	121.8 (only 1 replicate tested)	39.1
		Dosing stock single 400,000	1,144.2	99.7
		Dosing stock mixture 400,000	1,160.2	99.7
		cisplatin		
		Control	0.3 (1 tank, other 2 <LOD)	
		Single 100	89.9 ±4.5	10.1
		Mixture 100	98.2 ±5.1	1.8
		Dosing stock single 200,000	162,462	18.8
		Dosing stock mixture 200,000	178,103	10.9

4.3.4 Fish steady state plasma concentration calculations

According to the output of the fish blood plasma model, to achieve blood plasma concentrations in fish, equal to the human therapeutic plasma concentrations, would require concentrations in the water in which the fish were exposed to be in the thousands of µg/l for 5-fluorouracil and cisplatin, and the tens of thousands of µg/l for cyclophosphamide. The environmental risk (ER) for all drugs, at experimental and measured and predicted environmental concentrations, were all greater than one. Cyclophosphamide, at the highest experimental nominal concentration of 2,000 µg/l, generated an ER of 39. However, the ER was recalculated as 242 using the actual concentration of 323.85 µg/l, measured in the aquaria water at the end of the cyclophosphamide concentration series experiment. The ERs for the highest

concentrations used in the experiments for 5-fluorouracil (500 µg/l) and cisplatin (100 µg/l) were 10 and 57 respectively. The ERs for 5-fluorouracil and cisplatin, at their mean MECs taken from hospital effluents, were in the tens and hundreds respectively, and the cyclophosphamide mean MEC from surface waters, was in the tens of thousands. The surface water mean PECs for all drugs were in the millions to tens of millions.

Table 28: Results of the fish blood plasma model for the three drugs tested on the zebrafish. The actual environmental concentrations are: Experiment = the highest concentration the fish were exposed to in this project, Mean MEC = the mean of the lowest and highest MECs found in the literature, Mean PEC = the mean of the lowest and highest PECs found in the literature. The H_TPC environmental concentration is the concentration of the drug required to achieve a therapeutic plasma concentration. * These values are based on measurements taken in hospital effluent, as no surface water measurements were found in the literature.

Drug	$P_{blood:water}$	Actual environmental concentration (ng/ml)	$F_{SS}PC$ (ng/ml)	H_TPC (ng/ml)	ER	H_TPC environmental concentration (µg/l)
5-fluorouracil	0.023735588	Experiment: 500 Mean MEC: 61* Mean PEC: 0.0045	11.87 1.44823 0.00011	120	10 83 1,134,837	5,056
cyclophosphamide	0.15595525	Experiment (nominal): 2000 Experiment (actual): 323.85 Mean MEC: 0.033 Mean PEC: 0.0021	311.91 50.51 0.033 0.002	12,210	39 242 2,407,123 37,193,202	78,292
cisplatin	0.140993795	Experiment: 100 Mean MEC: 50* Mean PEC: 0.00052	14.10 7.05 0.00007	800	57 113 10,911,555	5,674

4.4 Discussion

4.4.1 Single drug and simple mixture morphological endpoints

The results of the pilot study suggested that all three drugs had the potential to produce significant effects in one or more of the morphological endpoints assessed (Figure 27). 5-fluorouracil and cisplatin appeared to cause these effects at concentrations measured in the environment, albeit in hospital effluents, where these drugs could be present at concentrations much higher than in surface waters (Table 22). Cyclophosphamide was tested at a concentration two orders of magnitude higher than the highest level reported in hospital effluents (Table 22).

However, these results were not repeated in any of the subsequent concentration series experiments for any of the drugs, even though these experiments included concentrations at least ten times higher than those used in the pilot experiment. Due to the magnitude of the response seen in the mean hepatosomatic index of pilot fish exposed to 5-fluorouracil, the decision was taken to repeat the concentration series experiment for this drug. Again, no significant response was observed in this endpoint, or any of the others tested.

Significant changes were observed for the single chemical groups in the simple mixture experiment. However, apart from the mean hepatosomatic index of male zebrafish exposed to 5-fluorouracil, these changes were not consistent with those observed in the pilot study. In addition, none of the significant effects observed in the single chemical groups were seen in the fish exposed to the simple mixture of these chemicals.

These observations are discussed in more detail for each of the drugs and the simple mixture in the following sections. Where available the results of this study will be compared to those relevant studies found in the literature.

4.4.1.1 5-fluorouracil

In the pilot study, both male and female fish exposed to 5-fluorouracil displayed a significantly reduced hepatosomatic index when compared to the control group (Figure 27). This would indicate that the livers of the exposed fish were smaller than those of the controls. It is known that this drug is mainly metabolised in the liver (Hansen *et al.*, 2001).

5-fluorouracil is generally used to treat colorectal and breast cancers (and also basal skin cancers when applied topically) (Chu and Sartorelli, 2012). However, it has also been used to reduce liver tumours caused by metastatic colorectal cancer, in order to allow their removal by surgery (Giacchetti *et al.*, 1999). It is therefore possible that this drug could have been taken up by the zebrafish liver cells. The cytotoxic effects of this drug could then have induced cell death, leading to a reduction in the size of the livers of the exposed fish. Chemical analysis of the aquarium water in the control and 5-fluorouracil tanks showed no detectable levels of the drug in the controls. The actual concentration of 5-fluorouracil was 72% of the nominal at the start of the experiment (Table 27), below the 80% limit defined by the guidelines (OECD, 1984). At the end of the experiment the actual concentration was measured at 120 µg/l, on the border of the 20% deviation allowed by the guidelines. This gave some confidence that the fish had been exposed to a concentration of the drug close to the desired concentrations.

However, the subsequent concentration series experiment did not replicate the results of the pilot study. There appeared to be no response in the hepatosomatic index of exposed fish, up to a concentration of 500 µg/l (Figure 28). Assessment of the boxplot of the pilot experiment data in Figure 27 did not reveal a large amount of variation in the hepatosomatic index of the exposed fish. The sample size, however, was small with only six female and six male fish in the 5-fluorouracil group. This was by design, due to this experiment being a pilot study. In addition, these factors had been taken into account when conducting the statistical analysis, with a less robust non-parametric test being used. Due to financial constraints only the samples of aquarium water taken at the beginning of the 5-fluorouracil concentration series were chemically analysed. All actual concentrations measured were close to the nominal values, discounting an issue with dosing as the cause of the discrepancy in the results between the experiments.

Based on these findings, it was decided to conduct a partial (only 100 and 500 µg/l concentrations) repeat of the 5-fluorouracil concentration series, but with only one replicate per concentration and also for the control. As with the full concentration series, no significant reduction in the hepatosomatic index was observed in the exposed fish; although there was a non-significant concentration-dependent reduction in hepatosomatic index. For this experiment chemical analysis was conducted on both the start and end

samples of aquarium water for all tanks. As with the concentration series, actual concentrations were close to the nominal values (Table 27).

Assessment of the three experiments has shed little light on to why such a strong response was seen in the pilot, but not replicated in the subsequent experiments. The same strain of fish from the same source was used for all three experiments (Table 20). The same drug manufacturer and batch of drugs was used for all three experiments. The same person was responsible for dissection in each experiment. Chemical analysis of all three experiments reported that actual chemical concentrations were close to nominal values (section 4.3.3), although some groups were outside the twenty percent difference between nominal and actual concentrations defined in the OECD guidelines (OECD, 1984). If the actual values for the two concentration series experiments had been appreciably lower than the nominal, this could have been a factor in the observed results. However, this was not the case.

One possible explanation is biological variability combined with the small sample size and lack of replicates in the pilot experiment. Unfortunately, due to stock availability and financial constraints, the repeat exposure only had six fish per group and no replicates. A more robust experimental design may have been able to provide more evidence as to the role of biological variability in the pilot study results.

The other significant result was the reduction in the mean hepatosomatic index of male fish exposed to 5-Fluorouacil in the mixture experiment (Figure 32). Although this used a different strain of fish and batch of drug (factors considered unlikely to influence the results to any great extent), this could be seen as supporting evidence for the pilot study results. However, due to the uneven sex ratio described in section 4.2, this result was generated by only five male fish. Whilst this is the minimum number required by the output of the power analysis (section 4.2.1), this was only two fish from one replicate and three from another. The non-significant result for the hepatosomatic index of females was based on fifteen female fish. In addition, no significant response was observed in the mixture group (discussed in more detail in section 4.4.1.4). As with the previous 5-fluorouracil experiments, biological variability within a small sample size could be considered as a potential explanation.

THE PROS AND CONS OF THE FISH BLOOD PLASMA MODEL

To provide additional data on the interaction of the drugs with the fish and their environment, the fish blood plasma model was used. Calculating the environmental risk (ER), using the fish blood plasma model served two purposes. For the experimental concentrations, this provided an indication of whether the drugs may have had an effect on the fish. For the MEC/PEC, it gave an indication of the risk the drugs posed to fish at these environmental concentrations.

For all drugs, CMax was used for the human therapeutic plasma concentration (H_TPC) as these were the only values found for all three drugs. Ideally, the area under the curve (AUC) values would have been used instead of CMax. AUC represents the total exposure a patient experiences of the drug over time, whereas CMax represents the peak blood plasma concentration (Schall *et al.*, 1994). The AUC value will be lower than CMax, and for chronic exposures studies such as those conducted on zebrafish in this project AUC could offer a better comparison to fish steady state plasma concentration ($F_{SS}PC$).

As with all models, assumptions are made in order to simplify a highly complex real world situation. In the case of the fish plasma model (equation 2), this assumes that no further metabolism, excretion or bioaccumulation occurs and that all the drug is biologically available in the plasma of the fish (Huggett *et al.*, 2003). Ideally, actual plasma concentrations would be measured in the fish. However, this is costly and time consuming, which often is often prohibitive. Models provide a cost effective way of predicting risk and can aid in the prioritisation of chemicals for further, more robust assessment.

The ER values produced from the fish plasma model for exposure to 5-fluorouracil, for both the highest concentration in the experiments and for the MEC and the PEC, were 10, 83 and 1,134,187, respectively. Huggett *et al.* (2003) proposed that an ER of 1 or less would suggest that a drug was present in a fish's plasma at a concentration sufficient to cause a therapeutic effect. Of course, this does not mean that adverse effects are not possible at lower, non-therapeutic concentrations. It does, however, allow a comparison with the well documented therapeutic effects of the drug in humans. In the case of 5-fluorouracil, an ER of 10 for the experimental concentration would suggest that exposure was below the

concentration required to elicit therapeutic effects. Indeed, the model calculated that the fish would need to be exposed to a concentration of 5,056 µg/l to achieve a human therapeutic plasma concentration. The very large ER values calculated for the environmental scenarios, suggest that there is little chance of observing any adverse effects in fish in freshwater environments, therapeutic or otherwise.

Morphological effects of 5-fluorouracil on fish in the literature

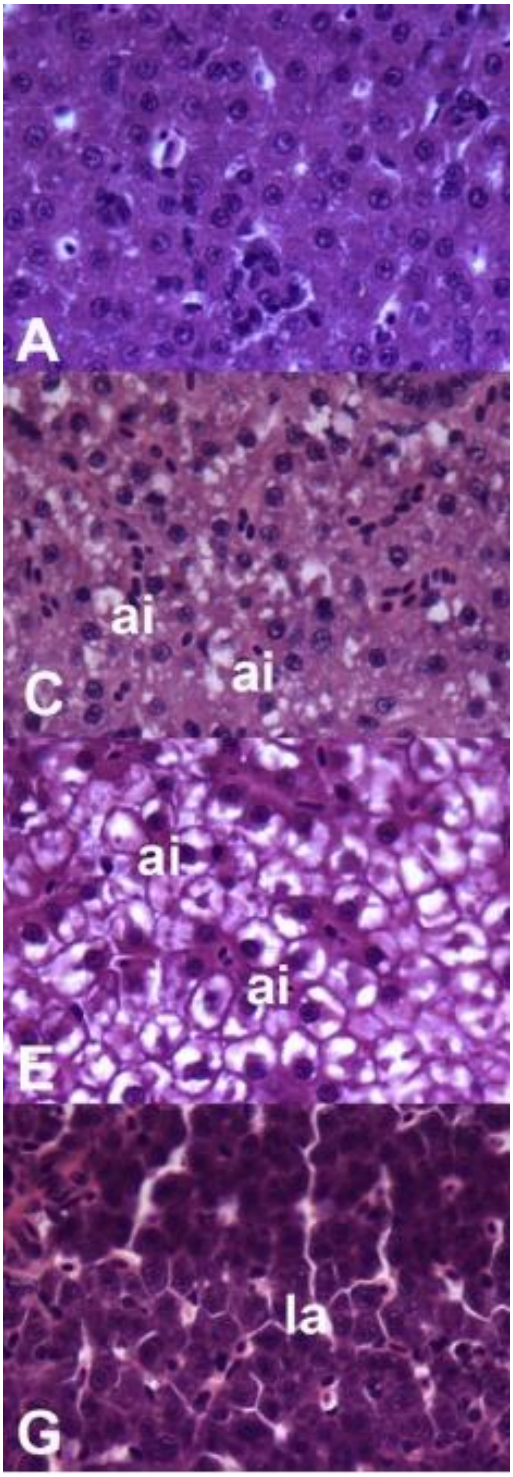


Figure 37: Histopathology of adult zebrafish exposed to control (A), 0.1 (C), 1.0 (E) and 100 µg/l (G) 5-fluorouracil. Ai = lipidosis la = atrophy. (Kovács et al., 2015)

There is little information in the literature concerning this drug's impact on zebrafish morphology. A recent study assessed low nominal concentrations of 5-fluorouracil at 0.01, 1.0 and 100 µg/l across two generations of zebrafish, examining a number of morphological endpoints at 7 months for the F1 generation and 33 dpf for the F2 (Kovács *et al.*, 2015). A significant reduction was observed in the mean weight of the fish in the lowest concentration, and a significant increase in the top two concentrations. No significant differences in the length of the fish was seen in any of the exposed groups and no malformations were reported in the embryos of either generation. Histopathology was conducted on the livers of the 7 month old F1 generation, in which lipidosis (Figure 37 C and E) and regressive degeneration of the liver was observed at all concentrations of 5-fluorouracil. In addition, liver atrophy (Figure 37 G) was observed at the highest concentrations. The authors claim that both the lower mean fish weight observed in the lowest concentration and the higher mean fish weight observed in the highest two concentrations could be attributed to the morphological changes observed in the liver (and also the kidney, where hyperplasia was observed in the lowest two concentrations and tubulonephrosis in the highest). To the untrained eye the amount of lipid appears to increase between the control, 0.1 and 1.0 µg/l samples. In the 100 µg/l sample there appears to be reduction in lipid

containing vacuoles but, according to the authors, an increase in evidence of atrophy. An increase in lipid content along with the enlargement of the kidney due to hyperplasia could be seen to support the increase in mean weight in the fish exposed to the middle concentration of 1.0 µg/l. However, these same changes were observed in the fish exposed to 0.01 µg/l, (albeit with smaller vacuoles) for which the mean weight was significantly less than the controls and where the authors also claim that the histopathological changes were the cause. With regards to the significant results seen in the pilot study, where fish were exposed to 100 µg/l of 5-fluorouracil, no significant change was observed in the condition factor but a significant reduction in the hepatosomatic index was seen. If the significant increase in mean fish weight of the group exposed to 100 µg/l 5-Flourouracil observed by Kovács *et al.* (2015) was due to the morphological changes in the fish's organs, this would appear to contradict the findings of this experiment. Experiment duration may be a factor, with chronic exposure required to produce the observed effects. However, the subsequent 5-fluorouracil experiments did not repeat the reduction in hepatosomatic index.

Only one paper examined the effects of 5-fluorouracil on adult fish. Following a Polish experimental protocol, Zaleska-Radziwill *et al.* (2011) exposed adult zebrafish to a concentration series of 5-fluorouracil for 96 hours, counting mortalities every 24 hours. No deaths were observed at the highest concentration tested of 100 mg/l. The same paper contained the results of a juvenile growth assay following the OECD 215 protocol. Juvenile zebrafish were exposed to a concentration series for 28 days. Fish were weighed at the start and end of the exposure period and growth rates for each concentration calculated. A NOEC of <1560 mg/l was recorded for this experiment, with the authors stating that growth inhibition was observed at 12.5 mg/l and 6.25 mg/l. They then go on to say that growth stimulation was observed at lower concentrations, although no specific values were given. Biological variability could be an alternative cause for the apparent growth stimulation attributed to 5-fluorouracil.

Egeler and Seck (2008) conducted the OECD 203 early life-stage toxicity test on zebrafish, exposing them to a concentration series from 1.0 to 100 mg/l for a period of 35 days. Endpoints such as mortality, time to start and end of hatch, deformed larvae, abnormal behaviour and average length and dry weight at the end of the exposure period were recorded. A LOEC of 100 mg/l (the highest concentration tested) was recorded for

mortality, deformed fish and average length. This value was the NOEC for all other endpoints. This protocol required exposure over a much longer period than the other studies described here, and is therefore likely to be more sensitive. In addition, the experiment was carried out under Good Laboratory Practice guidelines, which if followed correctly, will ensure a robust outcome.

Studies using other species of freshwater fish were also found. Two studies assessed the effect of 5-fluorouracil on the mortality of adult Rainbow trout (*Oncorhynchus mykiss*). No effect on mortality was observed at 1000 mg/l, in an acute toxicity exposure study with 48 hours of exposure, followed by a 48-hour post exposure observation period. The reported NOEC was the only concentration to which these fish were exposed (Gröner, 1983). A 96-hour acute toxicity test using the OECD 203 protocol reported a NOEC of 867 mg/l in Rainbow trout exposed to the 5-fluorouracil pro-drug Capecitabine (ABC, 1997c). Both of these studies were reported in Straub (2010). However, the original publications could not be found, so no further assessment of this experiment could be done.

A study using early life-stage Fathead Minnow (*Pimephales promelas*) exposed to 5-Fluororuacil for 120 hours (DeYoung *et al.*, 1996) produced an LC_{50} of 2,420 mg/l and EC50 of 400 mg/l for deformed larvae. The study used static-renewal, with the medium being refreshed every 24 hours, a method judged to be less robust than continuous renewal. Also, it was not clear how many concentrations were used to calculate the endpoints. It appeared to be just two (plus the control), which would give a concentration-response curve with a low resolution for calculating the endpoints.

Despite the differences between these studies, all reported that 5-fluorouracil has no detectable effects on fish at environmentally relevant levels. All significant effects were observed at concentrations in the tens to hundreds of milligram range, much higher than the concentrations used in this project. These studies provide further support to the argument that the significant results observed in this project, could be due to biological variability.

4.4.1.2 Cyclophosphamide

In the pilot study, significant differences were observed in female condition factor and male hepatosomatic index, with both these endpoints reduced in comparison to the control groups. Cyclophosphamide is mainly metabolised in the liver, but is used to treat cancers in other tissues such as breast, ovarian and bone. As described above in the 5-fluorouracil section, the hepatosomatic index reduction may be due to cyclophosphamide's cytotoxic mechanism of action, which reduced the number of liver cells. Cyclophosphamide is used to treat tumours in the liver as a result of breast cancer metastasising (Estape *et al.*, 1990). More relevantly to healthy liver cells, cyclophosphamide metabolites have also been shown to cause liver toxicity in humans (McDonald *et al.*, 2003).

Why only significant results were observed in the hepatosomatic index of male fish is unclear. The mean of the hepatosomatic index of female fish was also reduced compared to the control group. However, there was much greater variability between individual females (Figure 27). The pilot study had no replicates and the number of fish in each group was below the number considered suitable for parametric statistical tests. However, the number of individuals was chosen using power analysis, albeit *a priori* due to the lack of experimental data available (which was one of the reasons the pilot test was conducted, to provide data for the concentration series experiments power analysis). An alternative explanation is that greater natural variability is generally observed in the size of the livers of female fish, because of the biological demands of egg production.

Causal links between cyclophosphamide and the significant reduction in only female condition factor are harder to define. Evidence of weight loss in humans as a side effect of chemotherapeutic regimens using cyclophosphamide was not found in the literature. A study exposing mice to continuous low doses of cyclophosphamide via their drinking water (a scenario more in common with the adult fish exposures than is generally seen in mammalian studies) observed no significant weight loss in the exposed groups (Man *et al.*, 2002). As with the other significant results, natural biological variability should also be considered. Due to financial constraints, it was not possible to conduct chemical analysis of the aquarium water of the cyclophosphamide tank, so this attribute cannot be assessed.

Due to the lack of repeatable results in the 5-fluorouracil, efforts were taken to improve the robustness of the cyclophosphamide concentration series, by increasing the number of control replicates to three. This change was made on the advice of Martin Shultz, a biostatistician at Brunel University. For this experiment, no significant differences were seen in any of the morphological endpoints observed. As noted previously, due to an error in dosing, fish were exposed to concentrations of cyclophosphamide at twice the planned concentration, with the highest concentration at twenty times that used in the pilot study. Chemical analysis of the aquaria water was carried out for this experiment. As discussed in detail below (see section "Chemical analysis of aquaria water containing cyclophosphamide"), this showed that while actual concentrations were 50%-80% lower than the nominal values, cyclophosphamide was still present at concentrations higher than the nominal value set for the pilot experiment. As the actual concentrations are likely to have been lower than the nominal, this would suggest that the results observed in the pilot study could be the result of biological variation.

The results for the single chemical group from the simple mixture experiment would appear to support the notion that the significant findings are due to biological variation. In this experiment female hepatosomatic and male gonadal somatic indexes were seen to be significantly larger than the control groups. Whilst it could be argued that this may have been due to inflammation of these organs, the fact that this finding is contrary to those from previous experiments would appear to lend greater support to the biological variation argument.

The biological variation argument is further supported by the results of the blood plasma model. The mean actual concentration of the highest nominal concentration of 2000 µg/l of cyclophosphamide in the concentration series was 207.5 µg/l at the beginning of the experiment and 323.85 µg/l at the end. When these values are entered into the fish blood plasma model, fish steady state plasma concentration values of 32.36 ng/ml and 50.51 ng/ml were generated. The human therapeutic plasma concentration (H_TPC) used for cyclophosphamide in the model was 12,210 ng/ml. The corresponding ER values were 377 and 242, as opposed to an ER of 39 for the nominal concentration of 2000 µg/l. This would suggest that the H_TPC was not achievable in the plasma of the exposed fish, as the concentration they were actually exposed to was not high enough. Additionally, no

significant results were observed in the group of fish exposed to the nominal 2000 µg/l concentration, for any of the endpoints assessed; whereas significant results were observed in the fish exposed to the highest concentrations in the pilot and simple mixture experiment of 100 µg/l and 200 µg/l respectively. This suggests that the drug was not a factor in the significant results observed and that biological variability could be a more likely explanation.

Finally, for cyclophosphamide, an ER of 2,407,123 was calculated for the mean MEC and 37,193,202 for the mean PEC. Even if the significant results in the cyclophosphamide experiments were due to this drug, the ER values suggest that it is extremely unlikely that these would be observed at environmental concentrations.

Chemical analysis of aquaria water containing cyclophosphamide

Due to the mistake in calculating the flow rate at the beginning of the cyclophosphamide series, samples were also taken at the middle of the study, when the mistake was recognised. At the time, it was decided that dosing should continue at the higher concentration, as no mortality or health issues were observed in the exposed fish. The midpoint samples were the ones sent for testing. However, from the results of the chemical analysis (Table 27), either the mistake was misdiagnosed or significant degradation of the drug occurred, as the actual concentrations in the tanks containing nominal concentrations of 20 and 200 µg/l, match the original nominal concentrations of 10 and 100 µg/l.

For the nominal 1000/2000 µg/l tanks, in either case, either significant degradation has occurred, or there was an error in making up the dosing stocks as actual concentrations were measured at ~320 µg/l. The latter scenario would not appear to be the case, because the dosing stocks for the tanks containing nominal concentrations of 1000 and 2000 µg/l were also used, once further diluted, to provide the drug to the tanks containing lower nominal concentrations. As described above, although the actual concentrations were approximately fifty percent lower than the nominal values of 20 and 200 µg/l, this was still not as great a reduction as the approximately eighty percent reduction measured in the nominal 2000 µg/l tanks. It could be expected that if the dosing stock of the 1000/2000 µg/l was made up incorrectly, the actual concentrations for the lower two concentrations would also be proportionally less. Flow rates for all concentrations were set at 0.1 ml/min and

were tested and adjusted if required, on a daily basis. The same tubing and therefore the same bore was used for all concentrations. The same peristaltic pump was also used for all concentrations. Water flow rates were all set for 12 l/hr and monitored daily. Although these were from individual taps, which can produce differences, there was a consistent and equal difference between the two highest concentration tanks and the rest of the exposed tanks. It therefore doesn't appear possible that a difference in flow rate could be the cause of the lower than expected actual concentrations in the tanks containing the high concentrations of the drug.

Another potential cause of the discrepancy is degradation of the chemical. Studies have shown that cyclophosphamide is resistant to photolysis (at visible and UV wavelengths) and biodegradation (Kim *et al.*, 2009; Buerge *et al.*, 2006; Steger-Hartmann *et al.*, 1997). However, the product information sheet for cyclophosphamide from the supplier notes that chemical degradation is an issue for this drug, with hydrolysis resulting in the removal of chlorine above 30 °C. Although the dosing stocks were never exposed to temperatures as high as this, this document also states that aqueous solutions can be kept at room temperature for only a few hours. As this was considered impractical, in an effort to reduce degradation the drug was stored in the dark at -20°C and fresh dosing stocks were made up from it every four days. The dosing stocks were held in brown glass bottles, during the four-day dosing period.

Importantly, the mixtures experiment tank results also saw a ~40-60 percent reduction between nominal (200 µg/l) and actual concentrations in both the single chemical and mixture aquaria. In addition, the dosing stocks for this experiment were also analysed, with the actual concentrations in both stocks measured at ~1,150 µg/l, a 99 percent reduction on the nominal value of 400,000 µg/l for both dosing stocks (Table 27). As the dosing stocks from the cyclophosphamide concentration series were not collected and analysed, it was not possible to assess if this very large reduction also occurred in that experiment. However, the same protocol for making up the dosing stocks was used for both the cyclophosphamide concentration series and mixture experiments, with only small changes to the dosing stock protocol to make up the mixture dosing stock.

Given that the mixtures exposure saw similar differences between nominal and actual concentrations, it seems likely that the nominal concentrations were calculated and dosed correctly in the previous experiments. Therefore, in light of the information above, the reduction seen in actual concentrations could be attributed to chemical degradation of cyclophosphamide. There is still the question of what caused the greater discrepancy between nominal and actual concentrations in the 2000 µg/l tanks (80 percent vs 50 percent in the lower two concentrations) and the much larger discrepancy of a 99 percent reduction between the nominal and actual concentrations in the cyclophosphamide dosing stocks in the mixture experiment. This could be due to a greater rate of degradation at higher concentrations, as there appears to be a concentration-dependent relationship. On re-reading the product information sheet it also became apparent that this degradation could have been largely curtailed by the addition of 0.9% sodium chloride to the dosing stock. This was reported to have reduced chemical degradation to 11.9% after one week at 25 °C. A lack of experience when first setting up the cyclophosphamide experiments meant that this solution to the degradation issue was not appreciated.

Morphological effects of Cyclophosphamide on fish in the literature

No information concerning cyclophosphamide and adult zebrafish morphology was found in the literature. There were several studies employing zebrafish embryos, using this early life-stage to develop teratogen (embryo malformation agent) assays. This type of assay classes drugs like cyclophosphamide as proteratogens, as they require metabolism to their active metabolites. As fish embryos do not appear to express sufficient quantities of the enzymes associated with metabolism during the first few hours post fertilisation (hpf), activation is carried out using a Mammalian metabolic Activation System (MAS). The MAS usually consists of liver microsomes from a mammal that contain the metabolising enzymes. The embryos are incubated in a medium containing the proteratogen and the MAS, allowing formation of and exposure to the active metabolites (Busquet *et al.*, 2008). Busquet *et al.* (2008) exposed zebrafish embryos at 1-2 hpf to a concentration series of cyclophosphamide with MAS as well as controls containing just cyclophosphamide and just MAS for a period of one hour. The embryos were then removed from the exposures and monitored and scored at 8, 24 and 48 hpf for a range of endpoints. Significant numbers of coagulated (mortality) and teratogenic eggs with a range of deformation endpoints were

observed at the highest concentration of cyclophosphamide plus MAS at 28mM (~8000 mg/l) (Figure 38). The majority of the deformities were observed in the embryos heads, (chart A, Figure 38).

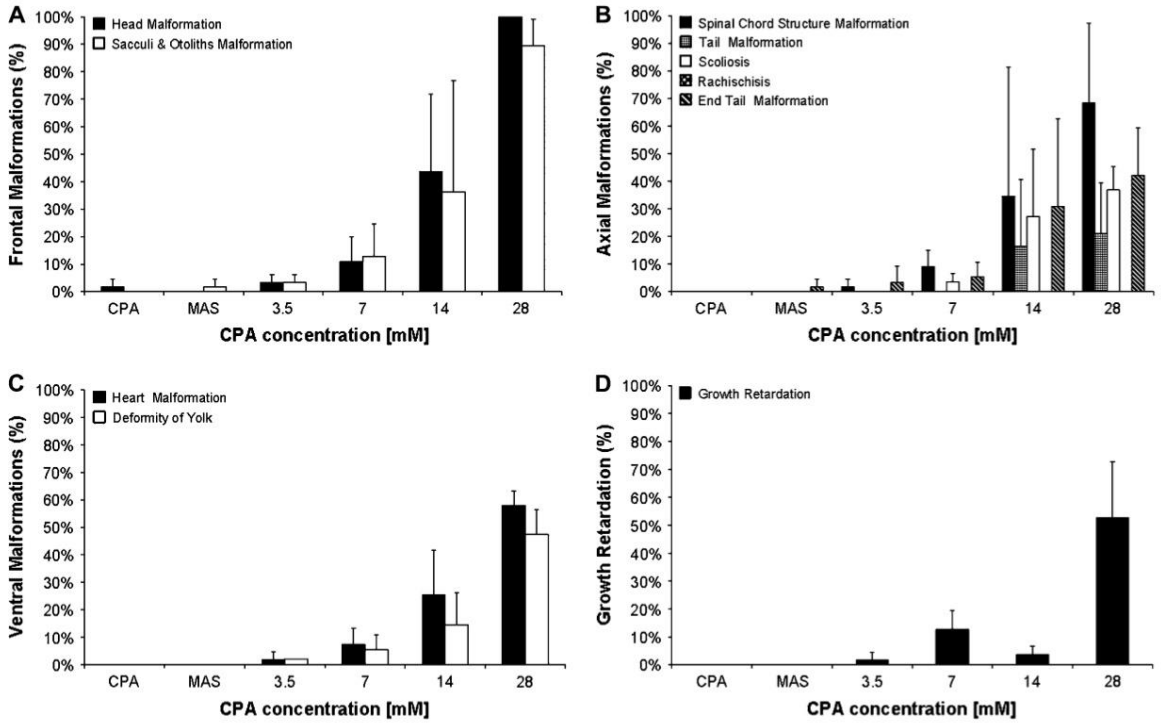


Figure 38: Results of the teratogenic screen experiment using zebrafish embryos exposed to cyclophosphamide (CPA), conducted by Busquet et. al. (2008). The four graphs show the mean percentage of each type of deformation and where relevant, where on the embryo body these were located. MAS is the mammalian metabolism activation system control (Busquet et al., 2008).

Little and no significant response was observed in the cyclophosphamide control (no MAS present), although it could not be established from the paper what concentration this was at. If the intention was to show that MAS was required for effects to occur, then it is assumed that it would be at the highest concentration of 28mM. The MAS control was required as this can be toxic to the embryos, which was why the exposure period was only one hour.

Similar experiments were conducted by Weigt *et. al.* (2010). They also used cyclophosphamide as a control under the same conditions. Their findings supported those of Busquet *et. al.* (2008), that this drug was only toxic to zebrafish embryos at 1-2 hpf if exposed in conjunction with MAS. In this experiment this was at a concentration of 10mM (~2800 mg/l). In a subsequent experiment Weigt *et. al.* (2011) exposed embryos for three days, from 1-3 hpf to 3 days post fertilisation (dpf), at which point the embryos generally

hatch. A concentration series of 1 – 10 mM (~280-2800 mg/l) with spacing of 2mM between concentrations of cyclophosphamide without MAS was used, with observations taken at 8 hpf and then every 24 hours. All concentrations produced teratogenic effects and mortality rates that were significantly different from the controls, in a concentration dependent manner (Table 29). Malformations were seen in the head, eyes, ears, spine, tail and yolks. This supports the position that zebrafish embryos in the first few hours post fertilisation do not possess the enzymes required to metabolise xenobiotic chemicals.

Table 29: Percentage effects of a concentration series of cyclophosphamide on malformations and mortality in zebrafish embryos, after three days of exposure.

	Normally developed embryos (%)	Embryos with teratogenic effects (%)	Embryos with lethal effects (%)	Affected embryos (%)	Number of replicates
Tris-buffer	98.0 ± 2.5[a]	1.3 ± 2.3	0.7 ± 1.8	2.0 ± 2.5	15
Cyclophosphamide					
1 mM	90.0 ± 0.0	10.0 ± 0.0	0.0 ± 0.0	10.0 ± 0.0**	3
3 mM	71.7 ± 2.9	25.0 ± 5.5	3.3 ± 2.9	28.3 ± 2.9**	
5 mM	11.7 ± 10.4	58.3 ± 7.6	30.0 ± 13.2	88.3 ± 10.4**	
8 mM	8.3 ± 7.6	56.7 ± 10.4	35.0 ± 8.7	91.7 ± 7.6**	
10 mM	0.0 ± 0.0	33.3 ± 10.4	66.7 ± 10.4	100.0 ± 0.0**	

With regards to the experiments conducted for this thesis, the above experiments used developmental endpoints that are unlikely to be relevant to adult fish. However, the highest concentration of 2000 µg/l in these experiments was two orders of magnitude below the lowest concentration (280,000 µg/l) used in the experiments reported in the literature. This would suggest that significant results were unlikely to be observed in the experiments in this project.

4.4.1.3 Cisplatin

The only significant result in the pilot study was an increase in the mean gill somatic index of female fish exposed to cisplatin. This drug is associated with kidney toxicity in patients (Arany and Safirstein, 2003). It has been shown to cause damage to kidney, liver and eye tissue in rats, with the mechanism of action attributed to oxidative stress (Nazıroğlu *et al.*, 2004). Whilst there are several papers in the literature on the effects of oxidative stress on the gills of freshwater fish, they tended to assess this damage from a morphological perspective using histopathology. In the experiments for this project, the gills were weighed and then flash-frozen in case the time was available to conduct PCR on these tissues. This meant that it was not possible to conduct histopathology on these tissues. The person dissecting these tissues was asked to report any abnormalities, of which none were

recorded. If oxidative stress was a factor, no reason was found in the literature as to why this would cause an increase in the gill somatic index. Chemical analysis was only conducted on the samples of the aquaria water taken at the beginning of the experiment, and showed actual concentrations to be 75% of the nominal (Table 27), 5% below the 80% limit defined in the OECD guidelines (OECD, 1984), but nevertheless still at actual concentrations relatively similar to the nominal values.

Again, there were no significant results observed in the cisplatin concentration series. As with the cyclophosphamide experiment, this used the more robust design of three replicates for the control group. Chemical analysis was done for all samples and the dosing stocks. The one and ten µg/l concentration tanks had actual values around 70% lower than the nominal, with the latter comparable to the actual value from the pilot experiment. The 100 µg/l was on the 80% limit defined by the guidelines. This was reflected in the concentrations of the dosing stocks. Regardless of whether these decreases were due to dosing errors or degradation, these results would not account for why the significant results observed in the pilot were not repeated in the concentration series. As with the other drugs, biological variability could explain this discrepancy.

In the cisplatin single chemical component of the simple mixture experiment, a significant increase in the mean condition factor of male fish and in the mean hepatosomatic index of the females was observed. Neither of these endpoints had displayed significant changes in any of the other cisplatin experiments. Furthermore, no associated change was observed in the simple mixture group. As with the pilot experiment results, oxidative stress could be a factor. However, as noted, there was no evidence in the literature to support this. Again these statistically significant results could be attributed to biological variability.

The results of the fish plasma model ($F_{SS}PC$ = 14.10 ng/ml and ER = 57 at the highest concentration of 100 µg/l in the experiment) suggest that the drug was not exposed at a concentration sufficiently high enough to cause significant effects, providing support to the biological variability argument for the significant effects observed. As with the other two

drugs, the ERs for the mean MEC and PEC (113 and 10,911,555)[4], along with the results from the experiments conducted in the project indicate that cisplatin is not likely to pose a threat to fish, at environmentally relevant levels.

Morphological effects of Cisplatin on fish in the literature

The literature that was found focused on cisplatin toxicity to the hair cells (ototoxicity) found in the inner ear and the lateral line of zebrafish larvae. It should be noted that ototoxicity has been attributed to reactive oxygen species damage (Rybak *et al.*, 2007), rather than the cytotoxic mechanism of action attributed to cisplatin. However, reviewing this literature was considered useful to understand at what concentrations of cisplatin this effect occurred.

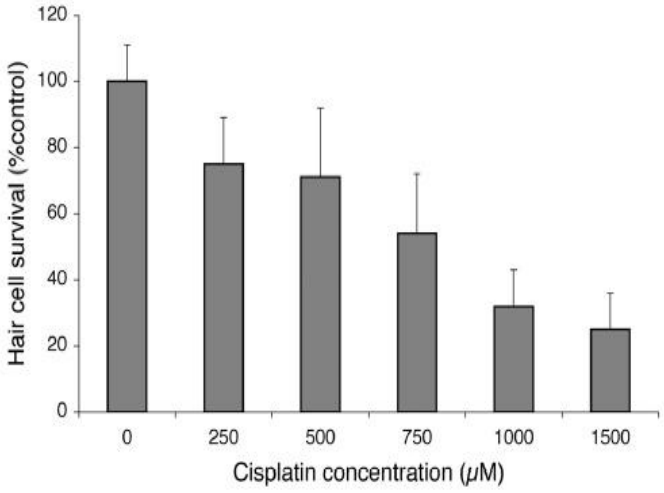


Figure 39: Percentage lateral line hair cell survival in 5 dpf zebrafish larvae exposed to cisplatin (Ou et al., 2007).

Ou *et. al.* (2007) visually observed lateral line hair cell death *in vivo* when fish were exposed to cisplatin. The lateral line hairs of 5 days post-fertilisation (dpf) fish were stained with a fluorescent dye before the larvae were exposed to a concentration series of cisplatin from 250 µM (~75 mg/l) to 1500 µM (~450 mg/l) in 0.25 mM steps, for four hours. The fish were then killed, fixed and the lateral line observed under a fluorescent microscope. The number of surviving hair cells in the lateral line was observed to decrease with concentration (Figure 39). No general toxicity, mortality or effects on other cell types were observed.

[4] The large difference between the two ER values is due to the only MEC values found in the literature being measured in hospital effluents, where concentrations are predicted to be far higher than those occurring in surface waters.

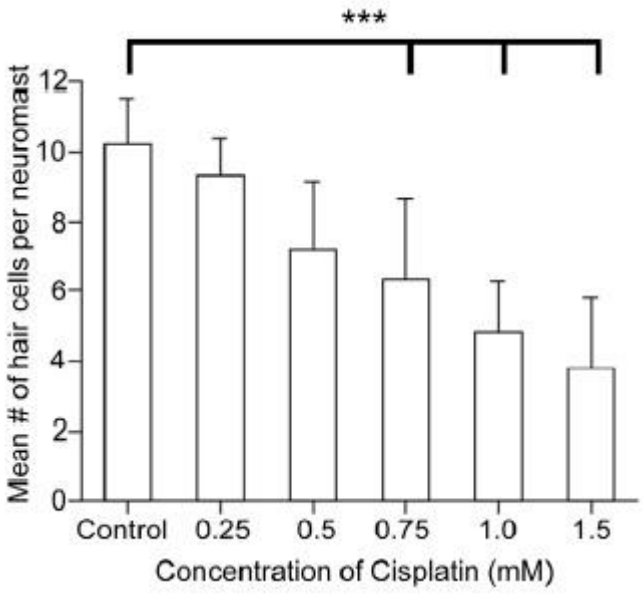


Figure 40: Mean number of hair cells per site on the lateral line of zebrafish larvae at 5 dpf exposed to a concentration series of cisplatin (Uribe et al., 2013)

Another study conducted a similar experiment involving exposing 5 dpf larvae to the same concentrations series of cisplatin (Uribe *et al.*, 2013). These larvae were from a transgenic strain that expressed green fluorescent protein under the control of the Brn3c promoter/enhancer. How this preferentially tagged hair cells was unclear. A cursory search of the literature suggested Brn3c is involved in retinal ganglion cell expression. However, this allowed visualisation of the lateral line hair cells in the same manner as Ou *et. al.* (2007). Again cisplatin was observed to be toxic to lateral line hair cells in a concentration-dependent manner. Statistical analysis identified that this decrease in the number of hair cells became significant at 0.75 mM cisplatin.

Loss of lateral line hair cells would impact on the fish's ability to orient itself in the water and to school with other fish. Although this was not empirically observed in the exposure experiments conducted in this project, fish behaviour was monitored on a daily basis before and after feeding. No unusual behaviour was observed in any of the fish exposed to cisplatin (or any of the other drugs or simple mixture). However, the highest concentration used in the thesis was 100 µg/l, whereas a reduction in lateral line hair cells was observed at ~75,000 µg/l, with statistical analysis finding this reduction to be significant at ~225,000 µg/l. Therefore, it would be unlikely that any behavioural response to a loss of lateral hair line cells would have been observed.

None of the significant effects observed in fish and reported in the literature occurred with exposure to cisplatin at anywhere near the concentrations of this drug likely to be present in the environment. This supports the findings of this project.

4.4.1.4 Mixture experiment

As described above, significant responses in several of the endpoints were observed in the single chemical exposure components of this mixture experiment. For example, significant increases were observed in the hepatosomatic and gonadal somatic indexes of fish exposed to cyclophosphamide and also in the hepatosomatic index and condition factor of fish exposed to cisplatin. However, no significant response in any of the endpoints was observed for either sex in the fish exposed to the simple mixture of the three drugs. Given the results of the other experiments in this project, biological variability could be attributed as the cause.

Another argument that is specifically relevant to a mixture, is that one or more of the drugs were acting antagonistically against each other, so that no significant effects were observed in the mixture. An initial step in assessing this would be to compare the metabolism pathways for each of the drugs. A thorough analysis is beyond the scope of this thesis but, for example, it is common for drugs to be enzymatically metabolised by one or more of the P450 enzymes (Hendriks-Balk *et al.*, 2007). However, of the three drugs tested, P450 enzymes only appear to be involved in the metabolism of cyclophosphamide (Hignite and Azarnoff, 1977). The 5-fluorouracil pathway is illustrated in Figure 3 and cisplatin requires two hydrolysis reactions to achieve its active form (Dickenson *et al.*, 2011). From this brief review, there would appear to be no evidence of competition for metabolism pathways between the three drugs. In terms of the mechanisms of action, both cyclophosphamide and cisplatin work by forming cross links in and across DNA strands, causing double strand breaks that are cytotoxic. However, unlike drugs that have mechanisms of action that require interaction with an active site of an enzyme, these two drugs do not have to compete for sites to form cross-link. One of 5-fluorouracil's mechanisms of action involves enzyme interaction (down-regulating the production of thymine by inhibiting thymidylate synthase). Nevertheless, this would not appear to be antagonistic to the other two drugs.

Further evidence that the lack of response in the mixture group is not due to antagonism is supplied by the fact that these drugs are often used together to treat cancer. Several of the drug regimens that use different combinations of two of the three drugs are described in section 1.5. An example of a regimen that includes all three (and three other anti-cancer

drugs) was found in the literature. Called CHAMP-5, it was used in a trial to treat ovarian cancer (Pasmantier *et al.*, 1985). Whilst it was found to be effective, this effectiveness was no more than occurred when three of the drugs (of which one was cisplatin) were administered singly. Without a full pharmacokinetics and pharmacodynamics assessment, it is unknown if antagonism was a factor in explaining why the combination tested was no more effective than the single drugs. However, given CHAMP-5 was effective, if the results observed in the single chemical component of the mixture experiment were due to the effects of the drug, then it would not be unexpected to see this in the mixture. As this was not the case and given the inconsistency in the occurrence of the significant morphological results in the other experiments, natural biological variability between the groups of fish are a more plausible explanation.

Morphological effects of anticancer drug mixtures on fish in the literature

No reference to fish morphology of any species with regards to exposure to mixtures of anticancer drugs was found in the literature.

4.4.2 Zebrafish morphology conclusions

The null hypothesis states that: "The selected cytotoxic pharmaceuticals, at the tested concentrations, will not cause significant effects on the adult zebrafish morphology observed".

For any of the single drugs or the mixture, it was not possible to reject the null hypothesis.

None of the drugs tested appeared to produce significant effects on the morphological endpoints observed in adult zebrafish, at the exposed concentrations. Although there were some significant results in the pilot experiment and in the single drug groups in the mixture experiment, these were not repeated in the single drug concentration series, which were of a more robust experimental design. Where significant results were observed, biological variability is a more plausible explanation. This appears to be supported by those studies found in the literature, where any significant effects on zebrafish morphology were observed at much higher concentrations.

4.4.3 Blood endpoints

For a variety of practical reasons, blood endpoints were only assessed in the pilot experiment and the 5-fluorouracil concentration series. Of the blood endpoints listed in Table 25, only blood cell viability was assessed.

4.4.3.1 Pilot experiment

Automated haemocytometry, the counting of the number of cells in a sample and the calculation of the percentage that were non-viable, was carried out for the pilot experiment. Statistical analysis showed no significant difference in the number of cells in the blood of the exposed fish, compared to that in the controls. This method of automated haemocytometry does not differentiate between cell types, so if any change in the number of cells had been observed, it would not have been possible to assess if this was due to a reduction in a particular type or types of blood cell. In addition, thrombocytes would have been too small to have been counted. In humans the relative proportion of each cell type is well known (Table 30). This is not the case in zebrafish, due to their small size and technical differences in the assessment techniques employed (Carradice and Lieschke, 2008).

Table 30: Normal range of the number of cells of each type in human whole blood (O'Sullivan, 2013).

Cell type	Male ($x10^9$/l)	Female ($x10^9$/l)
Red Blood Cells	4.5-6.5	3.8-5.8
Neutrophils	2.0-7.5	2.0-7.5
Monocytes	0.2-0.8	0.2-0.8
Lymphocytes	1.0-4.5	1.0-4.5
Eosinophils	0.04-0.40	0.04-0.40
Thrombocytes	<0.1	<0.1

Cytotoxic drugs have been shown to cause a range of haematoxic effects in humans (Table 31). If a reduction in cell number had been observed in the zebrafish in these project experiments, this may have indicated one or more of these effects had occurred. However, haematopoiesis varies greatly between these two species. Blood cells are formed in the bone marrow in mammals, whereas this occurs in the kidney in zebrafish (Davidson and Zon, 2004). For cyclophosphamide at least, the relevant conditions in Table 31 are due to myleosuppression (Emadi *et al.*, 2009), a process that would not affect blood cell production in zebrafish.

What was observed was a significant increase in the number of non-viable cells in those fish exposed to 5-fluorouracil, when compared to the control group. This was not accompanied by a reduction in the number of cells, so did not appear to suggest any of the conditions in Table 31. As the majority of blood cells, including erythrocytes, are nucleated in zebrafish, it could be argued that this reduction in cell number was due to the cytotoxic mechanisms of action associated with 5-fluorouracil. However, due to the relatively weak design of the pilot (only one concentration and no replicates), the results of the 5-fluorouracil concentration series were required to further evaluate these findings.

Table 31: Haemotoxic effects of the three cytotoxic drugs reported in humans.

Haemotoxic effect	Description	Drug
Anaemia	Low number of red blood cells	5-fluorouracil (Kennedy, 1999) cyclophosphamide (Emadi *et al.*, 2009) cisplatin (Wood and Hrushesky, 1995)
Leukopenia	Low number of white blood cells	5-fluorouracil (Kennedy, 1999) cyclophosphamide (Emadi *et al.*, 2009)
Neutropenia	Low number of neutrophils	cyclophosphamide (Emadi *et al.*, 2009)
Thrombocytopenia	Low number of platelets	cyclophosphamide (Emadi *et al.*, 2009)

The only other significant result in the pilot experiment was a reduction in the percentage of non-viable cells (i.e. an increase in the percentage of healthy blood cells compared to the control fish) in the fish exposed to cisplatin. It could be that another side effect of

cisplatin, nephrotoxicity (Arany and Safirstein, 2003), could interfere with zebrafish blood cell production. However, no clear argument could be made as to why this could produce a reduction in non-viable cells (Figure 33). As with the significant results observed for the morphology endpoints, natural biological variability could also be the cause, which could also be argued for the 5-fluorouracil results.

No information on the effects of these three drugs on fish blood could be found in the literature. For comparison, the significant effects on fish morphology that were found in the literature occurred at much higher concentrations than were used in this project. A lack of effects, for cyclophosphamide at least, could be due to this factor. According to Emadi *et. al.* (2009) in humans, neutropenia is occasionally observed at what constitutes a 'low' therapeutic dose of 1-3 mg/kg, although frequency of the occurrence of neutropenia increases with dose. Leukopenia, thrombocytopenia and anaemia are commonly observed at high doses, defined as >120 mg/kg. To provide some comparison an overly simplistic calculation, using the average weight of 0.85 g for the zebrafish exposed to cyclophosphamide in the Pilot experiment, this would equate a high human dose of 120 mg/kg to a dose of 0.102 mg (102 µg) for a zebrafish. In the Emadi *et. al.* (2009) paper this dose was administered intravenously over 2-4 days. In this project, zebrafish were exposed to a maximum actual concentration of 323.85 µg/l (2000 µg/l nominal) continuously for 14 days, three times the equivalent human dose calculated above. However, this would have required transfer of cyclophosphamide into the blood stream via the gills, a process far less efficient than intravenous administration. Uptake via the gills is dependent on the physiochemical parameters of the drugs, in particular the drug's hydrophobicity/hydrophilicity. The fish blood plasma model takes this into account by using the $LogD_{7.4}$ of the drug, when calculating the fish steady state plasma concentration. According to Joy *et al.* (2012) the mean Cmax achieved in that study of 12,210 ng/ml (The value used for the H_TPC in the fish blood plasma model calculations) was as a result of a dose of 0.7 g/m^2. Using the average surface area for a UK adult of 1.79 m^2 (Sacco *et al.*, 2010) and the average European adult weight of 70.8 kg (Walpole *et al.*, 2012), this dose was equivalent to 17.7 mg/kg. The fish blood plasma model calculated that to achieve the H_TPC of 12,120 ng/ml in fish would require a water concentration of 78,292 µg/l. As this concentration is far higher than the highest actual concentration of 323.85 µg/l of

cyclophosphamide that zebrafish were exposed to in this project, it is extremely unlikely that any haemotoxic effects would have occurred in the exposed fish.

4.4.3.2 5-fluorouracil

The decision was made to change the blood cell viability assay to use imaging flow cytometry, as this method is more accurate. Fish exposed to 4 µg/l of 5-fluorouracil were observed to have a significantly lower mean number of nucleated blood cells, compared to the control group. However, this difference was not seen in any of the higher concentrations. Given the low dose and the literature findings that significant effects on morphological endpoints were only observed at much higher concentrations, this reduction is more likely to be due to biological variability than exposure to 5-fluorouracil.

The mean number of non-viable cells was significantly higher for all concentrations, when compared to the control group. This difference increased in a concentration dependent manner. These findings agreed with the results for 5-fluorouracil from the pilot experiment. However, the percentage of non-viable cells in the pilot experiment was eleven percent compared with around 0.04 percent in this experiment. This difference could be attributed to the way that a non-viable cell is marked in each assay. In automated haemocytometry, the cytoplasm of the non-viable cell is stained. With imaging flow cytometry, it is the nucleus that is stained. The latter technique is considered more accurate as it counts every cell in a given volume of sample, rather than estimating this from an image as occurs with automated haemocytometry.

Observing these results in a concentration series, conducted with two replicates per treatment, gives more confidence in the findings of this experiment. It also lends credibility to the complementary results in the less robust pilot study. As with the pilot study, these results would suggest it is the cytotoxic mechanism of action of 5-fluorouracil that is the cause. Damage to the nuclear DNA is overwhelming the DNA repair enzymes ability to repair the damage. This can lead to apoptosis (programmed cell death) due to an accumulation of double-strand breaks (Longley *et al.*, 2003). However, the dominant mechanism of action of 5-fluorouracil is the inhibition of thymidylate synthase. This enzyme

is crucial to the production of thymine. Through its inhibition, the pool of available thymine falls below the cells ability to repair and replicate, leading to the death of the cell.

Only one study involving the effects of 5-fluorouracil on fish blood cells was reported in the literature. Kovács *et al.* (2015) reported significant DNA damage in the nucleated blood cells of F1 generation adult zebrafish from a two generation study exposing to 0.1, 1.0 and 100 µg/l of 5-fluorouracil. However, the significance was only observed in the 1.0 µg/l group, although significant damage was seen in the liver of fish in the highest two concentrations. Whether the amount of DNA damage observed in the 1.0 µg/l was high enough to produce non-viable cells is unknown. The lack of significant results observed in the highest concentration is also in contrast with the concentration-dependent response seen in the 5-fluorouracil concentration series experiment in this project (Figure 41).

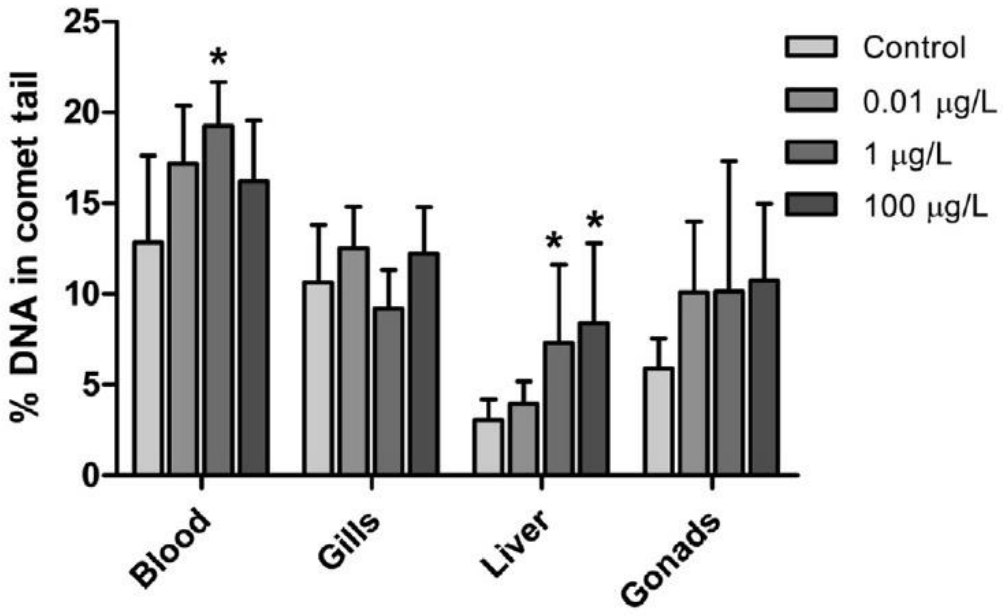


Figure 41: DNA damage in the nucleus of a range of tissues from F1 adult zebrafish exposed to a concentration series of 5-fluorouracil as part of a two generation study, sampled at seven months old. (Kovács et al., 2015)

Imaging flow cytometry analysis was also conducted on whole blood samples from the repeat 5-fluorouracil partial concentration series. The mean number of nucleated blood cells was significantly smaller in the control than either of the two exposed groups. Conversely, the mean percentage of non-viable cells was significantly higher in the control than in the exposed groups (Figure 35). The boxplot in Figure 36 illustrates the variability

in the percentage of non-viable cells between the samples in the control group. The control tank was sampled first on the sampling day. Analysis of the raw data (not shown), revealed that the largest number of non-viable cells was observed in the first control sample analysed. This consistently reduced in the subsequent samples in an exponential manner so that the last control fish to be sampled had a percentage of non-viable cells at the same order of magnitude as the exposed groups of fish that were sampled subsequently. The pattern of data observed in the two exposed groups, showed very little intra or inter-group variation. This pattern of intragroup variation is similar to that observed in the previous 5-fluorouracil concentration series experiment.

To have such a high percentage of non-viable cells in the control group is highly unlikely to be due to the experimental conditions, and could more likely be attributed to a technical artefact of the sampling process. A possible cause is contamination of the samples during the Draq7 staining process. Several assays were being initiated on the sampling days. The adjacent work station was producing whole blood smears on microscope slides. Part of the protocol required the washing of slides in ethanol. Ethanol has been shown to affect the permeability of cell membranes. It is possible that samples could have in some way been contaminated with ethanol, as this was being used on an adjacent work station, during sampling at end of the experiment. Cells with permeable membranes would have been able to take up the Draq7 stain and would have been identified as non-viable cells during the Imaging Flow Cytometry analysis.

4.4.3.3 Cyclophosphamide

Samples of whole blood were both prepared for the micronucleus assay and processed through the ImagestreamX. As described above, the partnership to aid in analysis of the micronucleus assay did not come to fruition. The Imaging Flow Cytometry analysis was not attempted until several months after the experiment was completed. At this time, it was discovered that the hard drive on which the data was stored had corrupted and in the meantime the backup on the computer connected to the ImagestreamX had been wiped. Therefore, there were no data available to assess cyclophosphamide's effects, if any, on zebrafish nucleated blood cells.

4.4.3.4 Cisplatin & simple mixture

Initial analysis of the PCR data suggested that there were significant differences in Vitellogenin RNA expression between control and exposed groups in some of the experiments run with the other two drugs, before the cisplatin and simple mixture. It was not possible to assess whether these changes were reflected in the abundance of Vitellogenin protein for the prior experiments as no suitable material remained. However, in case these findings were repeated for the cisplatin and/or simple mixture experiments, blood plasma was collected for use in ELISA assays that could quantify Vitellogenin abundance in exposed fish compared to the controls. Due to the small volume of zebrafish whole blood, the decision was made to only collect samples for Vitellogenin protein analysis and not conduct the imaging flow cytometry assay (the micronucleus partnership negotiations had failed by this point). Unfortunately, time and cost constraints meant it was not possible to complete the ELISA assay and data analysis before the Ph.D. deadline.

4.4.4 Zebrafish blood endpoint conclusions

The null hypothesis states that: “The selected cytotoxic pharmaceuticals, at the tested concentrations, will not cause significant effects on the zebrafish blood endpoints observed”.

For the impact of 5-fluorouracil on the viability nucleated blood cells it was possible to reject the null hypothesis.

For all other end points for 5-fluorouracil and all end points for the other single chemicals and the simple mixture, it was not possible to reject the null hypothesis.

5-fluorouracil appears to increase the number of non-viable cells in a concentration-dependent manner. Although the results of the pilot and 5-fluorouracil concentration series experiment support each other, the former was a less robust design and used a different assay to assess this endpoint. The lack of supporting evidence from the repeat 5-fluorouracil partial concentration series is disappointing, albeit most likely due to a technical error rather than a biological response to the experimental conditions. An ideal next step would be to repeat this exposure and assay to see if the results are repeatable.

Although no significant results were observed for the other drugs in the pilot experiment, the lack of data from the relevant concentration series experiments was not available to allow adequate testing of the null hypothesis. It would be useful to repeat the cyclophosphamide, cisplatin and simple mixture experiments and conduct the imaging flow cytometry assay on the viability of nucleated blood cells in the exposed fish.

5 Impact of cytotoxic drugs on mRNA expression in adult zebrafish

5.1 Introduction

DNA is common to all known life forms, providing the blueprint for both the replication of cells within an organism and the production of offspring, and as such is critical to all organisms. There are many naturally occurring processes that can damage DNA, such as exposure to ultra-violet light and interaction with reactive oxygen species. To counter this, cellular mechanisms have evolved to identify and repair the damage. In the worst case these processes ensure that an irreparably damaged cell is destroyed in a controlled way using processes such as apoptosis – programmed cell death (Kaina, 2003). This prevents the cell from producing mutated daughter cells with potentially harmful effects on the organism or its offspring.

As described in section 1.4, the mechanisms of action of all three of the cytotoxic drugs in some way damage DNA. 5-fluorouracil primarily does this by preventing the production of thymine, one of the four amino acids that are the building blocks of DNA. This prevents the cell from being able to repair or replicate. An additional mechanism of action of 5-fluorouracil is the ability of its metabolites to replace thymine in RNA and DNA strands, which can also lead to double strand breaks. Cyclophosphamide and cisplatin form cross-links in and between the two strands of DNA. These must be removed to allow replication to proceed. Removal results in breaks in the DNA strands that require repairing. Too many of these cross-links overwhelm the repair machinery, resulting in breaks in both of the DNA strands. These are known as double strand breaks (DSB) and are highly toxic to cells. Just one double strand break can be fatal for a cell if it deactivates an essential gene, causing the cell to initiate apoptosis (Khanna and Jackson, 2001).

There are many enzymes involved in the repair of DNA, with multiple processes for conducting this, depending on what type of damage has been caused. Two of these were chosen as targets for the approach used in this chapter. RAD51 is an enzyme associated with the repair of double strand breaks. It tags the site of a break, allowing other enzymes to locate and repair them. p53 is primarily known as a tumour suppressor gene, a process it achieves by identifying sites of DNA damage (in certain circumstances in co-ordination with RAD51), arresting the cell cycle to provide more time for repair or, if damage is

unsurmountable, initiating cell death to prevent replication. More information on these enzymes and why they were selected for study can be found in section 5.2.

It is known that when a cell is exposed to a DNA-damaging agent, there are a large number of genes that are up-regulated, resulting in an increase in the proteins they code for in the cell (Friedberg, 2003). It was hypothesised that RAD51 and p53 would be members of this group, given their functions. It was confirmed that the genes for these proteins were present in the genome of zebrafish (section 5.4.3). A well-established technique (qPCR, section 5.4.5) exists for quantifying the amount of RNA that is transcribed from these genes, in order to produce the proteins. Using qPCR, the relative amounts of mRNA for the genes of interest between the control and exposed groups is examined, and it is assumed that differences in amount of transcript also indicate a difference in the amount of the expressed protein.

As a significant proportion of these drugs were likely to accumulate and be metabolised in the liver of the zebrafish, the expression of the genes in this organ were measured. As it was considered possible that cytotoxic drugs could affect the liver morphology of the zebrafish, an additional protein that is normally produced in this organ was included for assessment. Vitellogenin is an egg yolk precursor protein produced in the liver of most oviparous species, including fish. While the amount produced in female fish is much higher, it is also found in the liver of male fish. If the cytotoxic drugs were to have effects on the liver, vitellogenin gene expression may have been affected.

5.1.1 Aims

- Assess effects of 5-fluorouracil, cyclophosphamide and cisplatin, both singly and as a simple mixture, on the expression of genes that codes for the DNA repair enzymes RAD51 and p53 and the egg yolk precursor protein vitellogenin.

5.1.2 DNA damage enzymes RNA expression hypothesis

“The selected cytotoxic pharmaceuticals, at the tested concentrations, will cause significant effects on the level of expression of the RNA that codes for the target proteins”.

5.2 Selection of target and housekeeping genes

The original intention for molecular assessment in this project was to adapt an immunohistochemistry protocol that used high-throughput imaging flow cytometry. This technique was originally developed to detect double strand breaks in human cells exposed to DNA damaging agents, such as gamma radiation and cytotoxic drugs. Primary antibodies (called primary as they bind to the protein of interest) were created that specifically bound to mammalian DNA repair enzymes in the nucleus of cells exposed in vitro to the DNA damaging agents. The cells are then incubated with a secondary antibody that contains a fluorophore such as Phycoerythrin (emits red light when stimulated by specific wavelengths of light), which bind specifically to the primary antibody. The samples are run through an imaging flow cytometer, which simultaneously takes images of each cell in the visible band of light, and at the specific wavelength that stimulates the fluorophore. Software analysis of the resulting data set allows gating out of unwanted cells and material and the identification of those cells that have been tagged by the antibodies. Furthermore, it is able to quantify the amount of bound DNA repair enzyme and display visually the locations of the enzymes within the cells nuclei.

RAD51 is a repair enzyme that is known to be involved in the repair of double strand breaks, as part of a process called Homologous Recombination (HR) (Mao *et al.*, 2008). Multiple copies of the RAD51 enzyme congregate at the site of a double strand break, forming foci that indicate the presence of the breaks to other repair enzymes (Figure 42). These repair enzymes then use the matching section of DNA from the homologous chromosome to repair the break. When the antibodies are applied and imaged, these RAD51 foci show up as bright dots within the cell nucleus. This allows the number of double strand breaks to be quantified (Figure 42).

Support for the choice of RAD51 as a DNA repair gene in zebrafish was found in the literature. Liu *et al.* (2012) assessed the repair processes and genes involved in Double Strand Break (DSB) repair in zebrafish embryos. As described previously, RAD51 is a key part of the Homologous Recombination (HR) repair pathway. Liu *et al.* (2012) were able to initiate specific DSBs that triggered three repair pathways including HR. It found that Non-Homologous End Joining (NHEJ) was the dominant repair pathway, but that HRs prevalence

would increase if NHEJ was impaired by the knockdown of a key repair enzyme in the NHEJ pathway. Of particular relevance to this thesis, HR was shown to decrease if RAD51 was the knockdown target. This gave some confidence that this enzyme played a role in DNA repair in zebrafish.

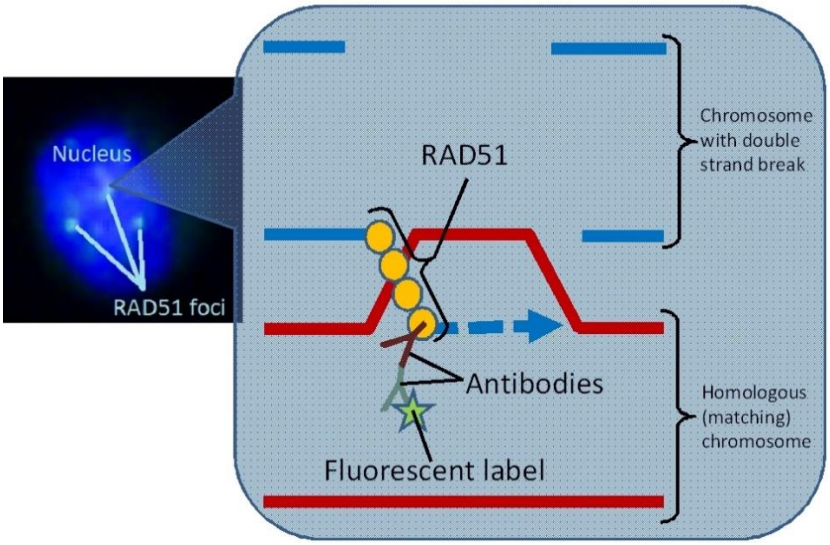


Figure 42: Visualisation of RAD51 foci within the nucleus of a human cell exposed to a DNA-damaging agent, and a diagrammatic representation of its role within the DNA damage identification and repair process known as Homologous Recombination.

The initial aim of this project was to assess if it was possible to use immunohistochemistry for all three species. Unfortunately, it was not possible to complete the necessary method development work, and all the other tasks required by the European Union project that funded the Ph.D., within the time available. Indeed, so much time elapsed that it became clear that only the zebrafish could be assessed in greater detail. As can be seen in the chapters covering the algae and daphnia, only the standard exposure tests were conducted. However, it was considered important to have a molecular technique that assessed whether DNA damage was occurring within the exposed zebrafish. The micronucleus assay was a potential solution but, as described in the zebrafish morphology chapter, the partnership required to conduct the assay was not successful. It was therefore decided to use qPCR, as expertise in this technique was available at Brunel University. RAD51 had already been identified as a good candidate gene marker for DNA damage

assessment, therefore assessing the expression of this gene by qPCR might be a good indicator of cell damage/cytotoxicity.

Many enzymes are involved in DNA damage repair, so it was felt prudent to include one other. p53 is often described as a tumour suppressor, due to its role in preventing mutations that can lead to cancerous cells, through the prevention of DNA synthesis after DNA damage has occurred. It appears to have several roles in this process. Initial research showed that p53 plays a role in holding the cell cycle at the G1 checkpoint on detection of DNA damage (Figure 3). This gives the cell additional time to repair the damage. Kastan *et. al.* (1991) exposed human bone marrow cells in vitro to a range of DNA damaging agents. Using flow cytometry, they were able to show a significant increase in p53, as a rapid spike within 1-2 hours of gamma radiation exposure, with the level falling to half this increase by 10 hours post exposure, followed by a gentler decline to a fifty-hour time point, remaining at a greater elevation than the control samples throughout the exposure period (Figure 43). This increase correlated with a greater number of exposed cells held at the G1 checkpoint of the cell cycle. These findings were replicated by other research groups (Fritsche *et al.*, 1993; Coates *et al.*, 1995). Further research elucidated p53's involvement in the DNA repair process known as nucleotide excision repair (NER) (Smith *et al.*, 1995). This process is key in repairing both single strand and the more cytotoxic double strand breaks (Wakasugi *et al.*, 2014). If the amount of DNA damage is too great to be repaired, p53 has an additional role in permanently preventing the cell from passing on any deleterious genes or behaviour by aiding in the initiation of cell death through the processes of apoptosis, senescence or autophagy (Speidel, 2010; Vousden and Prives, 2009).

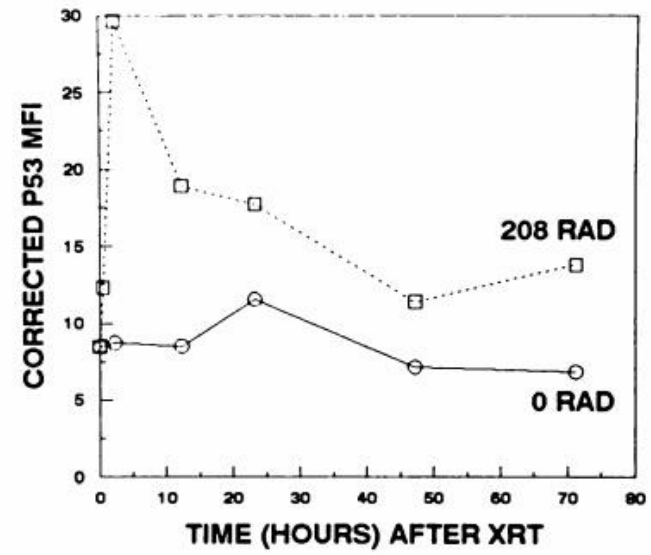


Figure 43: Amount of p53 tumour suppressor protein (dashed line) in human bone marrow cells over a 70-hour time period after initial exposure to gamma radiation (XRT), relative to a control group (solid line). The y axis is an arbitrary value that denotes a Mean Fluorescence Intensity (MFI) (Kastan et al., 1991).

Due to their vital functionality, DNA repair mechanisms were expected to be highly conserved across species. Indeed, p53 has been identified in a wide range of species, including several fish (Cachot *et al.*, 1998). However, it is more highly conserved in mammalian species (74%-90% similarity between species), whereas in vertebrates such as amphibians and frogs the similarity is much lower at between 38-53% (Embry *et al.*, 2006). Nevertheless, the areas of the gene coding for parts of the protein that are considered functionally critical are more highly conserved between fish species (Kazianis *et al.*, 1998).

The zebrafish liver was chosen as the source of mRNA for the repair enzymes. A large proportion of metabolism was expected to occur in this organ and therefore the greatest concentration of active metabolites would be present here. This had the potential to increase the opportunity for DNA damage to occur. In addition, this organ is large, providing adequate amounts of tissue, is relatively easy to dissect and was also being examined for morphological changes in this project. As was discussed in section 4.4.1, there was some indication that 5-fluorouracil might cause a reduction in the hepatosomatic index of exposed fish. To assess if the cytotoxic drugs were having an impact on the liver cell functions, it was decided to include Vitellogenin as another target for PCR analysis. This protein is an egg yolk precursor and is produced in the majority of egg-laying species, including fish. This protein is well studied in this group of organisms, due to the deleterious effects of environmental endocrine disrupting chemicals, which have been observed in freshwater species in developed countries over the last few decades.

As part of the quantification process, a way of normalising the expression of the genes of interest to take into account the natural biological variation in expression between fish was required. This is usually done by quantifying the expression of a gene that codes for a protein that is stably expressed within a cell. There are a range of genes that meet these criteria, which are often referred to as ‘house-keeping’ genes. They tend to take part in fundamental processes within the cell. For example, 18S is a protein that forms part of the ribosome, the organelle responsible for translating mRNA into proteins. Care must be taken to ensure that the experimental conditions do not perturb expression of the chosen housekeeping gene. It is good practice to select several genes and assess their expression level under all the experimental conditions. The stability of their expression can then be assessed visually by looking at a scatter plot of their Threshold Cycle (Ct) values or, more

robustly, using software to compare all housekeeping genes with the expression of each of the target genes.

Based on their use as house-keeping genes in other qPCR studies on zebrafish, 18S (Cooper *et al.*, 2006), along with ß-actin (Li *et al.*, 2012), which forms part of the cell's cytoskeleton and elongation-factor 1α (ef1α) (Chen *et al.*, 2005), that aids in routing tRNAs to ribosomes, were chosen as candidate housekeeping genes for this study.

5.3 Quantifying proteins of interest

When a cell experiences conditions that require an increase in a certain protein, the gene that codes for this protein is transcribed into messenger RNA (mRNA). The mRNAs are then translated into the associated proteins by organelles called ribosomes. The more of these proteins that are required, the more mRNA is produced. It should be noted that it cannot be considered a simple one-to-one relationship between the number of mRNA strands and the number of proteins, due to downstream processes that supress or enhance the amount of protein translated from the mRNA (Maier *et al.*, 2009). However, it still provides a relative comparison between the level of mRNA expression in control and exposed groups. It is therefore an indicator of the difference in amounts of target protein in the two groups.

Polymerase Chain Reaction (PCR) is a well-established molecular technique that has many variants and uses (Bartlett and Stirling, 2003). One application is that it allows the amplification of a specific gene from very small amounts of starting material, into an amount that allows quantification of the amount of gene transcript in the original samples, a technique known as quantitative PCR (qPCR). The qPCR process amplifies DNA, however for gene expression analysis the material available is mRNA. Therefore, the total RNA (mRNA, tRNA etc.) that is extracted from the zebrafish liver must first be reverse transcribed into DNA. This is conducted using a DNA polymerase that was isolated from a retrovirus, a family of viruses that replicate via reverse transcription of RNA from a host cell. This reverse transcribes a corresponding length of single strand DNA (ssDNA), known specifically as complementary DNA (cDNA). The RNA is then degraded to remove it from the cDNA using an RNase enzyme. The sample then contains cDNA corresponding to all the RNA that was in the liver sample. It is then ready for the qPCR step of the assay.

The cDNA sample contains strands of DNA that were reverse transcribed from all the RNA in the liver sample. To amplify just the DNA that codes for the protein of interest requires a way of specifically identifying it. Primers are short sections of synthetic DNA, also known as oligonucleotides. The nucleotides that form the primers are designed to be uniquely complementary to a section of DNA within the target sequence. These primers anneal (attach) to this section and provide a start position for the replication of the other strand of DNA. The resulting product is sections of double stranded DNA that contain sections of the gene of interest that are unique to this gene. The strands are denatured (separated) using heat and the process begins again. The solution in which the PCR processes are carried out contains the nucleotides that are required to build the DNA and another type of polymerase isolated from a thermophilic bacterium, which is stable at the high temperatures used in the PCR technique (Bartlett and Stirling, 2003). It also contains a dye called SYBR Green that binds strongly to double stranded DNA and emits a green light when stimulated with blue light. At the end of every replication cycle the amount of green fluorescence is measured. Around forty cycles of replication are completed, by which time the pool of primers and/or the other reaction components has been depleted and no further replication can occur.

The fluorescence detected is plotted to produce an amplification plot and takes the form of a sigmoidal curve (the top of the curve plateauing due to the depletion of primers and/or other reaction components described above). In the initial cycles of replication, the level of fluorescence remains unchanged, due to the very small amounts of DNA produced from the initially equally small amount of cDNA. There reaches a point where the amount of DNA produced and therefore the fluorescence detected, increases exponentially. The cycle at the point of the curve where exponential growth begins is the key piece of information from this technique. This is called the threshold cycle (Ct) and is the value that allows a relative comparison between samples. The lower the cycle number, the greater the amount of mRNA of the target gene that was present in the sample.

To account for biological variability between samples, the Ct values of the target gene are normalised against that of the housekeeping gene. In this project three housekeeping genes were selected: 18S, β-actin and ef1α. Their levels of mRNA expression were also assessed in the liver samples by qPCR and their stability of expression was then assessed *in*

silico. The housekeeping gene that was calculated as being most stable under all experimental conditions (control and increasing concentrations of drug) was used to normalise the levels of the target mRNA expression. This process is repeated for each drug, and may result in different housekeeping genes being selected for different drugs. As each sample is generally run through the qPCR stage in triplicate, the resulting value is described as the mean normalised expression of the target gene. The values for all samples in a group are themselves averaged and these values compared to the control group (and other concentrations if a concentration-dependent response is being assessed), as can be seen in section 5.5.

More information on the protocols and procedures outlined above are described in more detail in the following section.

5.4 Methods & materials

The following procedures were conducted to assess the level of the expression of the mRNA of the target proteins:

- Extract total RNA from the zebrafish livers collected from the experiment discussed in Chapter 4
- Design & optimise primer pair's specific to the genes of interest, using online tools
- Produce cDNA sequences for the target genes
- Run PCR assays to amplify cDNA of target genes
- Quantify relative gene expression between control and exposed zebrafish.

The following sections describe these procedures in more detail, including data from method development where relevant.

5.4.1 RNA extraction

Total RNA was extracted from the zebrafish livers collected after the single chemical and simple mixture experiments (Chapter 4). Extraction utilised the RNeasy Plus Universal Tissue kit (Qiagen Ltd.), following the manufacturer's protocol. Only the control and the group exposed to the highest concentration of drug from each experiment were assessed. The exceptions were the pilot and repeat 5-fluorouracil partial concentration series, where

all groups were sampled. Tissue samples were removed from the -80 °C freezer and kept on dry ice until use. Samples were homogenised with steel beads in the supplied lysis buffer in a TissueLyser II (Qiagen Ltd.) for 4 minutes at 20 Hz (rotating the caddy after 2 minutes to ensure equal homogenisation). Chloroform and genomic DNA (gDNA) eliminator solution were added and the solution centrifuged at +4 °C for 15 minutes. The resulting upper aqueous phase was removed to a separate tube and 70% ethanol added to precipitate the RNA. The solution was transferred to the supplied spin column and put through several short centrifugation cycles at room temperature, washing between each spin with the supplied buffers, before eluting in 50 µl of RNase-free water.

5.4.2 RNA quality assessment

An equal amount of RNA (0.3 µg) was required from each sample, so quantification was carried out using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc.). If a sample contained RNA at a concentration greater than the upper limit of detection of the NanoDrop, samples were diluted and reanalysed to ensure accurate measurement of RNA concentrations. Any samples with a 260:280 ratio <1.8 were excluded, as this ratio indicated potential contamination with extraction reagents, which could lead to overestimation of RNA concentration (Thermo Scientific, 2011)

Two samples from the control and each exposure concentration in the 5-fluorouracil, cyclophosphamide, cisplatin and Mixture experiments were selected for further assessment; one with the highest and one with the lowest 260:280 ratio. 1 µg of RNA per sample was made up to 20 µl in 6 x loading buffer containing 70% glycerol and Bromophenol blue. The samples were loaded into wells in 0.5% agarose non-denaturing Tris/Borate/EDTA (TBE) electrophoresis gels, containing Gelred stain. The use of gel red stain allowed visual assessment of RNA quality under UV light in a BioDoc Imaging System (UVP, LLC) (Figure 44). The gels were run for 45 minutes at 80V in 1 x TBE buffer. RNA quality was judged satisfactory if:

- The two bands relating to 28S and 18S were well defined and clearly visible.
- The upper 28S band was approximately twice as bright as the lower 18S band.
- There was no visible smearing in the lane below the 18S band, which would have signified the presence of degraded RNA.

Lane five of the cisplatin sample required additional dilution prior to cDNA synthesis, which was verified using the Nanodrop. Lane two of the 5-fluorouracil sample contained too little RNA, and was therefore not included in the experiment.

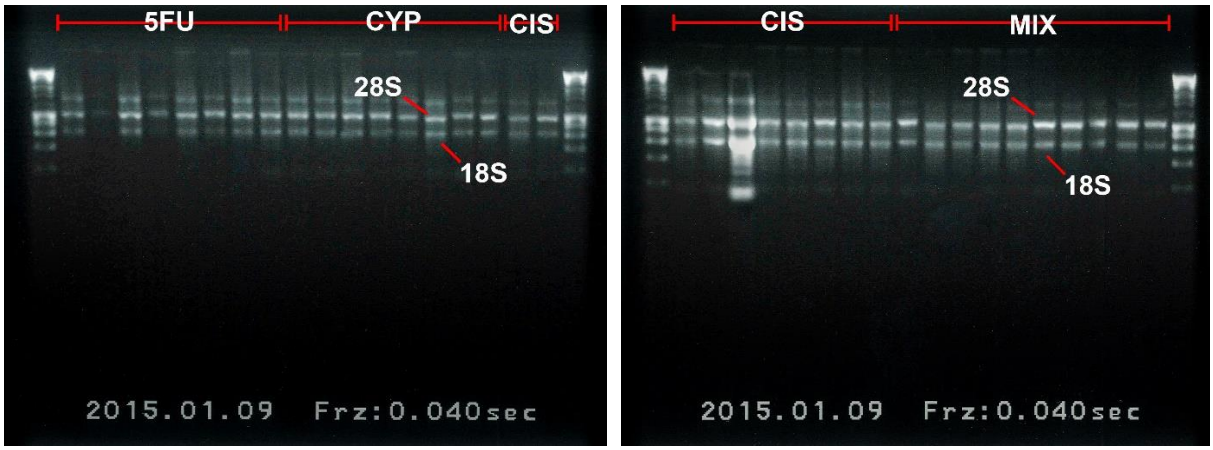


Figure 44: Photograph of an electrophoresis gel, run to assess RNA quality of the samples with the highest and lowest 260:280 ratios. The outside lanes of both gels contain 3 µl HyperLadder I (Bioline Reagents Ltd.). 5FU = 5-fluorouracil, CYP = cyclophosphamide and CIS = cisplatin.

5.4.3 RNA Primer design & optimisation

In order to design primers for the selected target and housekeeping genes, the gene sequence in the target organism must be known. In some cases, primers already existed in the literature, in others they had been created by a PHARMAS project partner (Table 32).

Table 32: Primer pair sequences used to amplify the target (p53, RAD51 & Vitellogenin) and housekeeping (ef1α, 18S & β-actin) genes. Primers for p53, RAD51 and β-actin were obtained from a PHARMAS partner institute, which had already produced these for their part of the research for the PHARMAS project.

Gene	Primer Pair	Source
p53	Forward: 5'- GGGCAATCAGCGAGCAAA Reverse: 5' - ACTGACCTTCCGAGTCTCC	Institute for Environmental Studies (IVM), VU University, Netherlands

RAD51	Forward: 5'- TGCTGCGTCTCGCTGA Reverse: 5' - GCCTCGGCCTCTGGTAA	Institute for Environmental Studies (IVM), VU University, Netherlands
Vitellogenin	Forward: 5'- AGCAACGAACAGCGAGAAA Reverse: 5' - ATGGGAACAGCGACAGGA	(Biales *et al.*, 2007)
ef1α	Forward: 5'- GCTTCTCTACCTACCCTCCT Reverse: 5' - ACCGATTTTCTTCTCAACGC	Designed in-house from Accession: AY422992.1
18S	Forward: 5'- GAGAAACGGCTACCACATCC Reverse: 5' - CACCAGACTTGCCCTCCA	(Xie *et al.*, 2011)
β-actin	Forward: 5'- CGAGCAGGAGATGGGAAC Reverse: 5' - CAACGGAAACGCTCATTGC	Institute for Environmental Studies (IVM), VU University, Netherlands

No good quality primers could be found for elongation-factor 1alpha (ef1α), so this primer pair was designed as follows. The ef1α gene sequence was searched for in the zebrafish genome stored in the National Centre for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore/). In this case the target gene had been annotated and was discoverable by searching using the gene name. If this had not been possible, the target gene would have been identified in another species (e.g. humans) and this sequence used to search the zebrafish database. As this gene codes for a protein that is involved in protein translation, it was likely to have been highly conserved across species.

The coding sequence for ef1α (as a FASTA file) was used to identify suitable primers by submitting it to the online primer design tool Primer3 (http://bioinfo.ut.ee/primer3/). The default settings were used, apart from those defined in Table 33.

Table 33: User defined settings for the primer design tool Primer3.

Setting	Variable
General Primer Picking Conditions	
Primer Tm Opt **(optimum primer annealing temperature)**	60
Max Tm Difference **(Maximum difference in annealing temperature between primers)**	1.0
Product Size Ranges **(Allowed range of primer lengths in base pairs)**	50-150
Use Thermodynamic Oligo Alignment	Unchecked
Old Secondary Structure Alignments	
Max Self Comp, Max 3' Self Comp, Max Pair Comp & Max 3' Pair Comp **(adjusted up in increments of 1 until primers found)**	0
CG Clamp	1

All primers, both those generated for ef1α and those for the other target/housekeeping genes, were then tested for annealing issues such as hairpins (primer annealing to self) and self or cross dimers (primer annealing to the same or opposite primer) (Figure 45), using the online software tool NetPrimer (http://www.premierbiosoft.com/netprimer/) with the default settings.

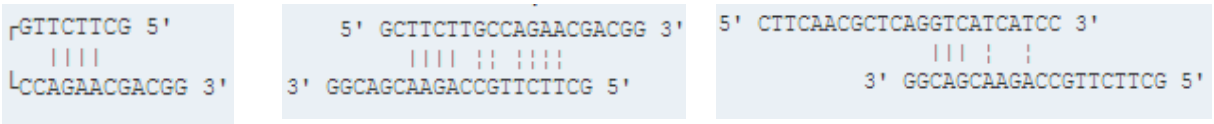


Figure 45: Examples of the types of issues that can be experienced with sub-optimal primer design. Left: Hairpin, where a primer anneals to itself. Middle: Self dimer, a primer that anneals to another copy of itself, e.g. a forward primer annealed to another forward primer. Right: Cross-dimer, a primer that has annealed to a copy of the opposite primer pair i.e. a forward primer annealed to a reverse primer. All images are screenshots from the NetPrimer tool.

Without this step, one or more of these design errors could lead to a reduction in the efficiency of amplification in the PCR stage. This would result in the misreporting of the amount of the target/housekeeping gene present in a sample. Ideally, any primer or primer pair found to have one or more of these issues would be rejected. However, the primer pairs supplied by IVM had one or more of these issues. As the intention was to compare their results with the ones generated in this project, IVMs primer design was not changed.

Table 34: Descriptions and illustrations of the primer annealing issues identified by the primer analysis software tool, NetPrimer.

Gene	Issue type	Primer	Locations
p53 (IVM)	Self-Dimer	Reverse	5' ACTGACCTTCCGAGTCTCC 3' 3' CCTCTGAGCCTTCCAGTCA 5' 5' ACTGACCTTCCGAGTCTCC 3' 3' CCTCTGAGCCTTCCAGTCA 5'
	Cross Dimer	Both 5' GCCTCGGCCTCTGGTAA 3' 3' AATGGTCTCCGGCTCCG 5'	5' GGGCAATCAGCGAGCAAA 3' 3' CCTCTGAGCCTTCCAGTCA 5' 5' GGGCAATCAGCGAGCAAA 3' 3' CCTCTGAGCCTTCCAGTCA 5'
RAD51 (IVM)	Self-Dimer	Forward	5' TGCTGCGTCTCGCTGA 3' 3' AGTCGCTCTGCGTCGT 5'
	Hairpin	Forward	┌TGCGTCGT 5' C └TCGCTGA 3'
	Self-Dimer	Forward	

Vitellogenin **(Biales *et al.*, 2007)**	None		
ef1α **(In-house)**	None		
18S **(Xie *et al.*, 2011)**	Cross Dimer	Both	5' GAGAAACGGCTACCACATCC 3' 3' ACCTCCCGTTCAGACCAC 5'
β-actin **(IVM)**	Self-Dimer	Reverse	5' CAACGGAAACGCTCATTGC 3' 3' CGTTACTCGCAAAGGCAAC 5'
	Cross Dimer	Both	5' CGAGCAGGAGATGGGAAC 3' 3' CGTTACTCGCAAAGGCAAC 5' 5' CGAGCAGGAGATGGGAAC 3' 3' CGTTACTCGCAAAGGCAAC 5' 5' CGAGCAGGAGATGGGAAC 3' 3' CGTTACTCGCAAAGGCAAC 5'

The optimum annealing temperature for each primer pair was then identified using the following process. A sample of cDNA was diluted 1:5 with molecular grade H_2O and loaded into a 96 well PCR plate, 1 µl per well, following the loading template in Table 35. Master mixes were made up for each primer pair, comprising of: molecular grade H_2O, the forward and reverse primer of the target/housekeeping gene and Fast SYBR Green (Applied Biosystems). SYBR Green contains dNTPs and a proprietary DNA polymerase that builds the DNA sequence of the target/housekeeping genes that is initiated by the primers. 19 µl of

master mix was added to each well containing cDNA. A further single well containing only 19 µl of master mix was included as a no template control (NTC) (Table 35).

Table 35: The PCR plate loading template, reproduced here to aid understanding of the protocol used for identifying the optimal annealing temperature for each primer pair. The letter 's' denotes a well containing sample and the letter 'x', an empty well. The No Template Control (NTC) wells contain only 19 µl of master mix, without any sample cDNA. This allows detection of any primer products that would artifically inflate the amount of target product produced by the PCR process.

Temp (C)	Empty	NTC	RAD51	p53	VIT	B-actin	ef1α	18S	NTC
65.0	x	x	s	s	s	s	s	s	x
64.5	x	x	s	s	s	s	s	s	x
63.3	x	x	s	s	s	s	s	s	x
61.4	x	x	s	s	s	s	s	s	x
59.0	x	RAD51	s	s	s	s	s	s	B-actin
57.0	x	p53	s	s	s	s	s	s	ef1α
55.7	x	VIT	s	s	s	s	s	s	18S
55.0	x	x	s	s	s	s	s	s	x

The plate was sealed and placed in a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories Ltd.). Amplification of the target/housekeeping genes were carried out using a protocol with the settings defined in Table 36. Each row of loaded wells was subjected to a different annealing temperature. The results of the PCR run were reviewed to identify the optimum annealing temperature for each primer pair, being the temperature that took the least number of cycles to achieve an exponential increase in the amount of DNA produced. For all primer pairs this temperature was 59 °C. In addition, for each primer pair, the amplification specificity was checked by reviewing the melt curve to ensure that only one peak was generated, relating to the gene of interest. Multiple peaks would indicate that non-target genes or primer dimers had been amplified. A small peak generated by the NTC sample was deemed acceptable providing it did not align with the peak of the target/house-keeping gene peak.

Table 36: The PCR protocol parameters used to identify the optimum primer annealing temperatures and the amplification specificity assessment of the resulting melt curves. The temperature used in step 4 is a range, with the specific temperature used dependant on which row in the PCR plate the sample is in, as defined in the PCR loading template in Table 35.

Protocol Step	Temperature (°C)	Duration (MM:SS)
1. Pre-warm block	50.0	2:00
2. Activate enzyme	95.0	10:00
3. Denature DNA	95.0	0:15
4. Anneal/Extend	55.0-65.0	1:00
5. Repeat steps 3 – 4	40 cycles	
6. Produce melt curve	65.0 to 95.0 in 0.5	0:05 per increment

5.4.4 cDNA synthesis

cDNA was synthesised using the QuantiTect Reverse Transcription kit (Qiagen Ltd.), following the manufacturers protocol. The kit included a genomic DNA (gDNA) elimination step, a buffer containing a DNase and an RNase inhibitor, which was added to the cDNA samples and heated at 42 °C for two minutes. Reverse transcriptases (two proprietary enzymes), a primer mix containing a combination of oligo-dT and random primers and a buffer containing deoxyribonucleotides (dNTPs) were added and the samples incubated at 42 °C for fifteen minutes for the synthesis step and then 95 °C for three minutes to deactivate the reverse transcriptases. This produced 20 µl of reaction product for each sample.

5.4.5 PCR

cDNA was diluted 1:5 before loading a 96 well plate (Bio-Rad Laboratories Ltd.) in triplicate per sample, 1 µl per well. Wells on the edges of the plates were not used to reduce the risk of uneven heating during PCR. A master mix was made up, comprising of molecular grade H_2O, the forward and reverse primer of the target/housekeeping gene and Fast SYBR Green (Applied Biosystems). 19 µl of master mix was added to each well containing cDNA, with a further single well containing only 19 µl of master mix as a no template control (NTC). The

plate was sealed and placed in a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories Ltd.). Amplification of the target/housekeeping genes were carried out using a protocol with the settings defined in Table 37.

Table 37: The PCR protocol parameters used in the amplification of the target/housekeeping genes.

Protocol Step	Temperature (°C)	Duration (mm:ss)
1. Pre-warm block	50.0	02:00
2. Activate enzyme	95.0	10:00
3. Denature DNA	95.0	00:15
4. Anneal/Extend	59.0	01:00
5. Repeat steps 3 – 4	40 cycles	
6. Produce melt curve	65.0 to 95.0 in 0.5 increments	00:05 per increment

5.4.6 Quantification of gene expression

To identify the most stable housekeeping gene, the raw cycle threshold values (Ct) for each target gene were compared to those of the equivalent housekeeping genes, using the Excel plug-in, NormFinder (Andersen *et al.*, 2004). To prepare these data for submission to NormFinder, the difference between each pair of target and housekeeping genes was calculated and linearly expressed by raising it to the power 2 (each cycle representing a doubling of the amount of target gene). In addition, the sex of the fish and its membership of either the control or treatment was defined. This allowed assessment of inter and intra group variation of the level of expression of each housekeeping gene. Under all conditions, ef1α was identified as the most stable housekeeping gene.

Raw Ct values were converted to values representing normalised expression of the target gene, using the q-gene excel spreadsheet (http://www.mybiosoftware.com/pcr-primer-design/3890) (Simon, 2003). As part of the conversion process, the amplification efficiency of each of the target/housekeeping genes was required. A sample of cDNA was loaded in triplicate as a dilution series at 100, 50, 10 and 1% of cDNA in molecular grade H_2O, repeated for each target/housekeeping gene. Master mixes were made up and loaded as above, the plate sealed and placed in the BioRAD C1000 Thermal Cycler. The plate was processed using a protocol with the settings in Table 37. The amplification efficiency graph was plotted and the value of the slope for the target/reference genes inserted in the relevant cell. The raw Ct values of each target gene (one per spreadsheet) and ef1α, for the control and exposed fish, were then copied to the "Data Analysis" worksheet. The values were processed using equation 2, which averages the triplicate raw Ct values before calculating the mean normalised expression for each sample. The acceptable variation in technical replicates was set at 20%. Any replicate set with a variation greater than this value was rerun through the PCR stage. An exception was if an obvious outlier was observed, in which case this was deleted and the normalised expression calculated from the remaining two Ct values.

5.4.7 Statistical analysis

The same process and tests were used as for the morphological endpoints. Please refer to section 4.2.7 for more details.

5.5 Results

This section presents the results of the PCR analysis, organised by experiment. The fish have been grouped by sex, due to allow the difference of expression, of at least vitellogenin, to be assessed. The results are normalised gene expression values, using ef1α as the control gene. For boxplots, open circles are outliers and thick black horizontal lines inside the boxes are the median value. The box contains the interquartile range, which are the values between the 25th and 75th percentile. The "whiskers" (vertical dotted lines with T bar terminators) display the adjacent values, defined as the value of the largest/smallest observation that is less than or equal to the upper/lower quartile plus 1.5 times the length

of the interquartile range. Any values that fall outside of the adjacent values are plotted as outliers.

5.5.1 Pilot experiment

No significant effects were seen in the level of RAD51 RNA expression for any of the drugs tested. A significant increase in the expression of p53 RNA in female fish exposed to 100 µg/l cyclophosphamide and a significant decrease in male fish exposed to 10 µg/l cisplatin was observed. The level of Vitellogenin RNA expression was significantly greater in both male and female fish exposed to 5-fluorouracil, whereas female fish exposed to cisplatin exhibited a significant decrease (Figure 46).

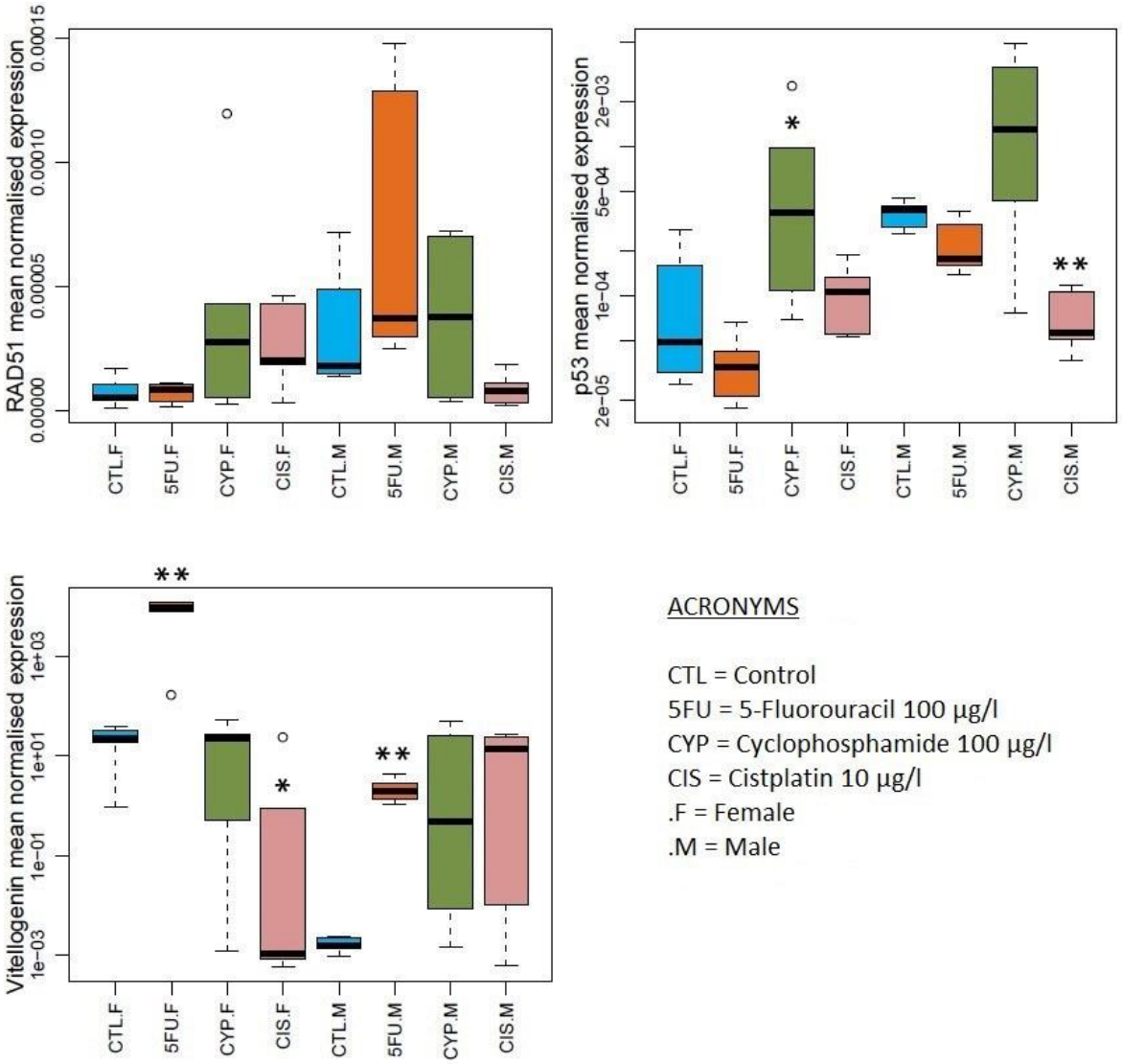


Figure 46: Boxplots of the zebrafish PCR results generated in the Pilot experiment to illustrate the amount of variability in and between groups. The y axis of the Vitellogenin chart is logarithmic (base 10) to help visualise the data.

5.5.2 5-fluorouracil concentration series

No significant changes in RNA expression for any of the target proteins was observed, when comparing the control and the group exposed to 5-fluorouracil at a concentration of 500 µg/l (Figure 47).

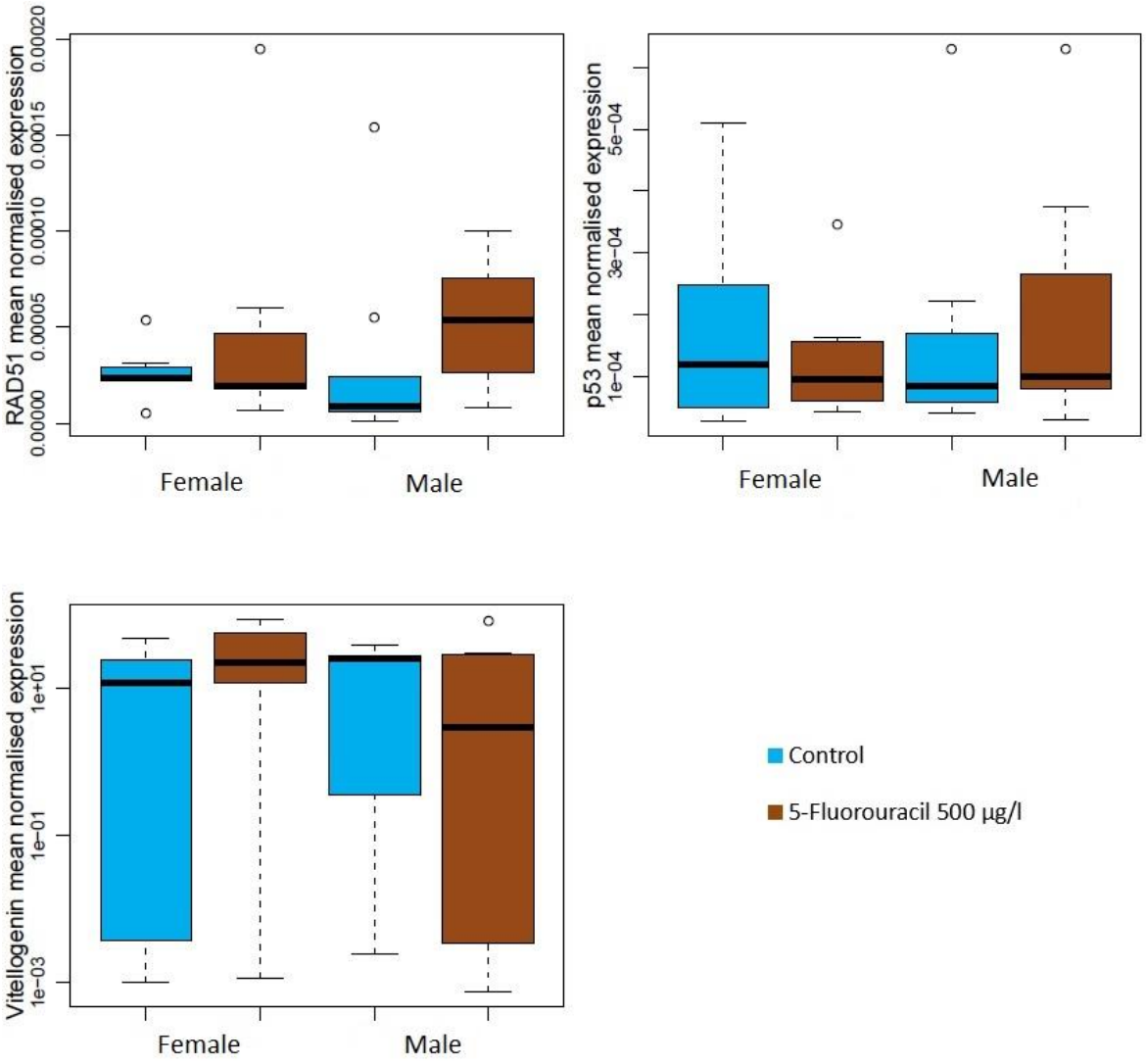


Figure 47: Boxplots of the zebrafish PCR results generated in the 5-fluorouracil concentration series experiment to illustrate the amount of variability in and between groups. In the x axis labels the number denotes the concentration of the drug and the letter after the full stop the sex of the group. The y axis of the Vitellogenin chart is logarithmic (base 10) to help visualise the data.

5.5.3 Repeat 5-fluorouracil partial concentration series

The only significant result observed was an increase in the level of Vitellogenin mRNA expression in female fish exposed to 500 µg/l of 5-fluorouracil (Figure 48). Compared to the vitellogenin results in the pilot study and previous 5-fluorouracil concentration series experiment, the level of vitellogenin expression was much lower.

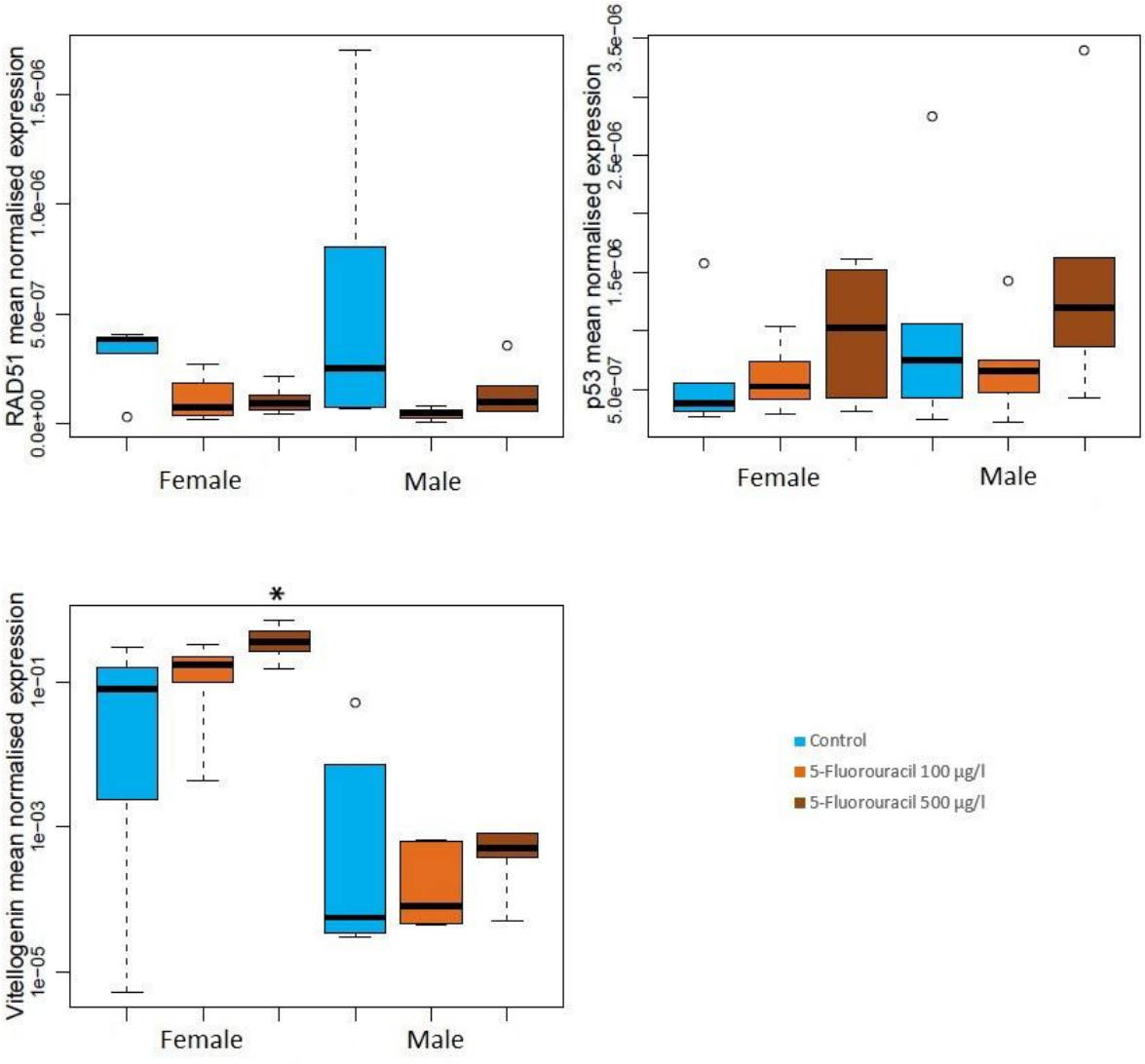


Figure 48: Boxplots of the zebrafish PCR results generated in the repeat 5-fluorouracil partial concentration series experiment to illustrate the amount of variability in and between groups. In the x axis labels the number denotes the concentration of the drug and the letter after the full stop the sex of the group. The y axis of the Vitellogenin chart is logarithmic (base 10) to help visualise the data.

5.5.4 Cyclophosphamide concentration series

RAD51 mRNA expression was significantly lower in female zebrafish exposed to 2000 µg/l of cyclophosphamide, when compared to the control group. The same trend was observed in the p53 mRNA expression of male fish exposed to the same concentration. No other significant results were observed in this experiment (Figure 49).

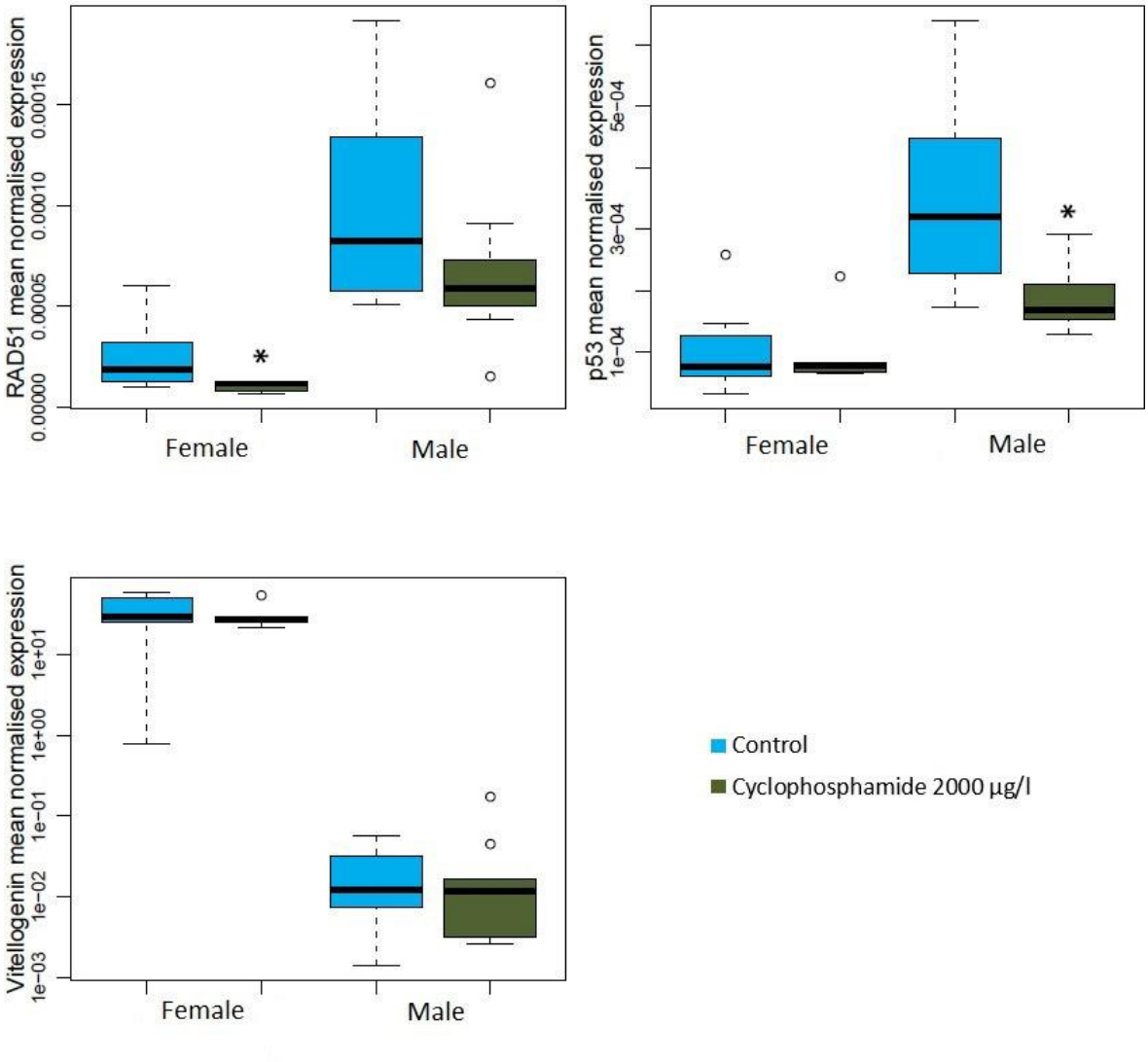


Figure 49: Boxplots of the zebrafish PCR results generated in the cyclophosphamide concentration series experiment to illustrate the amount of variability in and between groups. In the x axis labels the number denotes the concentration of the drug and the letter after the full stop the sex of the group. The y axis of the vitellogenin chart is logarithmic (base 10) to help visualise the data.

5.5.5 Cisplatin concentration series

No significant change in mRNA expression for any of the target proteins was observed, when comparing the control and the group exposed to cisplatin at a concentration of 100 µg/l (Figure 50).

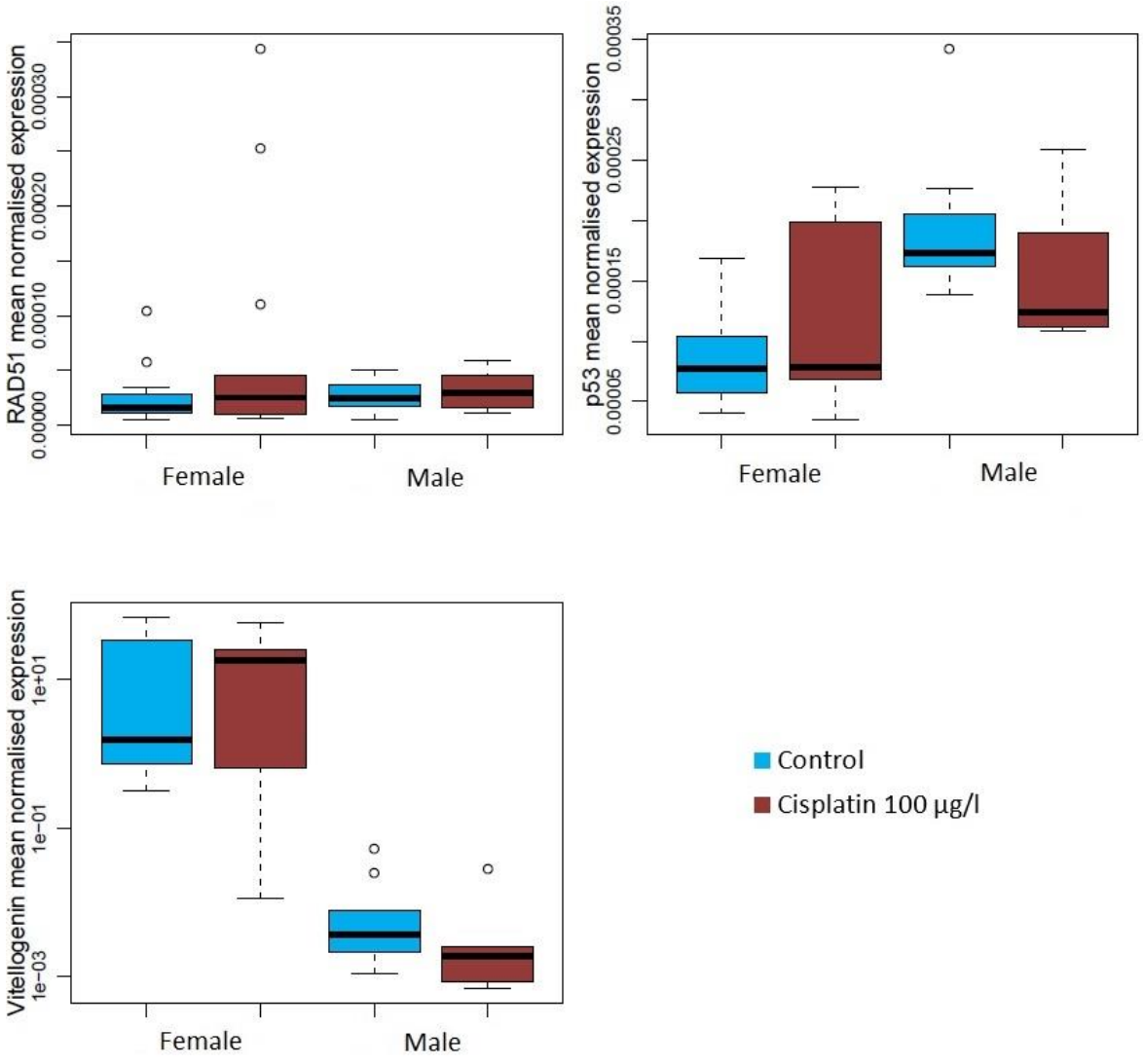


Figure 50: Boxplots of the zebrafish PCR results generated in the cisplatin concentration series experiment to illustrate the amount of variability in and between groups. In the x axis labels the number denotes the concentration of the drug and the letter after the full stop the sex of the group. The y axis of the Vitellogenin chart is logarithmic (base 10) to help visualise the data.

5.5.6 Simple mixture

The expression of RAD51 mRNA in female fish exposed to 50 µg/l of 5-fluorouracil was significantly greater than that of the control group. The only other significant result was a higher level of p53 RNA expression in the female fish exposed to the simple mixture (Figure 51).

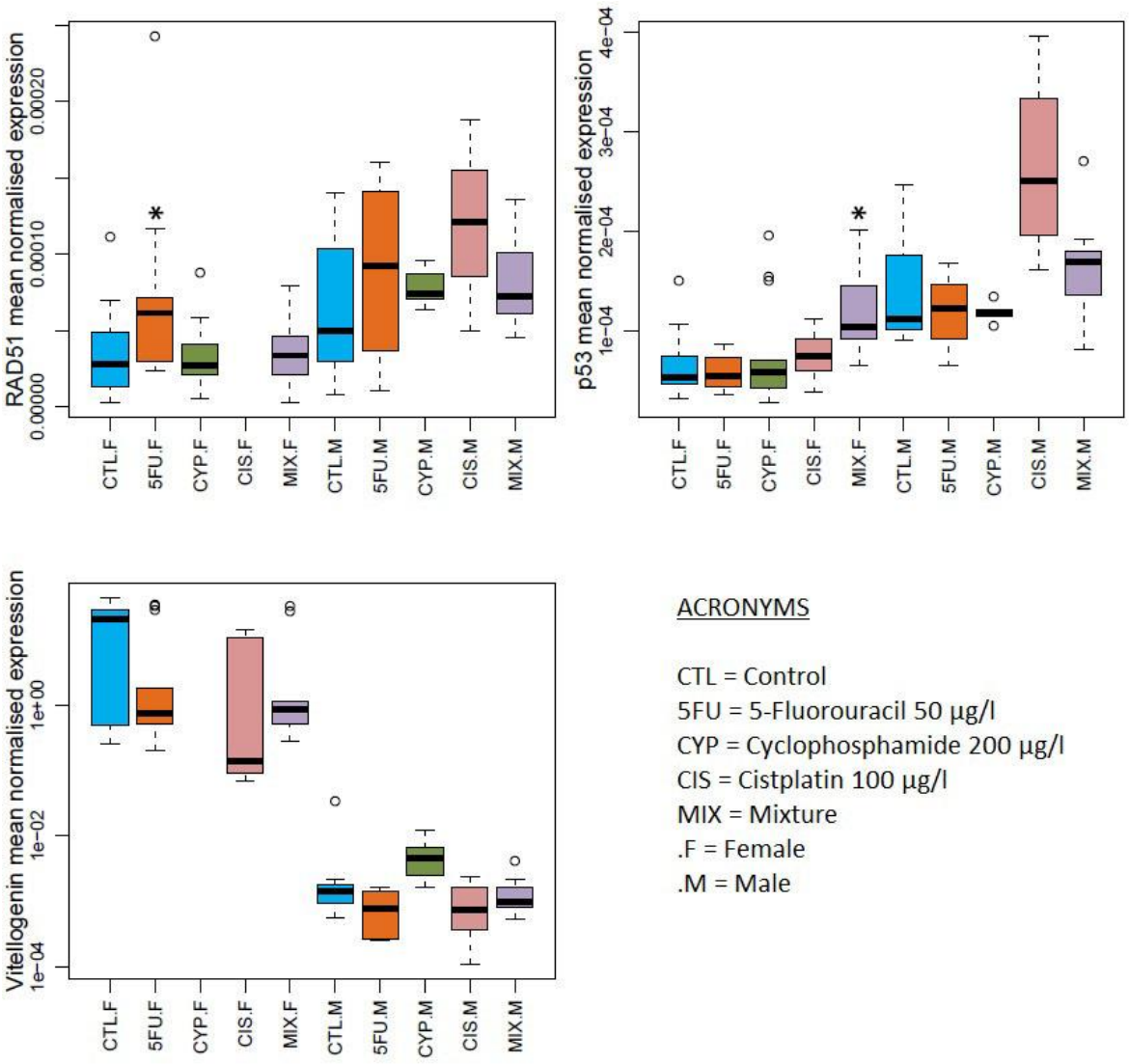


Figure 51: Boxplots of the zebrafish PCR results generated in the simple mixture experiment to illustrate the amount of variability in and between groups. The y axis of the Vitellogenin chart is logarithmic (base 10) to help visualise the data.

5.6 Discussion

As with the results of the morphology endpoints, although there were some statistically significant findings, they generally formed no coherent pattern. The only results that showed signs of a response pattern was seen in the groups of fish exposed to 5-fluorouracil, where a statistically significant increase in vitellogenin RNA expression was observed in some cases.

One of the weaknesses of this endpoint is that it is only possible to assess a snapshot of RNA expression for the proteins of interest. The cell is a dynamic environment, with constant fluctuations in the proportions of transcripts, depending on multiple internal and external factors. As can be seen in the results of Kastan *et al.* (1991) (Figure 43), acute exposure causes a sharp spike in p53 expression, followed by a gentle decline over a longer period of time. It is not known if chronic exposure would cause sustained changes in expression of p53 or RAD51, and if it did whether or not this would also reduce over time, due to the involvement of other repair processes or perhaps increased tolerance of the DNA damaging agents.

Factors other than the effect of the drug may also affect mRNA expression of the target proteins. Also in Figure 43, the results for the control group show a small increase in the level of p53 protein. The cause of this is unknown, but if variations like this occur as part of the normal functions of the cells or due to unknown external stimuli, this could lead to false positives or negatives if they occur at the point the fish are sampled. The significant results observed could be due to variability between individuals, of which a great deal can be seen in the boxplots in section 5.5. In the case of vitellogenin, variability due to normal biological processes required for egg production, could account for some or all of the significant results observed.

The concentration of drugs that the fish were exposed to may not have been high enough to cause a measurable increase the mRNA expression of p53 and/or RAD51. For RAD51 there is some doubt cast by the findings of Reinardy *et al.* (2013), which observed no change in RAD51 expression when DNA damage was induced in zebrafish larvae using hydrogen peroxide, a well-established DNA damaging chemical shown to cause double strand breaks. They did, however, observe upregulation of the gadd45a gene, which is

associated with DNA damage repair, which give some confidence that DNA damage did occur in their experiment. However, the mechanism of action of hydrogen peroxide (strand breaks caused by hydrogen peroxide generated free radicals) and the cytotoxic drugs in this project differ, so the findings of Reinardy *et al.* (2013) cannot rule out RAD51 from the repair of DNA damage caused by one or more of the drugs in this project.

Alternatively, DNA repair activity could have been stimulated but it involved other repair proteins, of which there are many. Embry *et al.* (2006) investigated the abundance of p53 in several fish cell lines (not including zebrafish). They could detect no increase in p53 protein in any of the cell lines when exposed to a range of cytotoxic drugs including 5-fluorouracil and cisplatin. They were, however, able to detect an increase in apoptosis in a concentration-dependent manner. This was significant for cisplatin, but not 5-fluorouracil, where there was a non-significant increasing trend. The work carried out by Embry *et al.* (2006) appears to be robust, with several different assays conducted to confirm that the antibody that recognises p53 was working as required in the fish cell lines. The authors noted that several of the drugs used in this study were shown to be dependent on p53 to induce apoptosis in mammals. This could suggest that p53 is still part of the apoptosis pathway, but does not require an increase in transcription to initiate it. In support of this hypothesis, Cachot *et al.* (1998) were able to detect consistent but low levels of p53 in flounder. Alternatively, lack of p53 induction could indicate that other proteins are responsible for apoptosis in fish.

Another factor which may have contributed to the lack of response observed in the target DNA repair genes is described in Sussman (2007). She found that zebrafish embryos appeared to have an enhanced ability to repair UV induced DNA damage, when compared to human lymphocytes, using the host cell reactivation (HCR) assay. This ability was assessed by transfecting embryos and lymphocytes with plasmids containing either undamaged or UV-damaged DNA that coded for reporter genes that fluoresce differentially. Fluorescence of the damaged reporter gene can only be observed if it is repaired by the cell it transfects. Sussman suggested that this enhanced ability may be due to the zebrafish home range being near the equator and therefore being exposed to high levels of UV light. She then goes on to say that, alternatively, because the zebrafish body is transparent, DNA damaging chemicals may have exerted the evolutionary pressure to

enhance damage repair. However, this seems counterintuitive as a transparent body could be expected to expose the zebrafish organs to higher levels of UV light, with this factor being responsible for the evolution of enhanced DNA damage repair. In either case, it should be noted that in mammals at least, UV damage is thought to be repaired by using the excision repair pathways (Sinha and Häder, 2002), different to those mechanisms that repair double strand breaks, of which RAD51 is a component.

The results of the single drugs and simple mixture experiments are discussed in more detail below. Where possible, these are compared to relevant literature results for the drug's effects on molecular endpoints in fish, or if not available, other species.

5.6.1 5-fluorouracil

In the pilot experiment, a statistical significant increase was seen in the vitellogenin mRNA expression in both sexes at 100 µg/l. However, this was not repeated in the more robust concentration series experiment, where the highest concentration was 500 µg/l. In the partial repeat of the concentration series, this increase was observed in the females only of the group exposed to 500 µg/l. Due to the small number of samples in this particular experiment, samples from the lower dose of 100 µg/l was also assayed, but no significant increase was observed. Finally, no significant findings were seen in the 5-fluorouracil single chemical component of the simple mixture experiment, where exposure was at 50 µg/l.

An increase in vitellogenin expression in fish exposed to 5-fluorouracil has not been reported in the literature. A consistent decrease could suggest that the cytotoxic nature of 5-fluorouracil would be having a negative impact on the normal functions of the livers of the exposed fish. A possible mechanism for this behaviour (had it been observed) is that one of the 5-fluorouracil metabolites (FUTP) intercalates into mRNA in place of uracil, negatively impacting translation into the corresponding protein (Longley *et al.*, 2003). However, the opposite behaviour could be attributable to underlying biological cycles. Within female zebrafish, vitellogenin production is not constant but is cyclical in relation to egg production (Peute *et al.*, 1978). It could therefore be possible that the different groups of fish in the pilot experiment were at different stages of the egg production cycle (a period of about 5 days). If the control fish were at the point of the cycle where vitellogenin is minimally expressed and the 5-fluorouracil at the point where it is maximally, this could

explain the significant results for the female fish. Nevertheless, vitellogenin levels in the male zebrafish exposed to 5-fluorouracil were also significantly increased.

An increase in vitellogenin has been shown to occur in male and female zebrafish under the influence of oestrogenic chemicals (Islinger *et al.*, 2003; Ortiz-Zarragoitia and Cajaraville, 2005), as would be expected. There is no indication in the literature that 5-fluorouracil is oestrogenic. In addition, this increase was only observed in one experiment. Experiments that expose fish to oestrogenic chemicals are conducted in the same facilities as this project was conducted, however, due to the highly sensitive response (significantly increased vitellogenin levels when exposed at concentrations in the nanograms per litre range) decontamination processes within the facility are robust so contamination is not considered to be an issue. Finally, as with the significant results observed in the morphological endpoints, these differences could be due to biological variability. This would explain why the results observed were not consistent between experiments.

Relevant to one of the mechanisms of action of 5-fluorouracil (section 1.4.1), Du *et al.* (2006) carried out sequence analysis of thymidylate synthase in zebrafish and found it to be highly similar to other species, including mammals. They also assessed its function in zebrafish embryos by disrupting its expression during development, after which they observed developmental defects. Whilst noting that this finding could allow the assessment of 5-fluorouracil's effects on thymidylate synthase, the authors did not carry out this work. However, this research gives some confidence that if 5-fluorouracil had been exposed and taken up by the zebrafish at a high enough concentration, and had been metabolised into the active metabolites that form the thymidylate synthase inhibition pathway, then it could have been possible for this mechanism of action to occur.

As described previously, the fact that no statistically significant change in the expression of p53 mRNA was observed may be related to the apparent lack of induction of the associated protein in fish. Alternatively, perhaps the concentration of 5-Fluororuacil that fish were exposed to was not high enough to alter expression of either p53 or RAD51. However, Kovács *et al.* (2015) carried out a two generation study on zebrafish exposed to 0.01, 1.0 and 100 µg/l of 5-fluorouracil. They observed significant upregulation of p53, along with several genes that are regulated downstream from p53 that are also involved in DNA

damage repair, at all concentrations of 5-fluorouracil. They also observed DNA damage in the 1.0 and 100 µg/l groups, using the comet assay. The exposure was conducted under semi-static renewal conditions, with the aquaria water replaced every three days. Chemical analysis was conducted, which showed the drug was present at the nominal concentrations at the beginning of the three-day period, with concentrations reducing to below the LOD of 0.003 µg/l by the end of the three-day period. The authors stated that while the fish were not continually exposed to 5-fluorouracil, this scenario is representative of the pulsed nature of environmental pharmaceutical exposure. The Kovács *et al.* (2015) experiment was a chronic exposure study, whereas the experiments in this project are considered acute exposures. Although the fish were exposed at higher concentrations, the far shorter exposure period of 14 days may not have been long enough to see changes in the regulation of the target genes.

Another possible explanation for the RAD51 results is that the level of expression of this gene did change due to 5-fluorouracil exposure, but that this increase was due to post-translational enhancement, which cannot be assessed by examining mRNA levels. However, this explanation is contradicted by the findings of Reinardy *et al.* (2013) reported earlier. A further alternative is that upregulation of one or both of these genes did occur, but as was observed by Kastan *et al.* (1991), upregulation is not consistent and may have been reduced at the time point at which the fish were sampled.

5.6.1.1 Molecular effects of 5-fluorouracil on fish reported in the literature

Two studies that investigated the molecular impact of 5-fluorouracil in other fish species were found. Julian *et al.* (1998) used 5-fluorouracil to cause apoptosis in rainbow trout retina cells at 0.2 mg per gram of fish, with the exposure conducted by intraperitoneal injection. Apoptosis was confirmed via the TUNEL assay (assesses presence of short stretches of DNA in cell nuclei that are characteristic of apoptosis) 2 days after exposure. Grisolia & Starling (2001) induced micronuclei in the red blood cells of Nile tilapia (*Oreochromis niloticus*), redbreast tilapia (*Tilapia rendalli*) and common carp using 5-fluorouracil at a concentration of 2.5mg/kg. Micronuclei were quantified at 2, 7, 14 and 30 days after a single “intra-abdominal” injection. The authors claim that a significant increase in micronuclei was only observed in common carp at the 7-day point.

To allow comparison of the concentrations of 5-fluorouracil used in the literature with those used in this project, the following rough calculations were made (summarised in Table 38, for use in the fish blood plasma model. Using the average weight of a zebrafish from the pilot experiment of 0.85g (as used in the previous chapter for comparison purposes), this would equate to acute doses of 170 µg (Julian *et al.*, 1998) and 2.1 µg (Grisolia and Starling, 2001) respectively, if applied to an average zebrafish in this thesis. The average extracted blood volume of zebrafish in the pilot study was 15 µl. For comparison purposes, if it is assumed that all the drug entered the bloodstream, this would give a plasma concentration of 11,333 µg/l and 140 µg/l respectively. The maximum concentration of 5-fluorouracil used in this project was 500 µg/l, and fish were exposed continuously over a 14-day period. However, this would have then had to have been transferred into the blood stream via the gills, a process far less efficient than administration by injection. Using the fish blood plasma model and the maximum actual concentration of 500 µg/l, this would give an estimated plasma concentration of 11.87 µg/l (Table 38).

Table 38: Data used to estimate blood plasma concentrations if the drug doses stated in the literature were administered to a zebrafish of average weight with average blood volume. *Value calculated using the fish blood plasma model.

Source	Drug Dose for average zebrafish weighing 0.85 g	Blood plasma concentration in a zebrafish with average whole blood volume 15µl	Therapeutic plasma concentration in humans (Higashida *et al.*, 2012)	Fish blood plasma concentration at 500 µg/l*
Julian *et al.* (1998)	$0.2\ \text{mg/g} \times 0.85\text{g} = 0.17\ \text{mg}$	$\frac{170\ \mu g}{15\ \mu l} = 11.33\ \mu\text{g/µl}$ $= 11{,}333\ \mu\text{g/l}$	120 µg/l	11.87 µg/l
Grisolia & Starling (2001)	$\frac{2.5\ \text{mg/kg}}{1000} = 0.0025\ \text{mg/g}$ $0.0025\ \text{mg/g} \times 0.85\text{g} = 0.00212\ \text{mg}$	$\frac{2.12\ \mu g}{15\ \mu l} = 0.141\ \mu\text{g/µl}$ $= 141\ \mu\text{g/l}$		

The results of the fish blood plasma model for 5-fluorouracil suggests that an environmental concentration of 5,056 µg/l would be required to achieve a human therapeutic blood plasma concentration of 120 µg/l. A therapeutic dose in anti-cancer treatment is one that kills tumour cells. The estimated dose used by Julian *et al.* (1998) is

far higher than the human therapeutic plasma concentration used in the model and did result in, at least the initiation of, apoptosis in the trout retinal cells. The plasma concentration estimated for Grisolia & Starling (2001) was slightly higher than the human therapeutic plasma concentration and resulted in an increase in micronuclei, a non-cytotoxic but potentially genotoxic outcome. A plasma concentration of 11.87 µg/l, however, is far below the human therapeutic or the two estimated plasma concentrations for the literature results. The calculations for the literature findings could indicate that the concentrations used in this project were too low to elicit an observable response in the zebrafish. This would support the suggestion that the significant results observed in the expression of mRNA for the DNA damage repair enzymes were due to natural biological variation.

5.6.2 Cyclophosphamide

No trend in the expression of mRNA for any of the genes of interest was observed across the experiments involving cyclophosphamide. A significant increase in p53 expression was observed in female fish in the pilot experiment, where fish were exposed to a nominal concentration of 100 µg/l. This was accompanied by a non-significant but notable increase in the male fish, where greater variability was observed between individuals (Figure 46). Given the results of the chemical analysis of the aquaria water samples for the subsequent experiments that used cyclophosphamide, the actual concentration is likely to be lower. In the more robust cyclophosphamide concentration series experiment, no response was observed in p53 mRNA expression in female fish exposed to an actual concentration of 323.85 µg/l (2000 µg/l nominal), but a significant decrease was seen in male fish exposed to the same concentration. In addition, a significant decrease in RAD51 expression was observed in the exposed females. When exposed singly to cyclophosphamide at an actual concentration of 101.35 µg/l (nominal 200 µg/l), as part of the simple mixture experiment, no significant response was seen for any of the target genes.

It is unlikely that any of the significant results could be attributed to cyclophosphamide. For example, the increase in p53 in the pilot experiment at 100 µg/l was not repeated for the female fish at the higher actual concentration of 323.85 µg/l. In male fish in the same circumstances a non-significant increase became a significant decrease. As p53 is involved

in DNA repair and apoptosis, it could be expected to have been seen at the higher concentration. It is possible that the higher concentration overwhelmed the cells repair machinery, which could have meant that less p53 was transcribed at the higher concentration. However, there is insufficient data to assess this possibility. Perhaps an assay such as TUNEL, which can detect apoptosis, could have been useful in investigating this line of reasoning. However, the more probable explanation is natural variability in gene expression between individuals.

Whether this method for detecting DNA damage would have worked at higher concentrations of cyclophosphamide is unclear. Indeed, it may have been that the concentrations used did elicit an increase in the DNA repair proteins, but this increase was due to post-translational enhancement of protein production (section 5.3). Without direct assessment of the DNA damage repair proteins, it was not possible to assess this. However, the fish blood plasma model calculations (section 4.3.4), predicted that the fish steady state plasma concentration of fish exposed to the actual water concentration of 323.85 µg/l (2000 µg/l nominal) was 50.51 ng/ml, far lower than the human therapeutic plasma concentration of 12,210 ng/ml. These calculations would suggest that it is unlikely that measurable amounts of DNA damage would have occurred.

5.6.2.1 Molecular effects of cyclophosphamide on fish in the literature

Although the effects of cyclophosphamide on gene expression in zebrafish have not been previously reported, there are studies using other fish species. Santos and Pacheco (1995) exposed wild caught Eels (*Anguilla anguilla L*) for 72 hours to a concentration range of cyclophosphamide of 6.25 to 100 mg/l. They then assessed the gill cells using the sister chromatid exchange (SCE) assay. This is a genotoxic assay that identifies exchange of genetic material between two identical sister chromatids during anaphase, when sister chromatids are separated prior to the formation of the two daughter cells during telophase. DNA damage causes breaks, which when repaired result in the exchange of material (Wilcosky and Rynard, 1990). A concentration-dependent response was observed, with all but the lowest dose of 6.25 mg/l causing an effect statistically greater than the control (Figure 52).

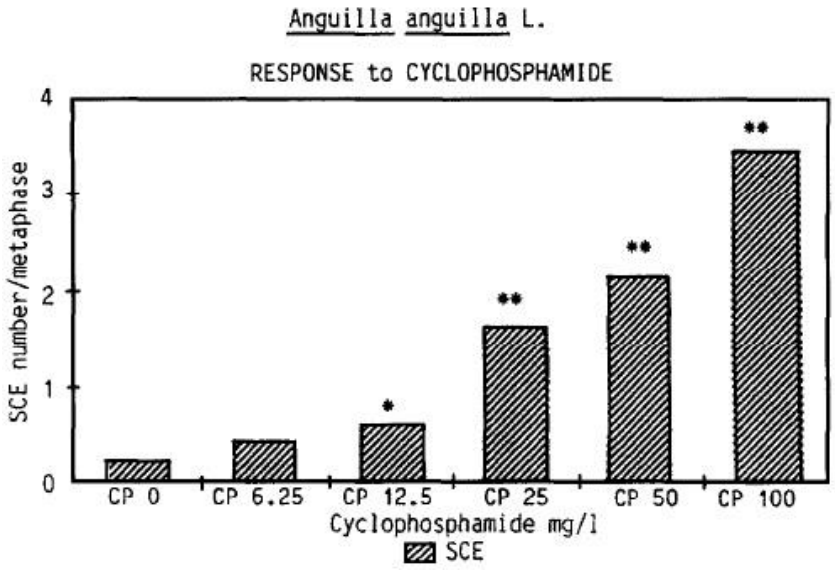


Figure 52: Mean number of sister chromatid exchanges observed in Eels exposed to a concentration series of cyclophosphamide. * & ** identify those concentrations that produced SCEs in significantly greater numbers than observed in the control group (Santos and Pacheco, 1995).

No baseline for genotoxic damage in wild caught fish can be made due to the potential for exposure to other environmental factors, which could also cause this kind of damage. However, the concentration-dependent increase is supportive of the argument that the observed results were due to cyclophosphamide exposure. The gills are continuously exposed to the environment and are a sensible endpoint for the assessment of the effects of cyclophosphamide exposure. Nevertheless, even the lowest concentration in which significant results were observed, were two orders of magnitude higher than the highest actual concentration of 323.8 µg/l (2000 µg/l nominal) used in this study. Using the fish blood plasma model, a water concentration of 12.5 mg/l (the LOEC from (Santos and Pacheco, 1995)) would result in a fish plasma concentration of 1,949 ng/ml, nearly six times less than the human therapeutic plasma concentration of 12,210 ng/ml. This would suggest DNA damage can occur below therapeutic levels, but that the concentrations of cyclophosphamide used in this project would be too low for this to occur.

Micronucleus assessment after cyclophosphamide exposure has been studied in the South American freshwater fish species, Twospot astyanax (*Astyanax bimaculatus*). Matsumoto and Colus (2000) exposed adult captive bred fish via intraperitoneal injection to 4, 8, 16 or 32 mg/kg of cyclophosphamide. 24 hours after exposure, whole blood samples were

collected and the samples prepared for micronuclei assessment, in a similar manner to this project (4.2.5.3). Only the 8 and 16 mg/kg treatments led to statistically greater numbers of micronuclei compared to the control. The authors claim that this is evidence that 16 mg/kg is an ideal concentration to be used in as a positive control when testing other genotoxic agents. However, there appear to be issues with the experiment that do not necessarily support this. It is stated in the paper that the total number of fish used was 48. It is not clear if this includes the control group, but in either case the sum of column N in Table 39 does not give 48, with or without the control. It could be assumed that not all fish in each group survived, but no mention of their fate is made in the paper. In addition, the paper states that 3000 cells per fish were analysed, but this does not hold true for the 8 mg/kg group, otherwise N should be 4, not 6. No mention of power analysis is made, so it is not clear if there were a sufficient number of fish in each group to achieve a desirable level of power. This is of particular concern for the 32 mg/kg group, which has the smallest N and in which no significant difference was observed. This leaves the reader with uncertainty as to whether there really is a response to cyclophosphamide exposure in this experiment. The small sample size for the 32 mg/kg group could be why no significant difference was seen for the highest concentration, as would generally be expected in a concentration-dependent response. Alternatively, the significant differences seen in the 8 and 16 mg/kg groups could be an artefact of natural variation, rather than evidence that cyclophosphamide is causing an increase in the number of micronuclei at these concentrations.

Table 39: Micronucleus frequency in the erythrocytes of Twospot astyanax exposed to a concentration range of cyclophosphamide. N is probably the number of fish per treatment, but there are some anomalies, see text for an explanation. The final column is the mean number of micronuclei per fish. Matsumoto and Colus (2000).

	Cyclophosphamide		
Treatment (mg/kg body weight)	N	No. of cells analyzed	$\bar{X} \pm$ SD*
Control	12	36,000	0.31 ± 0.27
4	7	21,000	0.57 ± 0.60
8	6	12,000	1.22 ± 0.66
16	10	30,000	1.53 ± 0.71
32	5	15,000	0.60 ± 0.37

To allow comparison to the results in this project, the same calculations as carried out for 5-fluorouracil for use in the fish blood plasma model yielded the following data on estimated plasma concentrations (Table 40). It should be re-iterated that these are estimates based on several assumptions. However, this model has been shown to provide reliable estimates in studies on other classes of pharmaceutical (Margiolta-Casaluci *et al.*, 2014; Runnalls *et al*., 2015)

Table 40: Data used to estimate blood plasma concentrations that might be achieved, if the drug doses stated in the literature were administered to a zebrafish of average weight with average blood volume. *Value calculated using the fish blood plasma model.

Source	Drug Dose for average zebrafish weighing 0.85 g	Blood plasma concentration in a zebrafish with average whole blood volume 15µl	Therapeutic plasma concentration in humans ((Joy *et al.*, 2012)	Fish blood plasma concentration at 2,000 µg/l*
Matsumoto and Colus (2000)	$\frac{32\ \text{mg/kg}}{1000} = 0.032\ \text{mg/g}$ $0.032\ \text{mg/g} \times 0.85\text{g} = 0.0272\ \text{mg}$	$\frac{27.2\ \mu\text{g}}{15\ \mu\text{l}} = 1.813\ \mu\text{g/µl}$ $= 1{,}813\ \mu\text{g/l}$	12,210 µg/l	311.91 µg/l

The estimated blood plasma concentration of 1,813 µg/l for the 32 mg/kg dose is nearly six times lower than the human therapeutic plasma concentration. This would suggest that DNA damage may not have occurred in the exposed fish, which may account for the lack of significant results observed by Matsumoto and Colus (2000).

5.6.3 Cisplatin

The only significant result observed in fish exposed to cisplatin was a decrease in p53 and vitellogenin mRNA expression in female fish at 10 µg/l in the pilot experiment. No other significant results were seen in the cisplatin concentration series or the single chemical component of the simple mixture experiment, even though these fish were exposed at a higher concentration of 100 µg/l. If the decrease in vitellogenin was due to the DNA damaging mechanism of action of cisplatin, then it could be expected to lead to an increase in the expression of DNA repair enzymes. No observed significant response from RAD51 and a significant decrease in p53 RNA expression are counter to these expectations. As with the other drugs, natural variability between individuals (as can be seen in the boxplots in

Figure 46) would pose a more likely explanation for the significant results observed. As was described in section 4.3.4, the fish blood plasma model predicted a fish blood plasma concentration of 14.10 ng/ml in a fish exposed to 100 µg/l, a concentration far lower than the mean therapeutic plasma concentration of 800 ng/ml. Although some DNA damage would be expected at doses lower than the therapeutic, a concentration this low would provide more support to the natural variability explanation for the statistically significant results.

The point at which the fish exposed to cisplatin were in the egg production cycle could also be a factor in the vitellogenin results. Assuming the 5-fluorouracil exposed fish were at the stage where vitellogenin is maximally expressed, cisplatin exposed fish could be considered to be at the stage associated with minimal expression. There were no replicate tanks in the pilot experiment, where the significant differences were observed. Whereas there were 2-3 replicates in the concentration series and simple mixture experiments. The pooling of replicate tanks during analysis may have masked the effects of egg cycle production in the multiple replicate experiments.

5.6.3.1 Molecular effects of cisplatin on fish reported in the literature

In the same paper that reported significant lateral hair line loss in zebrafish larvae exposed to increasing concentrations of cisplatin (section 4.3.1.5), Ou *et al.* (2007) also observed nucleic acid damage in lateral hair line cells. This team conducted time-lapse imaging of the lateral hair line cells of 5 dpf larvae, exposed to 1mM (~300,000 µg/l) during a 12-hour observation period. Evidence of apoptosis (nuclear condensation and fragmentation) was first seen 30 minutes after the start of the exposure. This effect continued to be observed throughout the 12-hour observation period. Although the exposure period was far shorter than the 14-day period used in this thesis, the exposed concentration was far higher. Even assuming that a chronic exposure to cisplatin may elicit greater effects, the very large difference in concentration could mean that the same effects would not have been observed in the zebrafish in this study.

The only other relevant source of information regarding zebrafish and cisplatin was found in Giari *et al.* (2012). Zebrafish larvae at 11 dpf were exposed to cisplatin for 24 hours at two concentrations of 10 and 50 µM (3,000 and 15,000 µg/l). Nuclear and mitochondrial

DNA damage was seen in lateral line and inner ear hair cells. This damage was concentration-dependent and was observed using a Tunnelling Electron Microscope (TEM). The damage was greater in the inner ear cells where vacuolation was also observed in the cytoplasm (Figure 53).

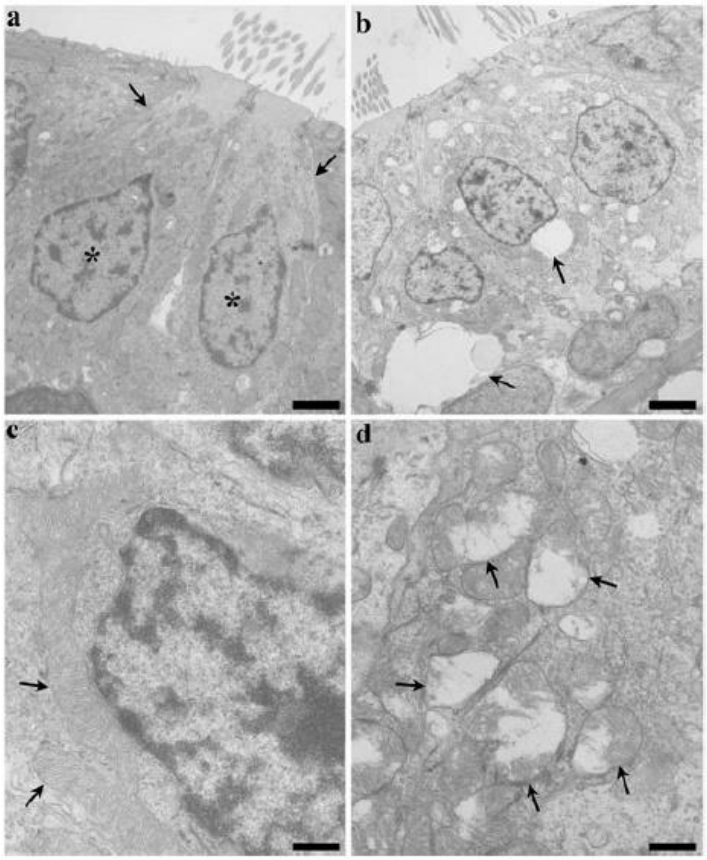


Figure 53: TEM images of zebrafish inner ear cells. a) Two inner ear cells in a fish from the control group. The arrows indicate the hair cells and the asterisks the nuclei; scale bar = 1.5 µm b) A hair cell from a fish exposed to 50 µM, the arrows indicating vacuoles that have formed in the cytoplasm; scale bar = 2.4 µm. c) A higher magnification Image from the control fish showing intact mitochondria;; scale bar = 0.6 µm. d) Damaged mitochondria in a fish exposed to 50 µM; scale bar = 0.6 µm.

Condensed chromatin observed in the nuclei of lateral hair line cells in exposed fish, with condensation increasing with concentration. In the same cells the number of cristae in the mitochondria can be seen to decrease with concentration (Figure 54). As with Ou *et al.* (2007), although the exposure period was shorter than the experiments in this thesis, the concentrations that the larvae were exposed to were far higher.

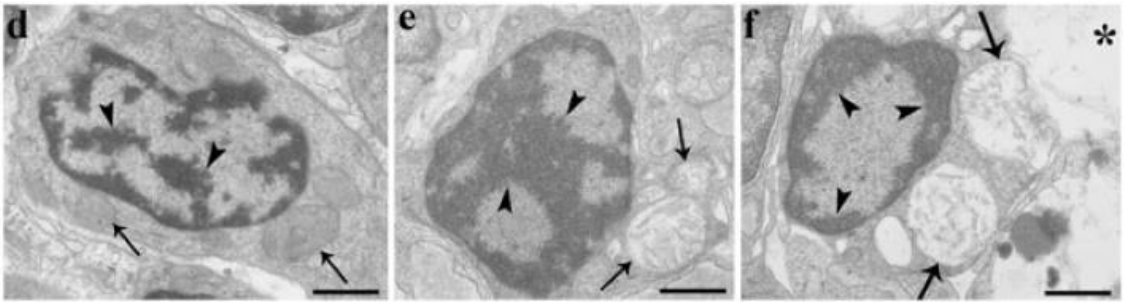


Figure 54: Chromatin condensation and mitochondrial damage in lateral line hair cells in d) control, e) 10 µM and f) 50 µM exposed zebrafish. Condensation (arrowheads) and loss of cristae (arrows) can be seen to increase with increasing concentrations of cisplatin.

5.6.4 Simple mixture

In the fish exposed to the simple mixture of the three drugs, the only significant result observed was an increase in p53 RNA expression in female fish. No corresponding increase was observed in the single chemical exposures in this experiment. The only corresponding response was in fish exposed to 100 µg/l of cyclophosphamide in the pilot experiment, whereas exposure to cyclophosphamide at the nominal concentration of 2000 µg/l, showed no significant increase. It could be argued that this is a response to the mixture of drugs, due to their combined effects. Ideally the mixture would have been designed by modelling the effects of the individual drugs and selecting their concentrations accordingly. As these data was not available, the arbitrary decision was taken to combine the drugs at their median concentrations from their respective concentration series experiments. There is no consistent indication that any of the drugs, when fish were exposed singly, produced an increase in p53 RNA expression. It is more likely that the increase observed in the mixture was due to biological variability between individuals.

5.6.4.1 Molecular effects of anticancer drug mixtures on other species reported in the literature

Molecular effects in fish due to exposure to cytotoxic mixtures have not been reported in the literature. In the same paper that assessed the effects of mixtures containing the anticancer drugs tamoxifen and cyclophosphamide and the antibiotic ciprofloxacin on algae (section 2.4.3), Mater *et al.* (2014) exposed HepG2 human liver cells to the same mixtures. This cell line was chosen as it retained the ability to metabolise drugs to their

active metabolites. The effects on cell viability and DNA damage were observed using the MTS cell proliferation and comet assays, on cells exposed for 24 and 72 hours. After 72 hours cell survival was significantly reduced, when exposed to the mixture at concentrations of ciprofloxacin 10, tamoxifen 1, cyclophosphamide 1 µg/l and ciprofloxacin 100, tamoxifen 10, cyclophosphamide 10 µg/l, the latter being the highest concentration of the mixture tested. When exposed to only cyclophosphamide, cell viability was also significantly reduced at 10 µg/l. DNA damage was detected in all mixture concentrations from the lowest of ciprofloxacin 0.01, tamoxifen 0.01, cyclophosphamide 0.01 µg/l, whereas no damage was observed in cells exposed to cyclophosphamide alone. Why cyclophosphamide alone would significantly reduce cell viability without appearing to damage DNA is unclear, given its mechanism of action.

Although conducted in mammalian cells in-vitro, the experiments were carried out at environmentally relevant concentrations. Significant results in cell viability were seen at concentrations two orders of magnitude lower than the highest concentration used in this thesis, for cyclophosphamide alone. This effect increased in the mixture, although as described in the algae chapter, this could be attributed to ciprofloxacin's ability to inhibit DNA repair and replication. The mixtures effect was most significant for DNA damage, as could be expected, with this being expected to occur before a reduction in cell viability. Although this logic does not fit with the reduction in cell viability without any corresponding increase in DNA damage, as was observed in cells exposed to only cyclophosphamide. The findings of Mater *et al.* (2014) could suggest that cyclophosphamide is more potent than the results of this project would suggest. A significant increase in p53 RNA expression was observed in the simple mixture in this project, which might be attributable to the presence of cyclophosphamide. There was also an increase in the expression of this RNA in the female fish exposed to cyclophosphamide at 100 µg/l in the pilot experiment. However, this increase was not observed in the males or repeated in the cyclophosphamide concentration series up to 2000 µg/l. If the cyclophosphamide and simple mixture experiment were to be repeated with the addition of the comet assay as one of the endpoints, this might provide additional data on which to assess this.

5.7 Zebrafish mRNA expression experiment conclusions

The null hypothesis stated that: “The selected cytotoxic pharmaceuticals, at the tested concentrations, will not cause significant effects on the RNA expression of the selected zebrafish genes”.

It was not possible to reject the null hypothesis for any of the single chemicals or the simple mixture.

None of the drugs tested appeared to produce significant effects in the mRNA expression of the target genes in adult zebrafish, at the concentrations that the fish were exposed to. Although there were some significant results, these were not consistent (i.e. not repeatable) and biological variability is the most plausible explanation. This appears to be supported by those studies found in the literature, where any significant effects on zebrafish morphology were observed at much higher concentrations than those used in this project. The one exception for 5-fluorouracil was the findings of Kovács *et al.* (2015), obtained under chronic exposure conditions, which could indicate that this drug has the potential to cause effects at environmentally relevant concentrations.

6 General Discussion

6.1 Species comparison

A comparison of the results obtained for the three species assessed in this project show some differences in their response to the cytotoxic drugs. A straight comparison between algae and daphnia results is possible as the same endpoint (EC_{50}) has been assessed in both species for all six drugs, albeit for different biological characteristics. The zebrafish results do not allow a straight comparison, so can only be discussed more generally in relation to the other two species.

6.1.1 Algae and daphnia comparison

For the single drug exposure experiments, both algae and daphnia appeared to be the least sensitive to cyclophosphamide, with both have in EC_{50} values in the millions of µg/l range. However, it is possible that algae may have been the least sensitive to carboplatin as this drug was not tested at a high enough concentration to elicit a response. The greatest difference in response to the single drugs was seen in the 5-fluorouracil exposure experiments. Algae was the most sensitive to this drug, with an EC_{50} for growth inhibition in the hundreds of µg/l range. Daphnia however were far less sensitive, with an EC_{50} for immobilisation in the hundreds of thousands of µg/l; the most toxic drug for this species being cisplatin with an EC_{50} in the thousands of µg/l range (Figure 55).

A large difference in response between the two species was also seen in the simple mixture experiment. As explained in section 2.2.2, the idea behind this experiment was to add the individual drugs to the mixture at a concentration that was the EC_{50} value divided by the number of the drugs in the mixture, in this case five drugs, so one fifth of their respective EC_{50} values. If the drugs exerted their effects on the species independently of each other, the mixture could expect to have an effect roughly equal to that caused by any of the five drugs at their individual EC_{50} concentration. It could be argued that this is what was observed in the algae simple mixture experiment with the mixture causing ~45% growth inhibition. In contrast, in the daphnia experiment, the percentage immobilisation caused by the simple mixture was much higher, at just under 90%. This could suggest that two or more of the drugs in the mixture were having an additive effect on daphnia immobilisation

(Figure 55). No response to the 'something from nothing' mixture was observed in the daphnia, compared to a ~2% growth inhibition observed in the algae (Figure 55). Whilst this does not corroborate the simple mixture results, this could be due to factors such as the increase in toxicity not being a linear relationship with the increase in drug concentration, and/or the potential problems with the 'something from nothing' experimental design described in section 2.2.2.

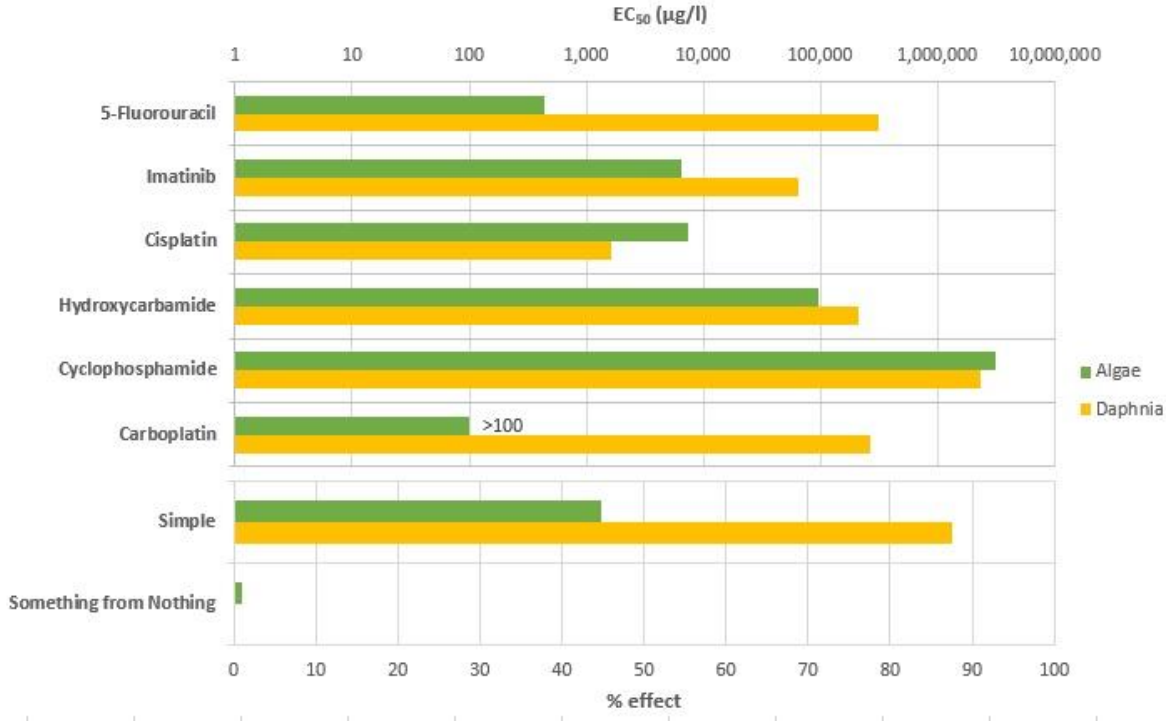


Figure 55: A comparison of the EC_{50} values for the effect of single drugs on algae growth inhibition and daphnia immobilisation (top) and the percentage of algae growth inhibition and daphnia immobilisation caused by the simple and something from nothing mixtures (bottom). For carboplatin, algae were tested up to 100 µg/l, with no observed growth inhibition. No immobilisation was observed in the daphnia 'something from nothing' experiment.

6.1.2 Zebrafish endpoint comparison

There was no consistent response in any of the endpoints assessed. Neither could any of the significant results observed be confidently attributed to the effects of any of the three drugs. It could be argued that the significant decrease in blood cell viability observed in the first two experiments involving 5-fluorouracil may be the exception, but given the conflicting results in the third experiment were far more likely to have been due to a technical error than a result of the drug. However, without a repeat of the third

experiment, it is not possible to discuss this further. All other significant results could more likely be attributed to biological variability, than to effects of the drugs (Table 41).

It can be argued that data from the pilot study should not be compared to the data generated by the concentration series experiments. In environmental toxicology, the aim of a pilot study is to give an indication of the effects, if any, caused by the substance being investigated. Often, it is also used to generate data that can then be fed into Power analysis, to aid the design of a robust experiment. To reduce the costs and for species whose use is regulated, such as the zebrafish, the number of organisms used is kept to a minimum. This can mean, as was the case in this study, that fewer concentrations are tested and no replicates are used. From a statistical perspective, this means that the output of a pilot study is not robust enough to give reliable results. This increases the risk of producing false positive or negative results, when statistically tested. Comparing these less reliable results with those generated from a well-designed and robust experiment can then be misleading as it is not a like for like comparison.

Table 41: A high level comparison of the morphology, blood and molecular endpoints assessed in the zebrafish drug exposure experiment. Some significant results were only observed in one sex, but to simplify, in order to simplify comparison, the sex has not been shown. ⇔ = no significant difference from controls, 🡻 = significant decrease compared to controls, 🡹 = significant increase compared to controls, X = endpoint not assessed in this experiment, CF = Condition Factor, HSI = Hepatosomatic Index, GoSI = Gondal Somatic Index, GiSI = Gill Somatic Index, Blood = the results from the imaging flow cytometry assay only for % of non-viable cells, RAD51, p53 and VIT are the three genes assessed for gene expression.

	CF	HSI	GoSI	GiSI	Blood	RAD51	p53	VIT
5-fluorouracil Pilot	⇔	🡻	X	⇔	🡹	⇔	⇔	🡹
5-fluorouracil 1	⇔	⇔	⇔	⇔	🡹	⇔	⇔	⇔
5-fluorouracil 2	⇔	⇔	⇔	⇔	🡻	⇔	⇔	🡹
5-fluorouracil Mixture	⇔	🡻	⇔	X	X	🡹	⇔	⇔
cyclophosphamide Pilot	🡻	🡻	X	⇔	⇔	⇔	🡹	⇔
cyclophosphamide	⇔	⇔	⇔	⇔	X	🡻	🡻	⇔
cyclophosphamide Mixture	⇔	🡹	🡹	X	X	⇔	⇔	⇔
cisplatin Pilot	⇔	⇔	X	🡹	🡻	⇔	🡻	🡻
cisplatin	⇔	⇔	X	⇔	X	⇔	⇔	⇔
cisplatin Mixture	🡹	🡹	⇔	X	X	⇔	⇔	⇔

Simple Mixture	⇔	⇔	⇔	⇔	X	⇔	🡅	⇔

As the zebrafish experiments were intended to be environmentally relevant, the concentrations of the drugs the fish were exposed to were far lower than the concentrations used in the algae and daphnia experiments. This fact, combined with the lack of consistent results across the zebrafish experiments, mean it is not possible to compare zebrafish sensitivity to the tested drugs, with that of the algae and daphnia.

6.2 Environmental relevance

No effects were observed in any of the species, at concentrations typically measured or predicted in the surface waters of European countries. All three species were tested at or above concentrations measured in environments with atypically high levels of these drugs, such as hospital effluents. While 5-fluorouracil was observed to inhibit algal growth at concentrations measured in hospital effluents, these concentrations would quickly be diluted to far lower concentrations within the sewerage systems of developed countries.

As was described in section 2.4.1, high levels of pharmaceuticals (hundreds of µg/l to tens of mg/l) were measured in surface waters near drug production facilities in some developing countries. No evidence was found in the literature that these waters have been tested for the presence of cytotoxic drugs, apart from Cyclophosphamide in once instance. However, an increase in the use of cytotoxic drugs, particularly off-patent drugs such as 5-Fluororuacil, has been predicted for low to middle income countries (Farmer *et al.*, 2010). As drug manufacturing facilities in these countries tend to focus on off-patent drugs, due to their comparative cheapness to produce, it is not implausible to suggest that manufacturing of cytotoxic drugs could increase in these facilities. Combined with the poor sewage infrastructure in low and middle income countries (Kookana *et al.*, 2014), this potential increase in manufacturing and consumption may increase the chance of these drugs occurring in the environment, at concentrations shown to cause effects in this project.

Although the algae protocol can be considered to replicate chronic exposure conditions, the daphnia and zebrafish exposure periods are best described as acute, as they only expose one generation for part of its lifecycle. In the environment, aquatic species are continuously exposed for generation after generation. Whilst environmental concentrations of the drugs in this project have been measured and/or predicted at concentrations far below those used in these experiments, the potential for chronic exposure effects have not been addressed.

The majority of this project focussed on the effects of the individual drugs, whereas in the environment these will be present along with a wide range of pharmaceuticals and other chemicals. Although simple mixture experiments were conducted on all three species, they could have been more robustly designed, in order to allow a better understanding of the mixture effects. In addition, the effects of these cytotoxic drugs in mixtures with other pharmaceuticals was not considered, but has been shown to be relevant in other studies (Backhaus 2014). Indeed, evidence that strongly additive effects can occur when cytotoxic drugs are included in a mixture with a different class of pharmaceutical was found in the literature (Mater *et al.*, 2014), as described in section 2.4.3.3.

As noted by Daniel *et al.* (2004) and confirmed by this project, experiments should be conducted on a diverse range of multiple species, to assess differences in response. As was seen in this project, and in the literature, some drugs were more toxic to crustaceans than algae and vice versa. The need to test on multiple diverse species is further supported by the findings of Gunnarsson *et al.* (2008). This research group used computer analysis to predict orthologs for human drug targets in a range of species, for which complete genome maps existed. In common with the three species used in this Ph.D. project, this included *D. rerio* and the closely related *Daphnia pulex* and green algae *Chlamydomonas reinhardtii*. Orthologs for 1,318 human drug targets were predicted, with 86% being detected in D. rerio, 61% in *D. pulex* and 35% in *C. reinhardtii*. In this paper Gunnarsson *et al.* (2008) highlight that at the relatively low concentrations of drugs found in the environment, it is the designated mechanism of action that is likely to cause adverse effects, rather than general toxicity. On the surface this would suggest that zebrafish could be expected to be more susceptible to human pharmaceuticals in the environment. However, all but two of the drugs tested exerted their main mechanism of action directly onto DNA, a molecule

that is shared by the vast majority of species. Interestingly, 5-Fluorouracil, one of the drugs that indirectly affected DNA as its main mechanism of action, was found to be most toxic to the green algae used in this Ph.D. project. Nevertheless, the concept of conservation of drug targets is relevant to DNA damaging drugs from a related perspective. All the drugs tested can be considered prodrugs, as they must be metabolised into their active forms. If a species does not have the enzyme orthologs for the relevant metabolism pathway, then it is less likely this drug can exert its mechanism of action in that species. Whilst the literature review for this project included researching the metabolism pathways of the target drugs, no work was conducted on identifying the required enzymes within the test species. This information would have been useful in assessing the risk these drugs posed for each of the test species, before experimental work had been conducted. It may have allowed a more comprehensive assessment of target drugs, identifying those more likely to be of risk to the test species.

A factor relevant to all drugs tested that was not considered, was the role of pH and pKa in their toxicity. pH is known to have a range of effects, including on the ability of the intestinal tract to absorb drugs and the distribution of a drug and metabolites between blood plasma and cells (Milne 1965). This ability is defined by the drug's PKa, which describes the ionisation and disassociation states of a drug, depending on the pH of the environment a drug is in (e.g. the stomach or intestine) and whether a drug is acidic or basic. These attributes affect the ability of the drug to be taken up by an organism. Furthermore, changes in the environmental pH can also affect the efficiency with which an organism can take up a drug. The pH of the water in a healthy river in the UK is between 6 and 9 (Carvalho *et al.* 2002) and the pH of freshwater fish is generally 7.4 (Wurts *et al.* 1992). The algae exposure protocol required the algae medium to be 8.1 (±0.2). No requirements for pH were defined for the daphnia exposure experiments and pH was not monitored. pH was monitored daily throughout the acclimatisation and exposure stages of the zebrafish experiments, with pH being required to be maintained between 6.0 and 8.5 on the instruction of the aquatics facility manager, with an actual range achieved of 7.2 to 8.5. A pH of 7.4 was used for the fish blood plasma. Where measured, pH was maintained within the boundaries of a healthy UK river. This gave some confidence that the experiments were conducted under conditions relevant to the UK freshwater environment. However, it would

be beneficial to know how the drugs behaved due to changes in environmental pH, within the range of 6.0 – 9.0. This may have impacted on the toxicity of the drug and could be a factor in the differences in toxicity observed between the species. Unfortunately, this was not possible within the time constraints of this PhD project.

6.3 General conclusion

No effects were observed above the environmental concentrations measured or predicted in surface waters in European countries. Whilst data on other developed nations was not collected, it is likely that these findings are likely to be applicable to their surface waters also.

In surface waters of some developing countries, close to drug manufacturing facilities, 5-fluorouracil and possibly Cisplatin have the potential to occur at concentrations that may produce adverse effects in the tested species. However, further research is required to establish if these drugs are actually present.

6.4 Next steps

If more funding could be found, the following research could be conducted:

- Multiple generation daphnia reproduction experiments, to assess the chronic effects of these drugs in this species
- Histopathology on the livers collected from the zebrafish exposure experiments.
- Micro nuclei assays on the blood cells collected from the zebrafish exposure experiments.
- Completion of the vitellogenin ELISA assays on the plasma collected from the zebrafish exposure experiments
- Completion of the method development for the immunohistochemistry technique, due to its great potential as a more sensitive and definitive assay for DNA damage.

7 References

Adams, N. and Dobbs, A. (1984) 'A comparison of results from two test methods for assessing the toxicity of aminotriazole to Selenastrum capricornutum', *Chemosphere,* 13(8), pp. 965-971.

Andersen, C.L., Jensen, J.L. and Ørntoft, T.F. (2004) 'Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets', *Cancer research,* 64(15), pp. 5245-5250.

Arany, I. and Safirstein, R.L. (2003) 'Cisplatin nephrotoxicity', *Seminars in nephrology.* Elsevier, 460-464.

Barsanti, L. and Gualtieri, P. (2006) 'Algae: anatomy, biochemistry, and biotechnology', .

Bartlett, J.M. and Stirling, D. (2003) 'A short history of the polymerase chain reaction', in *PCR protocols.* Springer, pp. 3-6.

Belt, R.J., Haas, C.D., Kennedy, J. and Taylor, S. (1980) 'Studies of hydroxyurea administered by continuous infusion. Toxicity, pharmacokinetics, and cell synchronization', *Cancer,* 46(3), pp. 455-462.

Besse, J., Latour, J. and Garric, J. (2012) 'Anticancer drugs in surface waters: What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs?', *Environment international,* 39(1), pp. 73-86.

Biales, A.D., Bencic, D.C., Lazorchak, J.L. and Lattier, D.L. (2007) 'A quantitative real-time polymerase chain reaction method for the analysis of vitellogenin transcripts in model and nonmodel fish species', *Environmental Toxicology and Chemistry,* 26(12), pp. 2679-2686.

Booker, V., Halsall, C., Llewellyn, N., Johnson, A. and Williams, R. (2014) 'Prioritising anticancer drugs for environmental monitoring and risk assessment purposes', *Science of the Total Environment,* 473, pp. 159-170.

Bound, J.P. and Voulvoulis, N. (2005) 'Household Disposal of Pharmaceuticals as a Pathway for Aquatic Contamination in the United Kingdom', *Environmental health perspectives,* 113(12), pp. pp. 1705-1711.

Brezovšek, P., Eleršek, T. and Filipič, M. (2014) 'Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga Pseudokirchneriella subcapitata and the cyanobacterium Synechococcus leopoliensis', *Water research,* 52(0), pp. 168-177.

Bristol-Myers Squibb Company (2010) *Hydroxyurea Safety Data Sheet*. Available at: http://www.msdsexplorer.com/PDFsFiles/7151.pdf (Accessed: 06/12 2015).

Brown, M.B. and Forsythe, A.B. (1974) 'Robust tests for the equality of variances', *Journal of the American Statistical Association,* 69(346), pp. 364-367.

Buerge, I.J., Buser, H.R., Poiger, T. and Muller, M.D. (2006) 'Occurrence and fate of the cytostatic drugs cyclophosphamide and ifosfamide in wastewater and surface waters', *Environmental science & technology,* 40(23), pp. 7242-7250.

Buser, H., Poiger, T. and Müller, M.D. (1999) 'Occurrence and environmental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and in wastewater', *Environmental science & technology,* 33(15), pp. 2529-2535.

Busquet, F., Nagel, R., von Landenberg, F., Mueller, S.O., Huebler, N. and Broschard, T.H. (2008) 'Development of a new screening assay to identify proteratogenic substances using zebrafish danio rerio embryo combined with an exogenous mammalian metabolic activation system (mDarT)', *Toxicological sciences : an official journal of the Society of Toxicology,* 104(1), pp. 177-188.

Cachot, J., Galgani, F. and Vincent, F. (1998) 'cDNA cloning and expression analysis of flounder p53 tumour suppressor gene', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 121(3), pp. 235-242.

Carlsson, G., Örn, S. and Larsson, D.G.J. (2009) 'Effluent from bulk drug production is toxic to aquatic vertebrates', *Environmental Toxicology and Chemistry,* 28(12), pp. 2656-2662.

Carradice, D. and Lieschke, G.J. (2008) 'Zebrafish in hematology: sushi or science?', *Blood,* 111(7), pp. 3331-3342.

Cave, T.A., Norman, P. and Mellor, D. (2007) 'Cytotoxic drug use in treatment of dogs and cats with cancer by UK veterinary practices (2003 to 2004)', *Journal of Small Animal Practice,* 48(7), pp. 371-377.

Chabner, B.A., Amrein, P.C., Druker, B.J., Michaelson, M.D., Mitsiades, C.S., Goss, P.E., Ryan, D.P., Ramachandra, S., Richardson, P.G., Supko, J.G. and Wyndham, H.W. (2006) 'Antineoplastic Agents', in Brunton, L.L., Lazo, J.S. and Parker, K.L. (eds.) *Goodman & Gilman's The Pharmacological Basis of Therapeutics.* 11th edn. McGraw-Hill.

Chen, J., Ruan, H., NG, S.M., Gao, C., Soo, H.M., Wu, W., Zhang, Z., Wen, Z., Lane, D.P. and Peng, J. (2005) 'Loss of function of def selectively up-regulates Delta113p53 expression to arrest expansion growth of digestive organs in zebrafish', *Genes and Development,* 19(23), pp. 2900-11.

Chu, E. and Sartorelli, A.C. (2012) 'Cancer Chemotherapy', in Katzung, B.G. (ed.) *Basic and Clinical Pharmacology.* 12th edn. New York: Lange Medical Books/McGraw Hill, pp. 949-975 (Chapter 54).

Coates, P.J., Save, V., Ansari, B. and Hall, P.A. (1995) 'Demonstration of DNA damage/repair in individual cells using in situ end labelling: association of p53 with sites of DNA damage', *The Journal of pathology,* 176(1), pp. 19-26.

Cooper, C.A., Handy, R.D. and Bury, N.R. (2006) 'The effects of dietary iron concentration on gastrointestinal and branchial assimilation of both iron and cadmium in zebrafish (Danio rerio)', *Aquatic Tox,* 79(2), pp. 167-75.

Daniel, M., Sharpe, A., Driver, J., Knight, A.W., Keenan, P.O., Walmsley, R.M., Robinson, A., Zhang, T. and Rawson, D. (2004) 'Results of a technology demonstration project to compare rapid aquatic toxicity screening tests in the analysis of industrial effluents', *Journal of environmental monitoring : JEM,* 6(11), pp. 855-865.

Daughton, C.G. and Ternes, T.A. (1999) 'Pharmaceuticals and personal care products in the environment: agents of subtle change?', *Environmental health perspectives,* 107 Suppl 6, pp. 907-938.

Davidson, A.J. and Zon, L.I. (2004) 'The 'definitive'(and 'primitive') guide to zebrafish hematopoiesis', *Oncogene,* 23(43), pp. 7233-7246.

Desesso, J.M., Scialli, A.R. and Goeringer, G.C. (1994) 'D-mannitol, a specific hydroxyl free radical scavenger, reduces the developmental toxicity of hydroxyurea in rabbits', *Teratology,* 49(4), pp. 248-259.

DeYoung, D., Bantle, J., Hull, M. and Burks, S. (1996) 'Differences in sensitivity to developmental toxicants as seen in Xenopus and Pimephales embryos', *Bulletin of environmental contamination and toxicology,* 56(1), pp. 143-150.

Di Lauro, L., Pizzuti, L., Barba, M., Sergi, D., Sperduti, I., Mottolese, M., Del Medico, P., Belli, F., Vici, P., De Maria, R. and Maugeri-Sacca, M. (2015) 'Efficacy of chemotherapy in metastatic male breast cancer patients: a retrospective study', *Journal of experimental & clinical cancer research : CR,* 34(1), pp. 26-015-0143-8.

Dickenson, E.R., Snyder, S.A., Sedlak, D.L. and Drewes, J.E. (2011) 'Indicator compounds for assessment of wastewater effluent contributions to flow and water quality', *Water research,* 45(3), pp. 1199-1212.

Drewes, J.E., Heberer, T., Rauch, T. and Reddersen, K. (2003) 'Fate of pharmaceuticals during ground water recharge', *Groundwater Monitoring & Remediation,* 23(3), pp. 64-72.

Du, C., Niu, R., Chu, E., Zhang, P. and Lin, X. (2006) 'Sequence analysis and functional study of thymidylate synthase from zebrafish, Danio rerio', *Journal of Biochemistry,* 139(5), pp. 913-920.

Eastman, A. and Barry, M.A. (1987) 'Interaction of trans-diamminedichloroplatinum(II) with DNA: formation of monofunctional adducts and their reaction with glutathione', *Biochemistry,* 26(12), pp. 3303-3307.

Ebert, D. (2005) 'Introduction to the Ecology, Epidemiology, and Evolution of Parasitism in Daphnia', .

Egeler, P. and Seck, C. (2008) '5-fluorouracil: A study on the toxicity to early-life stages of Danio rerio (zebrafish)', *ECT 08AZ1FV,* 7, pp. 1009.

Elledge, S.J., Zhou, Z. and Allen, J.B. (1992) 'Ribonucleotide reductase: regulation, regulation, regulation', *Trends in biochemical sciences,* 17(3), pp. 119-123.

Emadi, A., Jones, R.J. and Brodsky, R.A. (2009) 'Cyclophosphamide and cancer: golden anniversary', *Nat Rev Clin Oncol,* 6(11), pp. 638-647.

Embry, M.R., Billiard, S. and Di Giulio, R. (2006) 'Lack of p53 induction in fish cells by model chemotherapeutics', *Oncogene,* 25(14), pp. 2004-2010.

Estape, J., Daniels, M., Viñolas, N., Santabarbara, P., Grau, J.J. and Gardella, S. (1990) 'Combination chemotherapy with oral etoposide plus intravenous cyclophosphamide in liver metastases of breast cancer.', *American journal of clinical oncology,* 13(2), pp. 98-100.

Farmer, P., Frenk, J., Knaul, F.M., Shulman, L.N., Alleyne, G., Armstrong, L., Atun, R., Blayney, D., Chen, L. and Feachem, R. (2010) 'Expansion of cancer care and control in countries of low and middle income: a call to action', *The Lancet,* 376(9747), pp. 1186-1193.

Fawley, M.W., Dean, M.L., Dimmer, S.K. and Fawley, K.P. (2006) 'EVALUATING THE MORPHOSPECIES CONCEPT IN THE SELENASTRACEAE (CHLOROPHYCEAE, CHLOROPHYTA) 1', *Journal of Phycology,* 42(1), pp. 142-154.

Fent, K., Weston, A.A. and Caminada, D. (2006) 'Ecotoxicology of human pharmaceuticals', *Aquatic Toxicology (Amsterdam, Netherlands),* 76(2), pp. 122-159.

Fichtinger-Schepman, A.M.J., van Dijk-Knijnenburg, H.C.M., van der Velde-Visser, S.D., Berends, F. and Baan, R.A. (1995) 'Cisplatin- and carboplatin-DNA adducts: is Pt-AG the cytotoxic lesion?', *Carcinogenesis,* 16(10), pp. 2447-2453.

Fichtinger-Schepman, A., Van, d.V., Den Hartog, J.H.J., Lohman, P.H.M. and Reedijk, J. (1985) 'Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation', *Biochemistry,* 24(3), pp. 707-713.

Fick, J., Söderström, H., Lindberg, R.H., Phan, C., Tysklind, M. and Larsson, D.G.J. (2009) 'Contamination of surface, ground, and drinking water from pharmaceutical production', *Environmental Toxicology and Chemistry,* 28(12), pp. 2522-2527.

Field, A. (2009) *Discovering statistics using SPSS.* Sage publications.

Finkel, T., Serrano, M. and Blasco, M.A. (2007) 'The common biology of cancer and ageing', *Nature,* 448(7155), pp. 767-774.

Fishbein, W.N. and Carbone, P.P. (1963) 'Hydroxyurea: Mechanism of Action.', *Science,* 142, pp. 1069-1070.

Fitzsimmons, P.N., Fernandez, J.D., Hoffman, A.D., Butterworth, B.C. and Nichols, J.W. (2001) 'Branchial elimination of superhydrophobic organic compounds by rainbow trout (Oncorhynchus mykiss)', *Aquatic toxicology,* 55(1), pp. 23-34.

Franquet-Griell, H., Gómez-Canela, C., Ventura, F. and Lacorte, S. (2015) 'Predicting concentrations of cytostatic drugs in sewage effluents and surface waters of Catalonia (NE Spain)', *Environmental research,* 138, pp. 161-172.

Friedberg, E. (2003) 'DNA damage and repair', *Nature,* 421(6921), pp. 436.

Fritsche, M., Haessler, C. and Brandner, G. (1993) 'Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents', *Oncogene,* 8(2), pp. 307-318.

Gambacorti-Passerini, C.B., Gunby, R.H., Piazza, R., Galietta, A., Rostagno, R. and Scapozza, L. (2003) 'Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias', *The lancet oncology,* 4(2), pp. 75-85.

Giacchetti, S., Itzhaki, M., Gruia, G., Adam, R., Zidani, R., Kunstlinger, F., Brienza, S., Alafaci, E., Bertheault-Cvitkovic, F., Jasmin, C., Reynes, M., Bismuth, H., Misset, J.L.

and Levi, F. (1999) 'Long-term survival of patients with unresectable colorectal cancer liver metastases following infusional chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and surgery', *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO,* 10(6), pp. 663-669.

Giari, L., Dezfuli, B.S., Astolfi, L. and Martini, A. (2012) 'Ultrastructural effects of cisplatin on the inner ear and lateral line system of zebrafish (Danio rerio) larvae', *Journal of Applied Toxicology,* 32(4), pp. 293-299.

Grisolia, C.K. and Starling, F.L. (2001) 'Micronuclei monitoring of fishes from Lake Paranoá, under influence of sewage treatment plant discharges', *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 491(1), pp. 39-44.

Grung, M., Källqvist, T., Sakshaug, S., Skurtveit, S. and Thomas, K.V. (2008) 'Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline', *Ecotoxicology and environmental safety,* 71(2), pp. 328-340.

Guengerich, F.P. (2008) 'Cytochrome P450 and Chemical Toxicology', *Chemical research in toxicology,* 21(1), pp. 70-83.

Guryev, V., Koudijs, M.J., Berezikov, E., Johnson, S.L., Plasterk, R.H.A., van Eeden, F.,J.M. and Cuppen, E. (2006) 'Genetic variation in the zebrafish', *Genome research,* 16(4), pp. 491-497.

Hansen, M.C., Nielsen, A.K., Molin, S., Hammer, K. and Kilstrup, M. (2001) 'Changes in rRNA levels during stress invalidates results from mRNA blotting: flourescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels', *J Bacteriol,* 183, pp. 4747-4751.

Harrap, K.R. (1985) 'Preclinical studies identifying carboplatin as a viable cisplatin alternative', *Cancer treatment reviews,* 12(Supplement 1), pp. 21-33.

Hartmann, J. and Lipp, H. (2003) 'Toxicity of platinum compounds', *Expert opinion on pharmacotherapy,* 4(6), pp. 889-901.

Hendriks-Balk, M., Michel, M.C. and Alewijnse, A.E. (2007) 'Pitfalls in the normalization of real-time polymerase chain reaction data', *Basic Res Cardiol,* 102, pp. 195-197.

Higashida, M., Matsumoto, H., Kubota, H., Murakami, H., Kawabe, Y., Nakashima, H., Oka, Y., Okumura, H., Nakamura, M. and Hirai, T. (2012) 'Evaluation of 5-FU plasma concentration by 13C breath test in patients treated with oral 5-FU analogs', *Anticancer Research,* 32(12), pp. 5407-5414.

Hignite, C. and Azarnoff, D.L. (1977) 'Drugs and drug metabolites as environmental contaminants: Chlorophenoxyisobutyrate and salicylic acid in sewage water effluent', *Life Sciences,* 20(2), pp. 337-341.

Homsi, J. and Daud, A.I. (2007) 'Spectrum of activity and mechanism of action of VEGF/PDGF inhibitors', *Cancer Control,* 14(3), pp. 285.

Huggett, D.B., Cook, J.C., Ericson, J.F. and Williams, R.T. (2003) 'A Theoretical Model for Utilizing Mammalian Pharmacology and Safety Data to Prioritize Potential Impacts of Human Pharmaceuticals to Fish', *Human and Ecological Risk Assessment: An International Journal,* 9(7), pp. 1789-1799.

Islinger, M., Willimski, D., Völkl, A. and Braunbeck, T. (2003) 'Effects of 17a-ethinylestradiol on the expression of three estrogen-responsive genes and cellular ultrastructure of liver and testes in male zebrafish', *Aquatic toxicology,* 62(2), pp. 85-103.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M. and Barcelo, D. (2011) 'Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment', *Water research,* 45(3), pp. 1165-1176.

Jiang, Y.L., McDowell, L.M., Poliks, B., Studelska, D.R., Cao, C., Potter, G.S., Schaefer, J., Song, F. and Stivers, J.T. (2004) 'Recognition of an Unnatural Difluorophenyl Nucleotide by Uracil DNA Glycosylaseâ€ ', *Biochemistry,* 43(49), pp. 15429-15438.

John, D., Whitton, B. and Brook, A. (2011) 'The freshwater algal flora of the British Isles: an identification guide to freshwater and terrestrial algae', .

Johnson, A.C., Juergens, M.D., Williams, R.J., Kuemmerer, K., Kortenkamp, A. and Sumpter, J.P. (2008) 'Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study', *Journal of Hydrology,* 348(1-2), pp. 167-175.

Joy, M.S., La, M., Wang, J., Bridges, A.S., Hu, Y., Hogan, S.L., Frye, R.F., Blaisdell, J., Goldstein, J.A. and Dooley, M.A. (2012) 'Cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics in patients with glomerulonephritis secondary to lupus and small vessel vasculitis', *British journal of clinical pharmacology,* 74(3), pp. 445-455.

Julian, D., Ennis, K. and Korenbrot, J.I. (1998) 'Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina', *The Journal of comparative neurology,* 394(3), pp. 271-282.

Jürgens, K. (1994) 'Impact of Daphnia on planktonic microbial food webs-a review', *Marine Microbial Food Webs,* 8(1-2), pp. 295-324.

Kaina, B. (2003) 'DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling', *Biochemical pharmacology,* 66(8), pp. 1547-1554.

Kanehisa Laboratories (2015) ***Organism group: Green algae***. Available at: http://www.genome.jp/kegg-bin/show_organism?menu_type=pathway_maps&category=Green%20algae (Accessed: 22/05 2015).

Kang, Y.K., Kang, W.K., Shin, D.B., Chen, J., Xiong, J., Wang, J., Lichinitser, M., Guan, Z., Khasanov, R., Zheng, L., Philco-Salas, M., Suarez, T., Santamaria, J., Forster, G. and McCloud, P.I. (2009) 'Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III

noninferiority trial', *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO,* 20(4), pp. 666-673.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. (1991) 'Participation of p53 protein in the cellular response to DNA damage', *Cancer research,* 51(23 Part 1), pp. 6304-6311.

Kazianis, S., Gan, L., Della Coletta, L., Santi, B., Morizot, D.C. and Nairn, R.S. (1998) 'Cloning and comparative sequence analysis of TP53 in Xiphophorus fish hybrid melanoma models', *Gene,* 212(1), pp. 31-38.

Kelland, L. (2007) 'The resurgence of platinum-based cancer chemotherapy', *Nat Rev Cancer,* 7(8), pp. 573-584.

Kennedy, B. (1999) '5-Fluorouracil toxicity', *Cancer,* 86(7), pp. 1099-1100.

Khanna, K.K. and Jackson, S.P. (2001) 'DNA double-strand breaks: signaling, repair and the cancer connection', *Nature genetics,* 27(3), pp. 247-254.

Kim, I., Yamashita, N. and Tanaka, H. (2009) 'Photodegradation of pharmaceuticals and personal care products during UV and UV/H 2 O 2 treatments', *Chemosphere,* 77(4), pp. 518-525.

Klavarioti, M., Mantzavinos, D. and Kassinos, D. (2009) 'Removal of residual pharmaceuticals from aqueous systems by advanced oxidation processes', *Environment international,* 35(2), pp. 402-417.

Knox, R.J., Friedlos, F., Lydall, D.A. and Roberts, J.J. (1986) 'Mechanism of Cytotoxicity of Anticancer Platinum Drugs: Evidence That cis-Diamminedichloroplatinum(II) and cis-Diammine-(1,1-cyclobutanedicarboxylato)platinum(II) Differ Only in the Kinetics of Their Interaction with DNA', *Cancer research,* 46(4 Part 2), pp. 1972-1979.

Koç, A., Wheeler, L.J., Mathews, C.K. and Merrill, G.F. (2004) 'Hydroxyurea Arrests DNA Replication by a Mechanism That Preserves Basal dNTP Pools', *Journal of Biological Chemistry,* 279(1), pp. 223-230.

Kookana, R.S., Williams, M., Boxall, A.B.A., Larsson, D.G.J., Gaw, S., Choi, K., Yamamoto, H., Thatikonda, S., Zhu, Y. and Carriquiriborde, P. (2014) 'Potential ecological footprints of active pharmaceutical ingredients: an examination of risk factors in low-, middle- and high-income countries', *Philosophical Transactions of the Royal Society B: Biological Sciences,* 369(1656).

Kosjek, T. and Heath, E. (2011) 'Occurrence, fate and determination of cytostatic pharmaceuticals in the environment', *TrAC Trends in Analytical Chemistry,* 30(7), pp. 1065-1087.

Kovács, R., Csenki, Z., Bakos, K., Urbányi, B., Horváth, Á, Garaj-Vrhovac, V., Gajski, G., Gerić, M., Negreira, N. and de Alda, M.L. (2015) 'Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in zebrafish (Danio rerio) two-generation study', *Water research,* 77, pp. 201-212.

Kovalova, L., McArdell, C.S. and Hollender, J. (2009) 'Challenge of high polarity and low concentrations in analysis of cytostatics and metabolites in wastewater by hydrophilic interaction chromatography/tandem mass spectrometry', *Journal of Chromatography a,* 1216(7), pp. 1100-1108.

Kruh, G.D. (2003) 'Lustrous Insights into Cisplatin Accumulation', *Clinical Cancer Research,* 9(16), pp. 5807-5809.

Kümmerer, K. (2001) 'Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources–a review', *Chemosphere,* 45(6), pp. 957-969.

Kümmerer, K. (2010) 'Pharmaceuticals in the Environment', *Annual Review of Environment and Resources,* 35(1), pp. 57-75.

Kümmerer, K. and Helmers, E. (1997) 'Hospital effluents as a source for platinum in the environment', *Science of The Total Environment,* 193(3), pp. 179-184.

Kümmerer, K., Helmers, E., Hubner, P., Mascart, G., Milandri, M., Reinthaler, F. and Zwakenberg, M. (1999) 'European hospitals as a source for platinum in the

environment in comparison with other sources', *The Science of the total environment,* 225(1-2), pp. 155-165.

Lampert, W., Fleckner, W., Rai, H. and Taylor, B.E. (1986) 'Phytoplankton control by grazing zooplankton: A study on the spring clear-water phase1', *Limnology and Oceanography,* 31(3), pp. 478-490.

Langford, K.H. and Thomas, K.V. (2009) 'Determination of pharmaceutical compounds in hospital effluents and their contribution to wastewater treatment works', *Environment international,* 35(5), pp. 766-770.

Larsson, D.J. and Fick, J. (2009) 'Transparency throughout the production chain—a way to reduce pollution from the manufacturing of pharmaceuticals?', *Regulatory Toxicology and Pharmacology,* 53(3), pp. 161-163.

Larsson, D.G.J., de Pedro, C. and Paxeus, N. (2007) 'Effluent from drug manufactures contains extremely high levels of pharmaceuticals', *Journal of hazardous materials,* 148(3), pp. 751-755.

Le Guenno, G., Mahr, A., Pagnoux, C., Dhote, R., Guillevin, L. and French Vasculitis Study Group (2011) 'Incidence and predictors of urotoxic adverse events in cyclophosphamide-treated patients with systemic necrotizing vasculitides', *Arthritis & Rheumatism,* 63(5), pp. 1435-1445.

Lester, Y., Avisar, D., Gozlan, I. and Mamane, H. (2011) 'Removal of pharmaceuticals using combination of UV/H(2)O(2)/O(3) advanced oxidation process', *Water science and technology : a journal of the International Association on Water Pollution Research,* 64(11), pp. 2230-2238.

Li, D., Yang, M., Hu, J., Ren, L., Zhang, Y. and Li, K. (2008) 'Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river', *Environmental Toxicology and Chemistry,* 27(1), pp. 80-86.

Li, Y., Huang, W., Huang, S., Du, J. and Huang, C. (2012) 'Screening of anti-cancer agent using zebrafish: Comparison with the MTT assay', *Biochemical and biophysical research communications,* 422(1), pp. 85-90.

Lin, Z., Kong, H., Nei, M. and Ma, H. (2006) 'Origins and Evolution of the recA/RAD51 Gene Family: Evidence for Ancient Gene Duplication and Endosymbiotic Gene Transfer', *Proceedings of the National Academy of Sciences of the United States of America,* 103(27), pp. pp. 10328-10333.

Liu, J., Gong, L., Chang, C., Liu, C., Peng, J. and Chen, J. (2012) 'Development of Novel Visual-Plus Quantitative Analysis Systems for Studying DNA Double-Strand Break Repairs in Zebrafish', *Journal of Genetics and Genomics,* 39(9), pp. 489-502.

Liu, S., Liu, Y., Li, J., Guo, M., Nie, L. and Yao, S. (2005) 'Study on the interaction between DNA and protein induced by anticancer drug carboplatin', *Journal of Biochemical and Biophysical Methods,* 63(2), pp. 125-136.

Longley, D.B., Harkin, D.P. and Johnston, P.G. (2003) '5-fluorouracil: mechanisms of action and clinical strategies', *Nat Rev Cancer,* 3(5), pp. 330-338.

Luecke, C., Vanni, M.J., Magnuson, J.J., Kitchell, J.F. and Jacobson, P.T. (1990) 'Seasonal regulation of Daphnia populations by planktivorous fish: Implications for the spring clear-water phase', *Limnology and Oceanography,* 35(8), pp. 1718-1733.

Macmillan Cancer Support (2010) *Carboplatin - Cancer Information - Macmillan Cancer Support*. Available at: http://www.macmillan.org.uk/Cancerinformation/Cancertreatment/Treatmenttypes/Chemotherapy/Individualdrugs/Carboplatin.aspx#DynamicJumpMenuManager_6_Anchor_2 (Accessed: August 24th 2011).

Mahnik, S.N., Rizovski, B., Fuerhacker, M. and Mader, R.M. (2004) 'Determination of 5-fluorouracil in hospital effluents', *Analytical and Bioanalytical Chemistry,* 380(1), pp. 31-35.

Maier, T., Güell, M. and Serrano, L. (2009) 'Correlation of mRNA and protein in complex biological samples', *FEBS letters,* 583(24), pp. 3966-3973.

Man, S., Bocci, G., Francia, G., Green, S.K., Jothy, S., Hanahan, D., Bohlen, P., Hicklin, D.J., Bergers, G. and Kerbel, R.S. (2002) 'Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water', *Cancer research,* 62(10), pp. 2731-2735.

Mao, Z., Bozzella, M., Seluanov, A. and Gorbunova, V. (2008) 'Comparison of nonhomologous end joining and homologous recombination in human cells', *DNA Repair,* 7(10), pp. 1765-1771.

Mark-Kappeler, C.J., Hoyer, P.B. and Devine, P.J. (2011) 'Xenobiotic Effects on Ovarian Preantral Follicles', *Biology of reproduction,* 85(5), pp. 871-883.

Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I. and Alonso, E. (2011) 'Simultaneous determination of a selected group of cytostatic drugs in water using high-performance liquid chromatography?triple-quadrupole mass spectrometry', *Journal of Separation Science,* 34(22), pp. 3166-3177.

Mater, N., Geret, F., Castillo, L., Faucet-Marquis, V., Albasi, C. and Pfohl-Leszkowicz, A. (2014) 'In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water', *Environment international,* 63(0), pp. 191-200.

Matsumoto, F. and Cólus, I. (2000) 'Micronucleus frequencies in Astyanax bimaculatus (Characidae) treated with cyclophosphamide or vinblastine sulfate', *Genetics and Molecular Biology,* 23(2), pp. 489-492.

McDonald, G.B., Slattery, J.T., Bouvier, M.E., Ren, S., Batchelder, A.L., Kalhorn, T.F., Schoch, H.G., Anasetti, C. and Gooley, T. (2003) 'Cyclophosphamide metabolism, liver toxicity, and mortality following hematopoietic stem cell transplantation', *Blood,* 101(5), pp. 2043-2048.

Menezes, M. (2010) 'Chlorophyceae', in Forzza, R.C. (ed.) *Catálogo de plantas e fungos do Brasil. Vol. 1.* Rio de Janeiro.: Instituto de Pesquisas Jardim Botânico do Rio de Janeiro., pp. 335--352.

Metzger, J.W. (2004) 'Drugs in municipal landfills and landfill leachates.', in K¨ummerer, K. (ed.) *Pharmaceuticals in the environment: Sources, Fate, Effects and Risks.* 2nd edn. Berlin/Heidelberg/New York: Springer, pp. 133-138.

Millington, L., Goulding, K. and Adams, N. (1988) 'The influence of growth medium composition on the toxicity of chemicals to algae', *Water research,* 22(12), pp. 1593-1597.

Monjanel-Mouterde, S., Ciccolini, J., Bagarry, D., Zonta-David, M., Duffaud, F., Favre, R. and Durand, A. (2003) 'Population pharmacokinetics of cisplatin after 120-h infusion: application to routine adaptive control with feedback', *Journal of clinical pharmacy and therapeutics,* 28(2), pp. 109-116.

Muenz, R. (2007) 'Aging and demographic change in European societies: main trends and alternative policy options', *Social Protection, The World Bank.Discussion Paper,* (703).

Muneoka, K., Shirai, Y., Yokoyama, N., Wakai, T. and Hatakeyama, K. (2005) '5-fluorouracil cardiotoxicity induced by alpha-fluoro-beta-alanine', *Chemosphere,* 10(6), pp. 441-443.

Nash, R.D., Valencia, A.H. and Geffen, A.J. (2006) 'The origin of Fulton's condition factor—setting the record straight', *Fisheries,* 31(5), pp. 236-238.

Nazıroğlu, M., Karaoğlu, A. and Aksoy, A.O. (2004) 'Selenium and high dose vitamin E administration protects cisplatin-induced oxidative damage to renal, liver and lens tissues in rats', *Toxicology,* 195(2), pp. 221-230.

OECD (2011) *Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.* Organisation for Economic Co-operation and Development.

OECD (2004) *Test No. 202: Daphnia sp. Acute Immobilisation Test.* Organisation for Economic Co-operation and Development.

OECD (1992) *Test No. 203: Fish, Acute Toxicity Test.* Organisation for Economic Co-operation and Development.

OECD (1984) *Test No. 204: Fish, Prolonged Toxicity Test: 14-Day Study.* Organisation for Economic Co-operation and Development.

Ortiz-Zarragoitia, M. and Cajaraville, M.P. (2005) 'Effects of selected xenoestrogens on liver peroxisomes, vitellogenin levels and spermatogenic cell proliferation in male zebrafish', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 141(2), pp. 133-144.

O'Sullivan, M. (2013) *Full Blood Count*. Available at: http://www.pathology.leedsth.nhs.uk/pathology/ClinicalInfo/Haematology/FullBloodCount.aspx (Accessed: 07/17 2015).

Ou, H.C., Raible, D.W. and Rubel, E.W. (2007) 'Cisplatin-induced hair cell loss in zebrafish (Danio rerio) lateral line', *Hearing research,* 233(1), pp. 46-53.

Pal, A., Gin, K.Y.H., Lin, A.Y.-. and Reinhard, M. (2010) 'Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects', *Science of the Total Environment,* 408(24), pp. 6062-6069.

Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., Fiumano, V. and Isidori, M. (2014) 'Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans', *Chemosphere,* 115, pp. 59-66.

Parrott, J.L. and Blunt, B.R. (2005) 'Life-cycle exposure of fathead minnows (Pimephales promelas) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males', *Environmental toxicology,* 20(2), pp. 131-141.

Pasmantier, M.W., Coleman, M., Silver, R.T. and Ballard, W.P. (1985) 'Six-drug chemotherapy (hexamethylmelamine, doxorubicin, cisplatin, cyclophosphamide,

methotrexate, and 5-FU; CHAMP-5) for ovarian carcinoma: alternating sequences of combination regimens', *Cancer treatment reports,* 69(6), pp. 689-693.

Peute, J., Van der Gaag, M. and Lambert, J. (1978) 'Ultrastructure and lipid content of the liver of the zebrafish, Brachydanio rerio, related to vitellogenin synthesis', *Cell and tissue research,* 186(2), pp. 297-308.

Pietri, E., Andreis, D., Fabbri, F., Menna, C., Schirone, A., Kopf, B., Rocca, A., Amadori, D. and De Giorgi, U. (2015) 'A phase II study of a dose-density regimen with fluorouracil, epirubicin, and cyclophosphamide on days 1 and 4 every 14 days with filgrastim support followed by weekly paclitaxel in women with primary breast cancer', *The oncologist,* 20(3), pp. 239-240.

Platt, O.S. (2008) 'Hydroxyurea for the treatment of sickle cell anemia', *New England Journal of Medicine,* 358(13), pp. 1362-1369.

Pruden, A., Larsson, D.J., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D.W., Lazorchak, J.M., Suzuki, S., Silley, P. and Snape, J.R. (2013) 'Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment', *Environ Health Perspect,* 121(8), pp. 878-885.

Reardon, D.A., Norden, A.D., Desjardins, A., Vredenburgh, J.J., Herndon II, J.E., Coan, A., Sampson, J.H., Gururangan, S., Peters, K.B. and McLendon, R.E. (2012) 'Phase II study of Gleevec® plus hydroxyurea (HU) in adults with progressive or recurrent meningioma', *Journal of neuro-oncology,* 106(2), pp. 409-415.

Reinardy, H.C., Dharamshi, J., Jha, A.N. and Henry, T.B. (2013) 'Changes in expression profiles of genes associated with DNA repair following induction of DNA damage in larval zebrafish Danio rerio', *Mutagenesis,* 28(5), pp. 601-608.

Richards, M. (2010) 'Extent and causes of international variations in drug usage', *A report for the Secretary of State for Health by Professor Sir Mike Richards CBE.London: Central Office of Information,* .

Richards, M. (2009) *Uptake of NICE approved cancer drugs.* London: Department of Health.

Rodrigues, L.H.R., Arenzon, A., Raya-Rodriguez, M.T. and Fontoura, N.F. (2011) 'Algal density assessed by spectrophotometry: a calibration curve for the unicellular algae Pseudokirchneriella subcapitata', *Journal of Environmental Chemistry and Ecotoxicology Vol,* 3(8), pp. 225-228.

Rosenberg, B., Van Camp, L. and Krigas, T. (1965) 'Inhibition of Cell Division in Escherichia coli by Electrolysis Products from a Platinum Electrode', *Nature,* 205(4972), pp. 698-699.

Ruhoy, I.S. and Daughton, C.G. (2007) 'Types and quantities of leftover drugs entering the environment via disposal to sewage—revealed by coroner records', *Science of the total environment,* 388(1), pp. 137-148.

Rutman, R.J., Cantarow, A. and Paschkis, K.E. (1954) 'Studies in 2-Acetylaminofluorene Carcinogenesis', *Cancer research,* 14(2), pp. 119-123.

Rybak, L.P., Whitworth, C.A., Mukherjea, D. and Ramkumar, V. (2007) 'Mechanisms of cisplatin-induced ototoxicity and prevention', *Hearing research,* 226(1), pp. 157-167.

Sacco, J., Botten, J., Macbeth, F., Bagust, A. and Clark, P. (2010) 'The average body surface area of adult cancer patients in the UK: a multicentre retrospective study', *PLoS One,* 5(1), pp. e8933.

Sanchez, W., Sremski, W., Piccini, B., Palluel, O., Maillot-Maréchal, E., Betoulle, S., Jaffal, A., Aït-Aïssa, S., Brion, F., Thybaud, E., Hinfray, N. and Porcher, J. (2011) 'Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges', *Environment international,* 37(8), pp. 1342-1348.

Sanderson, B.J.S., Ferguson, L.R. and Denny, W.A. (1996) 'Mutagenic and carcinogenic properties of platinum-based anticancer drugs', *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 355(1-2), pp. 59-70.

Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A. and Solomon, K.R. (2003) 'Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening', *Toxicology letters,* 144(3), pp. 383-395.

Santos, M.A. and Pacheco, M. (1995) 'Mutagenicity of cyclophosphamide and kraft mill effluent and sediment on the eel Anguilla anguilla L.', *Science of The Total Environment,* 171(1–3), pp. 127-130.

Schall, R., Hundt, H.K. and Luus, H.G. (1994) 'Pharmacokinetic characteristics for extent of absorption and clearance in drug/drug interaction studies', *International journal of clinical pharmacology and therapeutics,* 32(12), pp. 633-637.

Schilsky, R. (1998) 'Biochemical and clinical pharmacology of 5-fluorouracil', *Oncology-New York,* 12(10), pp. 13-18.

Schwartz, P.S. and Waxman, D.J. (2001) 'Cyclophosphamide Induces Caspase 9-Dependent Apoptosis in 9L Tumor Cells', *Molecular pharmacology,* 60(6), pp. 1268-1279.

Silva, E., Rajapakse, N. and Kortenkamp, A. (2002) 'Something from "nothing"-eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects', *Environmental science & technology,* 36(8), pp. 1751-1756.

Simon, P. (2003) 'Q-Gene: processing quantitative real-time RT–PCR data', *Bioinformatics,* 19(11), pp. 1439-1440.

Sinha, R.P. and Häder, D. (2002) 'UV-induced DNA damage and repair: a review', *Photochemical & Photobiological Sciences,* 1(4), pp. 225-236.

Smith, M.L., Chen, I.T., Zhan, Q., O'Connor, P.M. and Fornace, A.J.,Jr (1995) 'Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage', *Oncogene,* 10(6), pp. 1053-1059.

Speidel, D. (2010) 'Transcription-independent p53 apoptosis: an alternative route to death', *Trends in cell biology,* 20(1), pp. 14-24.

Spence, R., Gerlach, G., Lawrence, C. and Smith, C. (2008) 'The behaviour and ecology of the zebrafish, Danio rerio', *Biological Reviews,* 83(1), pp. 13-34.

Steger-Hartmann, T., Kümmerer, K. and Hartmann, A. (1997) 'Biological Degradation of Cyclophosphamide and Its Occurrence in Sewage Water', *Ecotoxicology and environmental safety,* 36(2), pp. 174-179.

Straub, J.O. (2010) 'Combined environmental risk assessment for 5-fluorouracil and capecitabine in Europe', *Integrated Environmental Assessment and Management,* 6(S1), pp. 540-566.

Sussman, R. (2007) 'DNA Repair Capacity of Zebrafish', *Proceedings of the National Academy of Sciences of the United States of America,* 104(33), pp. pp. 13379-13383.

Tallarida, R.J. (2006) 'An overview of drug combination analysis with isobolograms', *The Journal of pharmacology and experimental therapeutics,* 319(1), pp. 1-7.

Tanaka, T., Masuda, H., Naito, M. and Tamai, H. (2001) 'Pretreatment with 5-fluorouracil enhances cytotoxicity and retention of DNA-bound platinum in a cisplatin resistant human ovarian cancer cell line', *Anticancer Research,* 21(4A), pp. 2463-2469.

Thermo Scientific (2011) *T042-Technical Bulletin - NanoDrop Spectrophotometers*. Available at: http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf (Accessed: 09/14 2015).

Uribe, P.M., Mueller, M.A., Gleichman, J.S., Kramer, M.D., Wang, Q., Sibrian-Vazquez, M., Strongin, R.M., Steyger, P.S., Cotanche, D.A. and Matsui, J.I. (2013) 'Dimethyl sulfoxide (DMSO) exacerbates cisplatin-induced sensory hair cell death in zebrafish (Danio rerio)', .

Van der Aa, N., Kommer, G., van Montfoort, J. and Versteegh, J. (2011) 'Demographic projections of future pharmaceutical consumption in the Netherlands', *Water Science & Technology,* 63(4), pp. 825-831.

Vannini, C., Domingo, G., Marsoni, M., De Mattia, F., Labra, M., Castiglioni, S. and Bracale, M. (2011) 'Effects of a complex mixture of therapeutic drugs on unicellular algae Pseudokirchneriella subcapitata', *Aquatic Toxicology,* 101(2), pp. 459-465.

Von Hoff, D.D., Schilsky, R., Reichert, C.M., Reddick, R.L., Rozencweig, M., Young, R.C. and Muggia, F.M. (1979) 'Toxic effects of cis-dichlorodiammineplatinum(II) in man', *Cancer treatment reports,* 63(9-10), pp. 1527-1531.

Vorhees, C.V., Butcher, R.E., Brunner, R.L. and Sobotka, T.J. (1979) 'A developmental test battery for neurobehavioral toxicity in rats: a preliminary analysis using monosodium glutamate calcium carrageenan, and hydroxyurea', *Toxicology and applied pharmacology,* 50(2), pp. 267-282.

Vousden, K.H. and Prives, C. (2009) 'Blinded by the light: the growing complexity of p53', *Cell,* 137(3), pp. 413-431.

Vyas, N., Turner, A. and Sewell, G. (2014) 'Platinum-based anticancer drugs in waste waters of a major UK hospital and predicted concentrations in recipient surface waters', *Science of The Total Environment,* 493(0), pp. 324-329.

Wakasugi, M., Sasaki, T., Matsumoto, M., Nagaoka, M., Inoue, K., Inobe, M., Horibata, K., Tanaka, K. and Matsunaga, T. (2014) 'Nucleotide excision repair-dependent DNA double-strand break formation and ATM signaling activation in mammalian quiescent cells', *The Journal of biological chemistry,* 289(41), pp. 28730-28737.

Walpole, S., Prieto-Merino, D., Edwards, P., Cleland, J., Stevens, G. and Roberts, I. (2012) 'The weight of nations: an estimation of adult human biomass', *BMC Public Health,* 12(1), pp. 439.

Weigt, S., Huebler, N., Braunbeck, T., von Landenberg, F. and Broschard, T.H. (2010) 'Zebrafish teratogenicity test with metabolic activation (mDarT): effects of phase I

activation of acetaminophen on zebrafish Danio rerio embryos', *Toxicology,* 275(1), pp. 36-49.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T. and Broschard, T.H. (2011) 'Zebrafish (Danio rerio) embryos as a model for testing proteratogens', *Toxicology,* 281(1), pp. 25-36.

Wilcosky, T. and Rynard, S.M. (1990) 'Sister chromatid exchanges', *Hulka BS, Wilcosky, TC.Griffith JD, eds.Biological Markers in Epidemiology,* 1, pp. 103-122.

Wood, P.A. and Hrushesky, W. (1995) 'Cisplatin-associated anemia: an erythropoietin deficiency syndrome.', *Journal of Clinical Investigation,* 95(4), pp. 1650.

Xie, X., Liu, J., Hu, B. and Xiao, W. (2011) 'Zebrafish foxo3b Negatively Regulates Canonical Wnt Signaling to Affect Early Embryogenesis', *PLoS ONE,* 6(9), pp. e24469.

Yamashita, K., Yada, H. and Ariyoshi, T. (2004) 'Neurotoxic effects of alpha-fluoro-beta-alanine (FBAL) and fluoroacetic acid (FA) on dogs', *The Journal of toxicological sciences,* 29(2), pp. 155-166.

Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., Seno, T., Garrett, C. and Wataya, Y. (1987) 'Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death.', *Journal of Biological Chemistry,* 262(17), pp. 8235-8241.

Zaharie, M. (2006) 'Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania', *Chemosphere,* 64(11), pp. 1808-1817.

Zaleska-Radziwill, M., Lebkowska, M., Affek, K. and Zarzeczna, A. (2011) 'Environmental Risk Assessment of Selected Pharmaceuticals Present in Surface Waters in Relation to Animals', *Archives of Environmental Protection,* 37(3), pp. 31-42.

Zhang, J., Tian, Q. and Zhou, S. (2006) 'Clinical Pharmacology of Cyclophosphamide and Ifosfamide', *Current Drug Therapy,* (1), pp. 55-84.

Zhou, J., Zhang, W.M., Chen, H., Xie, B., Zheng, J.H., Xu, Z.Y. and Lin, J.R. (2009) 'Analysis of responses and prognostic factors in different chemotherapy regimens for metastatic nasopharyngeal carcinoma: a report of 171 cases', *Zhonghua zhong liu za zhi [Chinese journal of oncology],* 31(11), pp. 867-872.

Zounkova, R., Kovalova, L., Blaha, L. and Dott, W. (2010) 'Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites', *Chemosphere,* 81(2), pp. 253-260.

Zounková, R., Odrá?ka, P., Dole?alová, L., Hilscherová, K., Mar?álek, B. and Bláha, L. (2007) 'Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals', *Environmental Toxicology and Chemistry,* 26(10), pp. 2208-2214.