The utility of trout hepatic cells in the prediction of xenobiotic bioaccumulation and environmental persistence

by

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Abstract

The need to protect aquatic organisms from the toxic potential of environmental contaminants with minimal use of animal testing has been highlighted by the introduction of a number of regulatory directives around the world. This thesis proposes the potential of 3D cell culture models to 'bridge the gap' between the use of existing subcellular and *in vivo* systems for the testing of compounds for potential persistence, bioaccumulation and toxicity, improving the predictive power of *in vitro* screening procedures. In this project, a novel trout hepatocyte spheroid model was optimised and using metabolic, fluorescence, transport and gene expression assays, the utility of this system in such studies as a superior alternative to currently used subcellular fractions and traditional hepatocyte monolayer culture was demonstrated. Hepatocytes cultured as spheroids remained viable for up to 40 days, exhibiting significantly greater functional xenobiotic metabolism and transport compared to other *in vitro* systems. Significantly greater levels of expression of genes involved in xenobiotic metabolism and efflux were also measured in spheroids compared to hepatocytes cultured using traditional techniques. These features of spheroids present a profile more closely representative of that of the whole liver. Together with the potential advantages of screening highly persistent compounds that require long term incubation and possible use in the assessment of the toxicity of compounds exhibiting chronic effects, this system successfully enriches current *in vitro* testing strategies.

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List of Abbreviations

3Rs	The principle of reduction, refinement and replacement of animal testing
4-MU	4-methylumbelliferone
ABC	ATP binding cassette
ADME	Absorption, distribution, metabolism and excretion
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
CAR	Constitutive androstane receptor
CLF	Cholyl-lysyl-fluorescein
CLh	Hepatic clearance
CLint	Intrinsic clearance
Ct	Cycle threshold
CYP	Cytochrome P450
DCDHFDA	2',7'-dichlorodihydrofluoroscein-diacetate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dpi	Days post isolation
DTT	Dithiothreitol
ECM	Extracellular matrix
ECOD	Ethoxycoumarin-*O*-deethylase

EDCs	Endocrine disrupting chemicals
EDTA	Ethylenediaminetetraacetic acid
ERA	Environmental risk assessment
EROD	Ethoxyresorufin-*O*-deethylase
FACS	Fluorescence activated cell sorting
H33342	Hoechst 33342
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFFRs	Halogen free flame retardants
HMDB	Human metabolite database
hpi	Hours post isolation
LC-MS	Liquid chromatography-mass spectrometry
LC_{50}	Acute median lethal concentration
LDH	Lactate dehydrogenase
MDR1 (P-gp)	Multidrug resistance protein (P-glycoprotein)
MRPs	Multidrug resistance associated proteins
MXR	Multixenobiotic resistance
m/z	Mass to charge ratio
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
OATP	Organic anion transporting polypeptide
OECD	Organisation for Economic Co-operation and Development
PAHs	Polyaromatic hydrocarbons

PAPS	5′-phosphoadenosine-3′-phosphosulfate
PBS	Phosphate buffered saline
PBT	Persistence, bioaccumulation and toxicity
PCA	Principal components analysis
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins
PCR	Polymerase chain reaction
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
REACH	Registration, evaluation, authorisation and restriction of chemicals
Rh123	Rhodamine 123
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Retention time
S9	9,000 *g* supernatant
SEM	Standard error of the mean
SULT	Sulfotransferase
T3	Triiodothyronine
TBE	Tris/borate/EDTA buffer
TCE	Trichloroethylene
UGT	UDP-glucuronosyltransferase
UDP	Uridine diphosphate
UDPGA	Uridine diphosphate glucuronic acid

Chapter 1

General Introduction

1.1 Rationale

This project addresses the requirements for the assessment of the environmental persistence and effects of pharmaceuticals and other chemical contaminants on aquatic organisms. Based on the increased desire to refine the procedures by which this is measured, new approaches to enhance the quality and utility of information obtained are presented, with a focus on the use of *in vitro* liver preparations from trout to assess metabolism and elimination potential. This chapter provides an insight into the need for testing, the processes which contribute to persistence and effects on organisms, the current testing strategy and alternative techniques that may be employed.

Rainbow trout (*Oncorhynchus mykiss*) is an Organisation for Economic Co-operation and Development (OECD) test species used in fish acute toxicity testing. For this reason and due to the increasing number of trout genes that have recently been sequenced, this project focuses on the use of the species in metabolic assessments.

1.2 Pharmaceuticals and other chemicals in the environment

Over the last 30 years, concerns about the environmental fate and effects of a vast number of chemical compounds have increased dramatically. It is estimated that well in excess of 100,000 different chemicals occur in the environment, covering a plethora of classes including, disinfectants, estrogenic compounds, flame retardants, pesticides and active pharmaceutical ingredients of human and veterinary origin (van Wezel and Jager, 2002, Sumpter and Johnson, 2005, Corcoran et al., 2010, Chen et al., 2012). The aquatic environment is a sink for these

compounds and as a result, the potential adverse effects on organisms that are constantly exposed to them, have stimulated research, with the topic receiving considerable attention in the scientific and general media and heavily influencing regulation and policy. Especially important are the potential long term effects on the aquatic flora and fauna as organisms are exposed to these chemicals throughout their lifetimes and the constant replenishment of compounds simulate chronic exposure. Indeed, a number of regulatory directives for the improved assessment of the potential effects of the use of chemicals on human health and the environment have been introduced around the world, including the Registration, Evaluation, Authorisation and restriction of Chemicals (REACH) which began in Europe in 2007.

1.2.1 Pharmaceuticals in the environment

Due to their extensive use, both human prescription and non-prescription drugs and compounds used in veterinary medicine, agriculture and aquaculture are now ubiquitous in the environment (Fent et al., 2006). These compounds have been detected in the ng/L to µg/L range in surface, ground and drinking waters worldwide (Sanderson et al., 2004, Gagne et al., 2006). A summary of the measured environmental concentrations of a number of pharmaceuticals covering a range of classes is given in Table 1.1. For example, ibuprofen, one of the most commonly used global pharmaceuticals (with an estimated annual consumption in the region of hundreds of tons (Daughton and Ternes, 1999)), has been detected at a range of concentrations in various environmental settings, including 0.4 µg/L in sewage effluent and 2.7 µg/L in surface waters (Corcoran et al., 2010). These concentrations are lower than those that induce therapeutic and acute toxicological effects in humans, however, very little about the potential effects of these

compounds on aquatic and terrestrial organisms is fully understood. In recent years, an increasing number of studies have shown a range of biological responses in aquatic species following laboratory exposure to pharmaceuticals, however reported effects have generally been at higher concentrations (at least an order of magnitude greater) than those typically occurring in the environment (Fent et al., 2006, Corcoran et al., 2010). The presence of a variety of compounds in the environment however, exposes organisms to the effects of mixtures and the potential for drug-drug interactions and synergistic effects (Daughton and Ternes, 1999).

Compound	Reported concentration (µg/L)	Environmental setting	Location	Reference
Clofibric Acid	1.6	Wastewater effluent	Germany	(Ternes, 1998)
	0.55	Surface water	Germany	(Ternes, 1998)
Diazepam	0.66	Wastewater effluent	-	(van der Hoeven, 2004)
	0.01	Drinking water	-	(Jones et al., 2001)
Diclofenac	33.9	Wastewater effluent	-	(Corcoran et al., 2010)
	1.2	Surface water	Germany	(Ternes, 1998)
	0.006	Drinking water	Brazil	(Stumpf et al., 1999)
Gemfibrozil	1.3	Wastewater effluent	Canada	(Metcalfe et al., 2003)
	0.052	Surface water		(Corcoran et al., 2010)
Ibuprofen	4.2	Wastewater effluent	UK	(Roberts and Thomas, 2006)
	2.4	Surface water	UK	(Roberts and Thomas, 2006)
Naproxen	12.5	Wastewater effluent	Canada	(Metcalfe et al., 2003)
	0.39	Surface water	-	(Thibaut et al., 2006)
Propranolol	0.37	Wastewater effluent	UK	(Roberts and Thomas, 2006)
	0.59	Surface water	Germany	(Ternes, 1998)

Table 1.1: Environmental concentrations of selected pharmaceutical compounds in wastewater effluent, surface and drinking waters worldwide. Environmental concentrations are heavily linked to human consumption with commonly used compounds most prominent in the environment.

Pharmaceuticals are marketed for their specific biological activity (targeting enzyme pathways, transporters, receptor mediated and ion channel mediated responses) in their target organisms and prolonged activity, reducing dosing requirements (Hutchinson, 2008). Drug targets however, are often conserved across non-target vertebrate organisms and as a result, pharmaceuticals designed to induce various effects in humans are highly likely to retain their biological activities in non-target organisms (Gunnarsson et al., 2008). Fish physiology is highly similar to that of mammals, thus the pharmacology is also thought be similar, with a potential for drug induced effects in non-target species. For example, rainbow trout possess β-adrenergic receptor mediated signalling mechanisms comparable to those of mammalian species. As a result this non-target fish species may be susceptible to the intended biological activity of human cardiovascular pharmaceuticals found in the environment (Gunnarsson et al., 2008, Owen et al., 2009, Winter et al., 2010). At the same time, non-target organisms may exhibit additional physiological differences which may be affected by the biological activity of a compound. This may lead to significant species differences in pharmacodynamic properties. Compounds targeting beta receptors are again an example, as many fish have additional beta-receptor subtypes linked to physiological functions other than the cardiovascular system, possibly resulting in compound induced effects not recognised in target species (Perry and Bernier, 1999, Seiler, 2002, Owen et al., 2009). It is also possible that irrelevant secondary pharmacodynamic effects in the target organism may cause significant pharmacological and toxicological effects in non-target species. For example, in 2004 diclofenac was attributed to the significant decline in population of Asian vultures, through renal failure. This resulted from the unusually high sensitivity of the organism to the compound (Oaks et al., 2004, Swan et al., 2006).

1.2.2 Routes of environmental exposure of pharmaceuticals

The pathways by which pharmaceutical products reach the environment are well described and summarised in Figure 1.1. The major route involved in the environmental fate of human pharmaceuticals is via effluent from sewage treatment plants following their administration and excretion. The parent compound and/or its metabolites are excreted via the urinary or biliary routes and transported in sewage to wastewater treatment works; where incomplete treatment and degradation (by adsorption and biodegradation) may result in the release of compounds into receiving waters via treated effluent (Fent et al., 2006). The rate of biodegradation during the wastewater treatment process is compound specific and has a direct effect on environmental fate (Jones et al., 2001).

In some cases, inactive metabolites in waste water can be reactivated through the cleavage of conjugate moieties by microorganisms in sewage treatment processes or via chemical degradation (Fent et al., 2006). This is a process known to occur with glucuronic acid and sulfate conjugates of natural and synthetic endocrine disrupting hormones (Fawell, 2012). When released in treated water, these compounds contaminate surface waters in their active form; so are potentially able to exert their therapeutic or toxicological effects on non-target aquatic organisms. The presence of pharmaceuticals in wastewater treatment works is exacerbated by the disposal of unused products in the toilet and through sinks and drains. Although this route is difficult to quantify, it is not considered to be as significant when compared to the human usage route (Loffler et al., 2005). Other sources of pharmaceuticals in the environment include leaching from solid waste landfill following household disposal and wastewater loss from drug production units during compound manufacture (Sanderson et al., 2004, Fent et al., 2006, Corcoran et al., 2010).

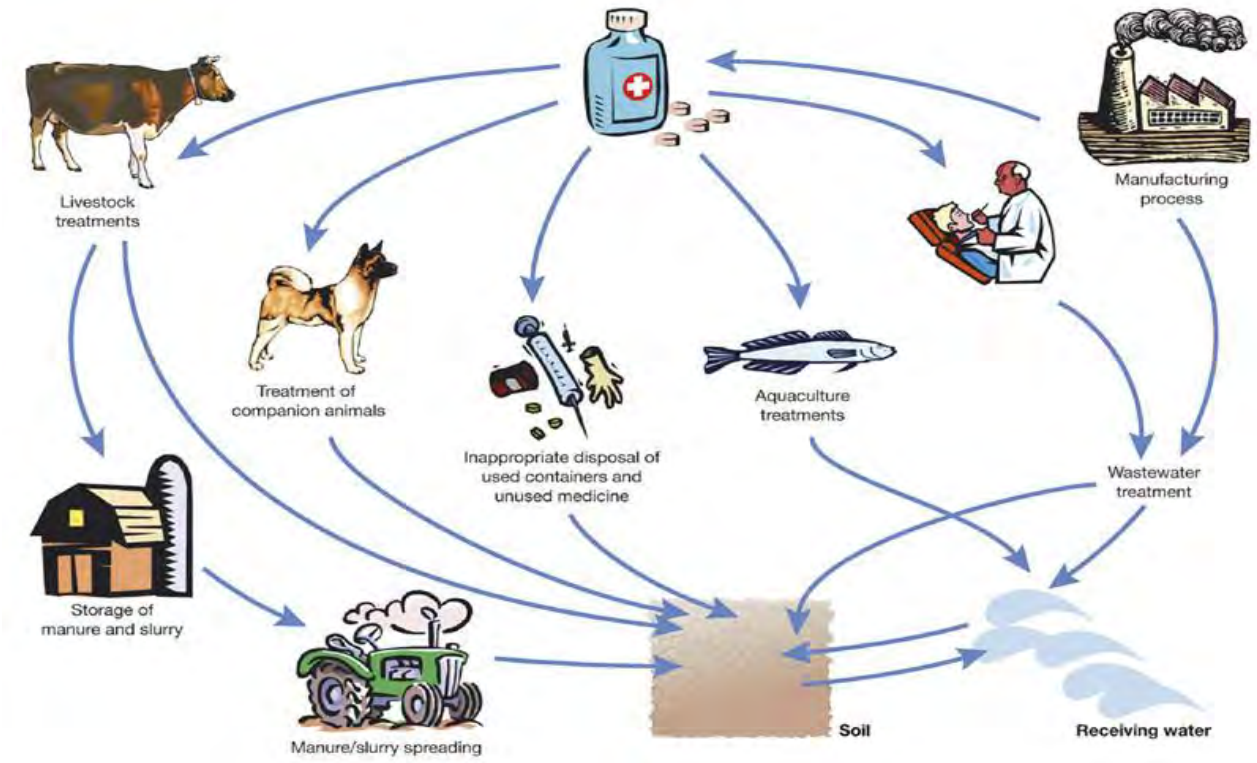


Figure 1.1: Routes of pharmaceutical entry into the environment. The major route for human pharmaceuticals is via the use, potential metabolism and excretion of compounds. Environmental exposure occurs through entry into receiving waters following incomplete degradation in wastewater treatment works. Less significant routes include inappropriate disposal of compounds, resulting in leaching from landfill and compound loss during manufacture. Direct application is an important route of environmental exposure for veterinary medicines and aquaculture treatments (Boxall, 2004).

Not only are human pharmaceutical products detected in the environment, but also those used in veterinary medicine and aquaculture. These enter aquatic ecosystems following the spreading of manure to farmland and its subsequent run-off into water systems. In the case of the deaths of Asian vultures highlighted previously, exposure to diclofenac was as a result of the consumption of the carcasses of pre-treated livestock (Swan et al., 2006).

1.2.3 Emerging non-pharmaceutical environmental contaminants

Despite the use of strict controls to regulate the use of industrial chemicals and reduce their potential impact, a wide range of chemicals of a variety of classes have persisted in the environment and newer emerging contaminants are being detected, especially in watercourses as a result of industrial discharges (Fawell, 2012). Traditionally, particular attention was given to selected priority groups including hydrocarbons from petroleum products, heavy metals, endocrine disruptors, organochlorine pesticides and chlorinated organic pollutants including trichloroethylene (TCE) and polychlorinated biphenyls (PCBs) and due to the effectiveness of imposed control measures, concentrations in effluents have declined (Clarke and Smith, 2011, Beceiro-Gonzalez et al., 2012, Petersen et al., 2012). Recently, newer classes of chemical contaminants have dominated, many of which have now been banned but remain detectable in many environmental settings. For example, polybrominated di-phenyl ethers, a subclass of the structurally diverse group of brominated flame retardants, used to reduce the flammability of combustible materials (Chen et al., 2012, Montory et al., 2012). These chemicals are both persistent and hydrophobic, rendering them highly amenable to bioaccumulation and were used extensively prior to comprehensive knowledge of these characteristics (Eljarrat et al., 2005). As a

result, they have been detected at concentrations up to 2,412 ng/L in waste water and 65 ng/L in river water in China (Chen et al., 2012) and in many other environmental settings. A range of alternative compounds including halogen free flame retardants (HFFRs) are being developed and will require extensive assessment of their environmental behaviour in the near future (Waaijers et al., 2013).

As new formulations of these industrial chemicals are manufactured and global consumption of pharmaceuticals increases, levels of environmental contamination will in turn escalate. Pharmaceutical compounds are designed to be highly lipophilic and relatively persistent, to reduce dosing demands and many of the emerging industrial contaminants are highly persistent chemicals. These are features which will enhance their accumulation in the environment (Jones et al., 2001). As a result, there is potential for these compounds to remain in the environment for extensive periods and so there has been an increase in focus on the impact of environmental pollutants that cause chronic toxicity, especially those that are poorly metabolised (Hutchinson, 2008). On account of this and the lack of knowledge of their long term impact and the effects of mixtures, the environmental risk assessment of these contaminants has become a research area of extreme importance (Owen et al., 2009).

1.3 Environmental risk assessment of compounds

As part of the approval procedure required for drug registration, the potential environmental risks related to the human use of new pharmaceutical products must be considered in the form of an Environmental Risk Assessment (ERA); in which "any risk of undesirable effects on the environment" must be assessed and "on a case by case basis, specific arrangements to limit the environmental impact should be envisaged" (European Union Directive 2004/27/EC, 2004). A similar process of assessment is conducted for non-pharmaceutical compounds and under the regulations of REACH, substances manufactured or imported into the EU in quantities of 1 tonne or more per year must be evaluated. The specific arrangements that result from ERA testing aim to manage any potential risks to protect the environment and exposed organisms through the introduction of precautionary and safety measures related to storage, administration and disposal of compounds (van Wezel and Jager, 2002, Laenge et al., 2006, Hutchinson, 2008). Although a requirement for product registration, in the case of pharmaceuticals, the environmental impact of a drug determined through ERA does not constitute a criterion for refusal of a marketing authorisation (European Union Directive 2004/27/EC, 2004).

The major parameters involved in the evaluation of environmental compounds are the intrinsic chemical properties of persistence, bioaccumulation and toxicity (PBT) and the testing battery is operated in a tiered approach, with ecological assays conducted on a variety of test organisms. The first phase analyses the exposure potential of the compound through calculations of the predicted environmental concentration (PEC), an estimate of the amount of the compound entering the environment. Outcomes of PEC calculations determine whether additional testing is required, should the environmental concentration be 1000 fold greater than the acute median

lethal concentration (LC_{50}) of the most sensitive species (most frequently daphnia) (Winter et al., 2009). A pharmaceutical product with a PEC in surface water below 0.001 µg/L and no other environmental concerns is considered unlikely to present a substantial risk to the environment following human use (EMEA Guideline, 2006).

The second phase of assessment considers the environmental fate of a compound and its effects. The physico-chemical properties of the compound and its biotransformation are paramount to this assessment. A biodegradability test is conducted on the parent compound and possible metabolites, to ascertain their fate and potential for bioaccumulation. Poorly degrading substances are further investigated in water sediment studies and if the potential for a compound to bioaccumulate is identified, a specific risk assessment is conducted. The pharmacodynamic effects of compounds on a number of aquatic species are studied in toxicity tests using algae, daphnia and fish. The predicted no effect concentration (PNEC) is calculated and the ratio of this and the PEC is used as a marker for compounds requiring environmental monitoring. Monitoring is required if PEC:PNEC >1 (Winter et al., 2010).

The metabolic potential of a compound is a key process affecting its PBT. The degree of metabolism of a pharmaceutical compound following ingestion and the rate of degradation of all compounds during the wastewater treatment process have an important bearing on concentrations reaching the environment (Corcoran et al., 2010, Winter et al., 2010). Compounds can accumulate in organisms following uptake by a number of routes. The process is mainly determined by rates of uptake and elimination and the potential of a compound to bioaccumulate is usually calculated directly from data obtained in uptake and elimination studies (van der Oost et al., 2003, Ehrlich et al., 2011). However, biotransformation can also reduce the

bioaccumulation potential of xenobiotics as the process alters the chemical structure of a compound through a variety of reactions to enhance its elimination from an organism (Mingoia et al., 2010). The metabolic process often (but not in all cases) alters the biological activity of a compound, reducing (and sometimes increasing) its toxic potential. Due to the impact of metabolism on these key features of the environmental testing of chemicals, the assessment of metabolic potential is of extreme importance. Together with measurements of uptake, accumulation and elimination, the metabolism of compounds is primarily assessed *in vivo*, a process which involves the use of considerable numbers of animals and can be technically difficult. In recent years, measures to establish alternative test systems to refine the bioaccumulation assessment have been sought, one of which is to use *in vitro* systems to assess the metabolic potential of compounds in fish (Ehrlich et al., 2011). The principle considers that there is an inverse relationship between degree of metabolism and the bioaccumulative potential of a compound.

1.4 Metabolism

As mentioned previously, biotransformation has an integral bearing on the potential of a compound to persist in the environment and to bioaccumulate within an organism.

In common for both mammals and fish, the fate of xenobiotics in the organism is highly dependent on the physico-chemical properties of a compound. A large proportion of environmental contaminants and especially pharmaceuticals, are highly lipophilic, thus not ideally suited for elimination. Several processes are involved in the metabolism of xenobiotics, generally converting nonpolar, lipophilic compounds into polar, inactive, or nontoxic metabolites that are readily eliminated (Venkatakrishnan et al., 2001). The outcomes of the wide variety of possible metabolic reactions are a decrease in biological half-life and duration of exposure, the avoidance of accumulation through a decreased elimination half-life and a possible change in biological activity through the formation of metabolites, which are often (but not always) less active than the parent compound.

The liver is the organ most commonly involved in xenobiotic biotransformation due to its position, blood supply and abundance of metabolic enzymes. A myriad of reactions are involved in the metabolism of exogenous compounds including oxidative, reductive and conjugative reactions (summarised in Table 1.2). These are often separated into two independent, but not necessarily sequential processes, phase I and phase II.

Phase I	Phase II
Oxidation	Acetylation (*N*-acetyltransferases)
Reduction	Amino acid conjugation
Hydrolysis	Glucuronidation (UDP-glucuronosyltransferases)
Hydration	Glutathione conjugation (Glutathione-*S*-Transferases)
Dehalogenation	Sulfation (Sulfotransferases)
	Methylation (Methyltransferases)

Table 1.2: List of the major phase I and phase II biotransformation reactions. Enzymes of the Cytochrome P450 super-family catalyse the majority of xenobiotic phase I biotransformation reactions. Enzymes which catalyse individual phase II reactions are given in brackets.

Phase I reactions are generally oxidation, reduction and hydrolysis reactions, resulting in a direct change in the structure of the parent compound, through the addition or exposure of functional groups. This process may however produce reactive metabolites that can infer an increased risk of toxicity in comparison to the parent compound. For example, the trichloromethyl radical ($CCl_3^{\cdot}$) and trichloromethylperoxy radical ($CCl_3OO^{\cdot}$) metabolites of carbon tetrachloride (CCl_4) are the cause of the hepatotoxic effects of the compound. The radicals, formed following microsomal enzyme mediated biotransformation bind to proteins and membrane phospholipids, causing lipid peroxidation, ultimately resulting in centrilobular hepatic necrosis (Manibusan et al., 2007). Prodrugs such as the lipid regulator clofibrate are effective in this way, as it is metabolised into the pharmacologically active form clofibric acid, through which therapeutic effects are produced (Buser et al., 1998).

Phase II reactions are conjugations, with endogenous compounds including glucuronic acid, sulfates, acetyl groups, amino acids (e.g. glycine) and peptides (e.g. glutathione). These conjugative reactions increase the water solubility of a compound, facilitating urinary excretion; or increase the molecular weight of the compound, facilitating biliary excretion (Nebbia, 2001). However, this is not always the case; for example when metabolites of some sulfonamides are acetylated, the resulting conjugates can be less water soluble than the parent and can cause renal toxicity through precipitation in the kidneys (Perazella, 1999). Phase II reactions can also protect against biological activation through the conjugation of functional groups prone to reactive intermediate formation with moieties that provide steric hindrance (Di-Giulio and Hinton, 2008).

Conjugations occur at sites where functional groups are added or exposed through phase I metabolism. However, these reactions can proceed directly if appropriate functional groups are

already present on a molecule. For example, ibuprofen can be directly conjugated with glucuronic acid without prior oxidation at the carboxylic acid group (Figure 1.2).

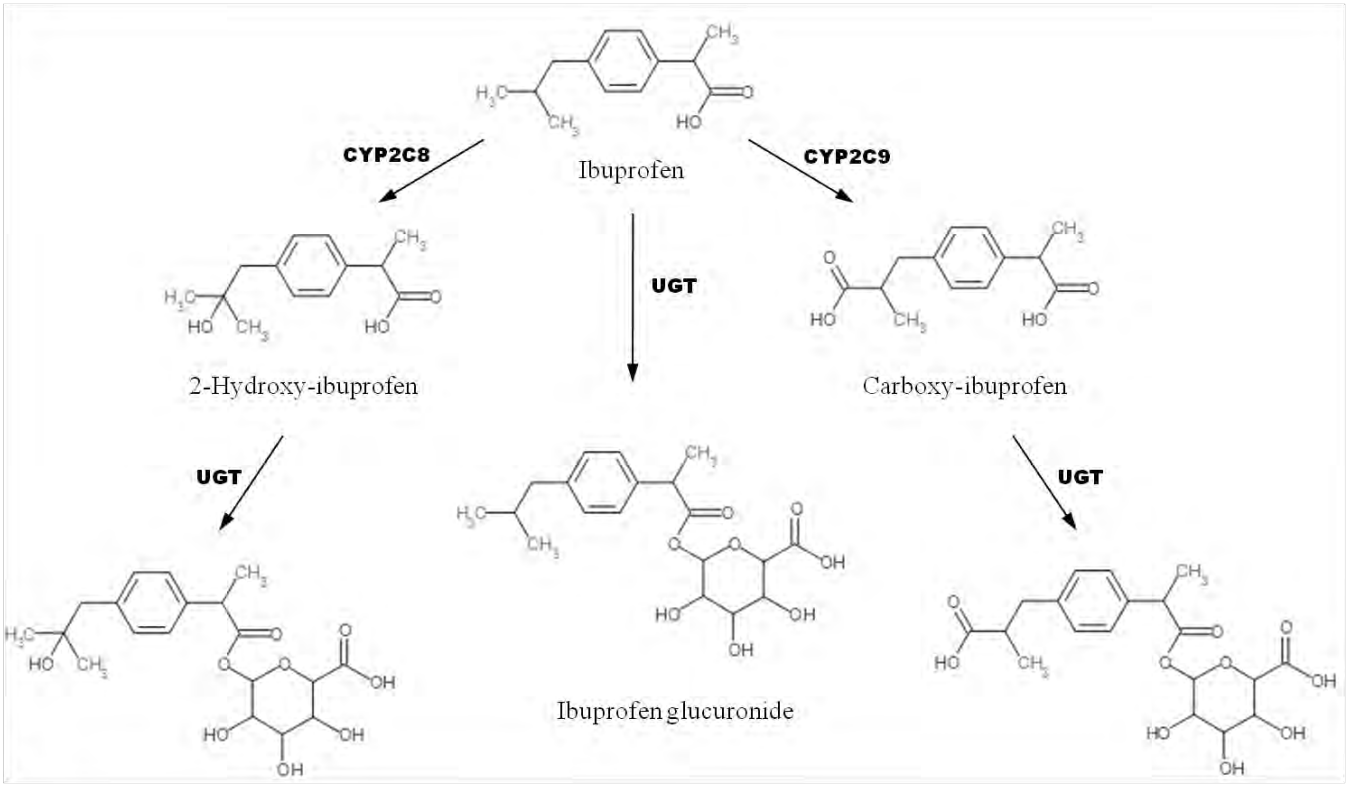


Figure 1.2: The major metabolites of ibuprofen formed in humans. Oxidative metabolism of ibuprofen to 2-hydroxy-ibuprofen and carboxy-ibuprofen is CYP2C8 and 2C9 mediated in humans. The 1- and 3-hydroxy-ibuprofen isoforms (not shown) are also produced but in smaller amounts. Elimination of the compound is primarily through conjugation of the parent (which can be directly conjugated without prior oxidation) and oxidative metabolites with glucuronic acid. Conjugative reactions are catalysed by UGT1A3, 1A9, 2B1 and 2B7 in mammals (Kepp et al., 1997, Kuehl et al., 2005).

1.5 Cytochrome P450s

A number of enzymes are involved in the phase I biotransformation of xenobiotics, however the cytochrome P450s (CYPs) are widely accepted to be the dominant enzyme system responsible for the majority of these reactions (Venkatakrishnan et al., 2001, Brown et al., 2008). CYPs make up a super-family of proteins, possessing a heme iron in the form of a protoporphyrin ring as the prosthetic group at the active site. The enzyme system is highly conserved across a range of species including plants, fungi, yeast, aquatic and terrestrial organisms (Anzenbacher and Anzenbacherova, 2001). CYPs catalyse a wide variety of reactions involving both endogenous and exogenous compounds and are membrane bound proteins in eukaryotes, predominantly located in the smooth endoplasmic reticulum of cells in the majority of tissues. They have been identified in all mammalian tissue types that have been investigated and are generally most abundant in the liver (Venkatakrishnan et al., 2001, Nelson, 2009). The CYP super-family of enzymes consists of a collection of isoforms with overlapping substrate specificities. This allows for multiple substrates to be metabolised by individual CYP isoforms, producing the same metabolites and for many substrates to be metabolised to more than one product by different isoforms. These features are particularly important in polypharmacy, as multiple compounds requiring metabolism by the same CYP isoform can compete for enzymatic metabolism, or one compound can alter the activity or expression of an isoform responsible for the metabolism of another compound, resulting in drug-drug interactions and potential adverse drug reactions, an effect also seen with mixtures of environmental contaminants (Pelkonen et al., 2008).

CYP genes are categorised in accordance to the amino acid sequence similarity of their coded proteins. Genes of the same family and subfamily have > 40% and > 55% amino acid sequence similarity respectively (Brown et al., 2008). In humans, the metabolism of the vast majority of xenobiotics is catalysed by isoforms of the CYP families 1–3. Specifically CYP3A4, 2D6, 2C9 and 1A2 are responsible for the metabolism of 60%, 25%, 15% and 5% of human pharmaceuticals respectively (Venkatakrishnan et al., 2001, Hemeryck and Belpaire, 2002).

1.5.1 Hepatic rainbow trout CYPs

Research on CYPs in fish is limited in comparison to the vast library of information on CYPs in mammalian species, however studies have identified a number of isoforms in rainbow trout, highly concentrated in the liver (Buhler and Wang-Buhler, 1998). However it has also been shown that other organs in fish, especially the gills (in which CYPs are also highly expressed) play an important role in xenobiotic metabolism (Jonsson et al., 2009).

As in mammals, multiple isoforms of CYPs are present in fish and although substrate specificities are not entirely understood, it is widely accepted that there is a broad overlap, allowing for the metabolism of a wide range of compounds (Di-Giulio and Hinton, 2008). However, studies are extremely limited and the metabolic fate and CYPs responsible for the metabolism of many pharmaceuticals in fish are largely unknown (Smith et al., 2010). CYP enzyme activities in fish have been shown to be generally lower than those seen in mammalian species, for example CYP2B activity towards 7-pentoxyresorufin and CYP3A activity towards testosterone was 14.0 fold and 11.8 fold greater respectively in isolated rat hepatocytes, in comparison to isolated trout hepatocytes (Nabb et al., 2006).

The regulation of CYP genes in fish is also diverse, including transcriptional and post transcriptional processes and the genes are also inducible with fish species possessing nuclear hormone receptor transcription factors including the aryl hydrocarbon receptor (AhR) and constitutive androstane receptor (CAR) responsible for the upregulation of genes related to xenobiotic metabolism. However, in comparison with those of mammalian species, relatively little is known about the mechanisms of induction of CYPs in fish (Sadar and Andersson, 2001).

As mentioned previously, CYP3A4, 2D6, 2C9 and 1A2 are the most important drug metabolising isoforms in humans. Of these, fish possess CYP1A and 3A homologs, however, no CYP2C or 2D isoforms have been identified (Di-Giulio and Hinton, 2008), although compounds known to be metabolised by these CYP families in mammals, e.g. ibuprofen, have been shown to be metabolised by fish (Gomez et al., 2011, Jones et al., 2012). Also, very little functional characterisation of fish CYPs has been conducted, as a result of which, knowledge of substrate specificities is limited and often assumptions are made of the ability of CYPs to metabolise the same compounds as in other vertebrates. Despite this, it has been shown that fish CYPs are susceptible to induction and inhibition by various compounds. EROD activity (widely accepted as a marker of CYP1A activity) in trout liver and gills has been shown to be induced by compounds such as benzo[*a*]pyrene and inhibited in hepatocytes *in vitro* by pharmaceuticals of a variety of classes (Laville et al., 2004, Jonsson et al., 2006). This is important in consideration of the wide range of mixtures to which fish are exposed in the environment. The lack of knowledge of substrate and inhibitor specificity also makes the selection of compounds for inhibition studies difficult.

1.5.1.1 CYP1A

The CYP1 family is the most studied in fish species due to its role of in the metabolism of a number of compounds of environmental importance. CYP1A isoforms are involved in the biotransformation of persistent polyaromatic hydrocarbons (PAHs), aromatic amines, polychlorinated biphenyls (PCBs) and some pharmaceutical compounds including paracetamol (Brown et al., 2008). There is much confusion and controversy over the designation and nomenclature of CYP1A in rainbow trout, as there is a possible expression of two closely related forms, 1A1 and 1A2 (Buhler and Wang-Buhler, 1998). There is only a 4% difference in the sequences of the identified clones and neither has been reported as an othologue of the mammalian CYP1A2 isoform (Di-Giulio and Hinton, 2008). Trout CYP1A1 has also been shown to be functionally different from its mammalian counterpart. In mammals, the metabolism of 7-methoxyresorufin (7-MR) is predominantly catalysed by CYPlA2, whereas EROD activity is predominantly a marker of CYP1A1 activity (Burke et al., 1994, Zamaratskaia and Zlabek, 2009). Studies using isolated CYP1A proteins from trout and zebrafish have shown that the different CYP1A isoforms metabolise both of these substrates equally (Gooneratne et al., 1997, Scornaienchi et al., 2010). As a result, CYP1As in fish are not provided with subfamily numbers (Di-Giulio and Hinton, 2008).

CYP1A protein expression in fish is relatively low, however it is known to be modulated by chemically induced processes. Expression has been reported to be induced via the AhR receptor with agonists including PAHs and polychlorinated dibenzo-*p*-dioxins (PCDDs) (Abnet et al., 1999, Grans et al., 2010). CYP1A expression is also inhibited by antifungal agents including clotrimazole and ketoconazole (Hegelund et al., 2004, Hasselberg et al., 2005). Studies

have also reported the presence of CYP1A not only in hepatocytes, but also in biliary epithelial cells and sinusoidal endothelial cells (Lester et al., 1993).

1.5.1.2 CYP2

The CYP2 family is the most diverse in fish with 13 known subfamilies, the majority of which are involved in the metabolism of endobiotics (Di-Giulio and Hinton, 2008). Five different CYP2 isozymes have been isolated from the liver of rainbow trout (Miranda et al., 1989); CYP2M1, is thought to have a high similarity to the activity of the mammalian CYP2B sub-family and its expression is highest in the liver (Yang et al., 1998). CYP2K1 is the dominant CYP isoform expressed in the liver of rainbow trout and its expression is thought to be sexually dimorphic, with increased levels in sexually mature males (Buhler et al., 1994). The isoform is involved in the metabolism of aflatoxin B_1 (Yang et al., 2000b).

Expression studies of CYP2 isoforms are increasing but information of their overall expression, distribution and function in fish remains relatively scarce (Smith et al., 2010). However, the CYP2 family in fish is not thought to be as sensitive to induction as seen in mammalian species, possibly due to functional differences in the activation and translocation of CAR. As a result, there is a lack of response to treatment with inducers such as phenobarbital. Slight decreases in CYP2 mRNA and protein levels have been seen in trout however, when treated with sex steroids especially testosterone and 17-β estradiol (Buhler et al., 2000). This is important in fish as many are exposed to endocrine disrupting chemicals (EDCs) in the environment at relatively high concentrations, throughout their lifetimes and it has been reported that fish are capable of metabolising some typical mammalian CYP2 substrates (Buhler and Wang-Buhler, 1998).

1.5.1.3 CYP3

Isoforms of the CYP3 family are the major CYP forms expressed in the liver in the majority of mammalian species and expression has been reported to be highly variable according to the species. Four CYP3 subfamilies exist in fish (3A–3D) with broad specificities for endogenous and exogenous compounds (Di-Giulio and Hinton, 2008). CYP3A27 is the predominant CYP3A isoform expressed in the liver and like such isoforms in mammals, it exhibits hydroxylase activity towards progesterone and testosterone. CYP3A27 activity is also affected by inhibitors of mammalian CYP3A4 (Lee et al., 1998). CYP3A45 is mainly expressed in the gastrointestinal tract and at a low level in the liver (Szklarz and Halpert, 1997).

An isoform of the CYP4 family has also been identified in rainbow trout. CYP4T1 has a 54.6% homology to rabbit CYP4B1 and 55.4% homology to rat CYP4B2 (Buhler and Wang-Buhler, 1998).

1.6 Glucuronide formation

The formation of glucuronic acid conjugates is a major phase II metabolic reaction in most vertebrate species and is the most common conjugative pathway involved in the metabolism of human pharmaceuticals, accounting for > 35% of all phase II drug metabolism (Kiang et al., 2005). Glucuronidation reactions are catalysed by the family of UDP glucuronosyltransferase enzymes (UGTs), a very well understood enzyme system in mammalian species; however research in lower vertebrates is relatively limited. UGTs are membrane bound proteins of the endoplasmic reticulum with the active site facing towards the lumen and are in close proximity to CYPs (Zhang et al., 2012). They are encoded by a large multi-gene family, producing proteins

with conserved membrane anchorage regions and different, but overlapping specificities, allowing individual isoforms to metabolise multiple substrates (Guillemette, 2003). The substrate specificity of UGTs is conferred at the amino terminus, with cofactor binding occurring at the carboxyl terminus (Kubota et al., 2007). UGT expression varies in tissues with expression in humans being greatest in the liver, intestines and kidneys and, due to the presence of multiple isoforms of the enzyme and its wide distribution, is the most versatile of the conjugation enzymes (Miners et al., 1988, Manevski et al., 2011).

The reaction catalysed is the transfer and covalent linkage of an activated sugar group to exposed functional groups of a wide variety of substrates (including alcohols, amines, carboxylic acids and phenols) both endogenous and exogenous, making glucuronidation an essential clearance mechanism for biological systems (King et al., 2000). The glucuronic acid moiety is derived from the cofactor uridine diphosphate glucuronic acid (UDPGA), which is synthesised in the cytosol from uridine triphosphate (UTP) and glucose-1-phosphate and then actively transported through the endoplasmic reticulum membrane to the active site (Bossuyt and Blanckaert, 1997).

Glucuronic acid conjugation facilitates biliary and urinary compound elimination due to a number of features; glucuronides have an increased molecular weight (an addition of MW 176), thus are well suited to biliary excretion (Luks et al., 1989); the glucuronic acid moiety increases the polarity of the substrate, facilitating urinary excretion (Clarke et al., 1992, Jemnitz et al., 2010); and enhances molecular recognition by specific efflux transporters (especially MRP2 and MRP3 in the liver) to aid transmembrane movement (Wang et al., 2011a).

Glucuronidation generally results in the detoxification of active compounds, however a small number of exceptions exist in which glucuronic acid conjugates retain biological activity (Uchaipichat et al., 2004). For example, several studies have shown that the therapeutic effect of morphine is more potent by the active glucuronide metabolite, morphine 6-*O*-glucuronide, in comparison to the parent compound due to a greater affinity of the former for the μ-opioid receptor (van Dorp et al., 2006, Joshi, 2008, Trecant et al., 2011).

As in mammals, a wide range of compounds, both exogenous and endogenous are substrates for UGTs in fish. The detoxification of phenolic compounds, bilirubin, secretion of bile and excretion of steroid hormones are known to be mediated by glucuronidation (Lambert and Resink, 1991, Clarke et al., 1992, Nichols et al., 2008, Gonzalez et al., 2009).

Glucuronidation activity towards *p*-nitrophenol in trout liver S9 (9.15 nmol/min/mg protein) has been shown to be significantly lower than measured in rat liver S9 fractions (27.5 nmol/min/mg protein) (Han et al., 2009). However, due to the range of substrates metabolised by UGTs, the importance of glucuronidation in the detoxification of xenobiotics is widely accepted to be just as great in fish species as compared to their mammalian counterparts (Sturm et al., 1999, Di-Giulio and Hinton, 2008, Gonzalez et al., 2009, Han et al., 2009).

1.7 Sulfate conjugation

Sulfotransferases (SULTs) catalyse the conjugation of endogenous and exogenous compounds with a sulfate moiety, derived from the donor 5'phosphoadenosine-3'-phoshosulfate (PAPS), formed from inorganic sulfate and ATP. Sulfotransferases are cytosolic enzymes abundant in the liver and kidney; however, the metabolic pathway can become saturated following the depletion of PAPS when large amounts of substrate, e.g. paracetamol, are present. This is mainly due to the fact that the rate of PAPS formation is slow and so cellular levels are often low (Kim et al., 1992). Compounds with exposed hydroxyl and amine groups are the major substrates of SULTs and the reaction generally yields a less toxic and more water soluble product that is readily-excreted in bile and urine (Kaptein et al., 1997, Coughtrie, 2002).

Very few studies of SULTs in fish have been conducted, however evidence of the conjugation of compounds such as 2-naphthol, 9-hydroxy-benzo(a)pyrene, estrogens and paracetamol, with sulfates is available (Kirk et al., 2003, Di-Giulio and Hinton, 2008). In common with other phase I and II metabolic activities of trout, rates of sulfate conjugation have been shown to be lower than those measured in mammalian species. Cytosolic fractions derived from rainbow trout livers exhibited SULT activities towards the thyroid hormone triiodothyronine (T_3) with the measured rate, 14.0 pmol/min/mg (Finnson and Eales, 1998), considerably lower than that measured in the cytosol of male Sprague-Dawley rat livers (530.9 pmol/min/mg protein (Gong et al., 1992)).

1.8 Excretion

Excretion is a key feature of the xenobiotic detoxification process and an important determinant in the manifestation of potentially toxic effects. Often referred to as phase III of drug disposition, the process allows newly formed intracellular water-soluble metabolites from phase I and II metabolism and unchanged compounds to be transported out of cells and these are eventually eliminated from an organism (Szakacs et al., 2008). Xenobiotic compounds and their metabolites can be eliminated from organisms by various routes, however excretion in the urine and faeces predominate. In fish, passive diffusion across the gills is also an important route (Streit, 1998). Impaired elimination of a xenobiotic can result in elevated plasma concentrations, leading to compound toxicity and liver damage. The potential for a compound to bioaccumulate within an organism greatly depends on the ability to eliminate the substance and compounds which are easily excreted have less potential to remain in the body (van der Oost et al., 2003).

Proteins of the ATP binding cassette (ABC) family facilitate the excretory function of organisms, transporting exogenous and endogenous compounds and/or their metabolites out of cells as part of the cellular multixenobiotic resistance process (Faber et al., 2003). This transport is effectively illustrated in hepatocytes, where ABC proteins are heavily expressed as the liver is a major site for the elimination of xenobiotics, via the biliary route (see Figure 1.3). This important role of hepatic transport systems in the xenobiotic detoxification process has recently gained significantly more attention, especially in terms of drug development (Chandra and Brouwer, 2004, Szakacs et al., 2008).

Biliary excretion is a major pathway in the elimination process of many compounds in a wide variety of species (Levine, 1978). Bile is produced by hepatocytes in the liver and is

secreted into the adjacent bile canaliculi through the action of transporters in what is generally an active process. Exogenous compounds and their metabolites, as well as inorganic ions, bile acids and water are present in bile which moves to the bile ducts by peristaltic contractions through to the gall bladder (Coleman and Roma, 2000). Xenobiotic compounds present in the bile pass out through the gut and are excreted in the faeces.

As with mammalian organisms, fish excrete a large number of xenobiotics via the biliary route and are capable of highly concentrating xenobiotics in bile, relative to plasma (Guarino and Lech, 1986). A wide variety of xenobiotics have been detected in fish bile including a number of pharmaceuticals (Samuelsen et al., 1995, Lahti et al., 2011), environmental chemicals (Arukwe et al., 2000), herbicides (Schlenk and Moore, 1993) and metals (Hauser-Davis et al., 2012).

Although not fully understood, it is thought that the mechanisms and criteria (molecular weight and polarity) applying to biliary excretion in fish are very similar to those identified in higher vertebrates (Di-Giulio and Hinton, 2008). Past studies suggest that in many fish species, biliary elimination is heavily influenced by molecular weight and charge, with compounds with a molecular weight greater than 600 excreted predominantly in bile (Gingrich et al., 1977). Enterohepatic recirculation occurs as a consequence of the biliary excretion of compounds and in fish, once secreted into the intestines, biliary conjugates are also subject to enzyme mediated hydrolysis, resulting in the possible cleavage of conjugates, releasing the less polar, free drug which can be re-absorbed. This has been illustrated in rainbow trout treated with 17α-ethylnylestradiol, where the measured plasma concentration profile exhibited multiple peaks, as a result of enterohepatic recirculation (Schultz et al., 2001). Bile flow rates recorded in fish species are substantially lower (up to 50 times) than in mammalian species, however, the bile acid

concentration in the bile of rainbow trout is reported to be similar to that detected in mammals (Di-Giulio and Hinton, 2008).

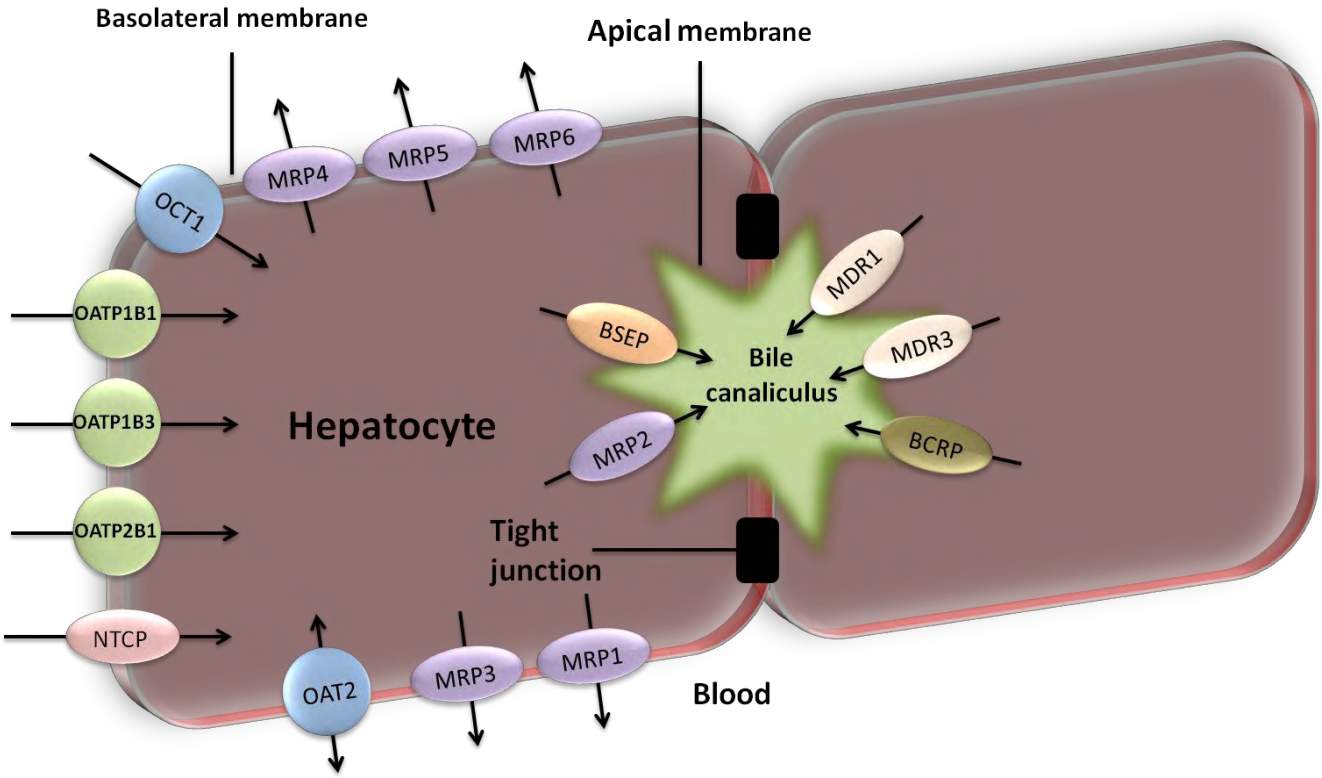


Figure 1.3: The cellular localisation of drug transporters in hepatocytes. Human hepatic uptake and efflux transporters are important determinants of drug disposition and also play a role in normal physiological processes. The key mediators of the efflux of xenobiotics and endogenous compounds into bile are MDR1, MRP2, BSEP and BCRP. MDR3 also plays a role in the export of phospholipids from hepatocytes.

1.9 ABC efflux transporters

Xenobiotic excretion into bile, a process facilitated by ABC efflux transporters, results in the reduced intracellular concentration and lower toxic potential of compounds, as well as a reduced potential for bioaccumulation (van der Oost et al., 2003, Caminada et al., 2008). However, despite the physiological importance of the process in fish, relatively little is known about the function and expression of efflux transporters, especially with respect to their substrate specificities, as efflux studies in fish are rare in comparison to their mammalian counterparts (Zaja et al., 2008b, Loncar et al., 2010).

ABC transporters are membrane bound proteins consisting of two transmembrane domains, which confer substrate specificity and form the transmembrane channel, and two nucleotide binding domains, which bind and hydrolyse ATP, resulting in the active transport of substrates from the cytoplasm and out of cells (Dean et al., 2001, Loncar et al., 2010, Jones and George, 2013).

These proteins are highly conserved and are expressed in a wide range of cell types of all known existing species, suggesting similar, fundamental physiological roles (Glavinas et al., 2004, Sarkadi et al., 2004). To date, 49 human ABC genes have been identified and are separated into seven subfamilies (A–G) based on their sequence homology (Barbet et al., 2012, Ieiri, 2012). In fish, an additional subfamily (H), with one member has also been identified (Loncar et al., 2010). As with many of the drug metabolising enzymes, ATP transporters exhibit a broad range of substrate specificities, allowing the transport of an array of compounds. ABC transporter subtypes are highly expressed in liver and kidney tissue and the most toxicologically relevant transporters in mammalian species have been identified as the multidrug resistance protein

(MDR1, abcb1), bile salt export pump (BSEP, abcb11), multidrug resistance-associated proteins 1–3 (MRP1–3, abcc1–3) and breast cancer resistance protein (BCRP, abcg2) (Chandra and Brouwer, 2004, Leslie et al., 2005). Table 1.3 provides a selected list of common substrates of these transporters, reported in the literature.

Recent publications have shown that a variety of toxicologically relevant ABC efflux transporters are expressed in rainbow trout, with a wide tissue distribution pattern. MDR1, MRP2, BSEP and BCRP have been identified as the efflux transporters with the highest levels of expression in trout liver (Zaja et al., 2008b, Zaja et al., 2008c, Hildebrand et al., 2009, Loncar et al., 2010, Fischer et al., 2011).

Interestingly, these transporters can be susceptible to inhibition by a number of compounds, affecting their ability to eliminate xenobiotics, resulting in the intracellular accumulation of compounds and possible toxic effects (see Table 1.3). This is a serious cause of potentially fatal adverse drug reactions in patients but is also a key issue in aquatic toxicology (Smital et al., 2004, Zhang et al., 2006, Zaja et al., 2008a, Zaja et al., 2008b). A wide range of environmental contaminants are specific inhibitors of ABC transporters and so can increase the sensitivity of organisms to further chemical insult; especially important in the aquatic environment due to the effects of the vast mixtures of chemicals to which organisms are exposed (Daughton and Ternes, 1999, Smital et al., 2004). This has been illustrated in a study using a fish hepatoma cell line, where 18 of the 33 environmentally relevant pharmaceuticals tested exhibited inhibitory activities towards MDR1 and proteins of the MRP subfamily (Caminada et al., 2008). These interactions will also affect the bioaccumulation potential of compounds, due to potential reductions in clearance efficiency. Inhibition of transporters can also affect normal physiological

features, for example, reduced functional activity can cause impaired bile secretion, potentially resulting in cholestatic liver disease (Kipp and Arias, 2002, Wang et al., 2003, Yamaguchi et al., 2010).

Efflux transporter	Endogenous substrates	Exogenous substrates	Inhibitors
MDR1	**Glucosylceramide** (Thomas and Brown, 2010)	**Antibiotics and antivirals** (Szakacs et al., 2008)	**Cyclosporin A** (Fischer et al., 2011)
	Testosterone (Szakacs et al., 2008)	**Antiepileptic drugs** (Luna-Tortos et al., 2008)	**PSC-833** (Fischer et al., 2011)
		Doxorubicin (Chandra and Brouwer, 2004)	**Reversine 205** (Zaja et al., 2007)
		Insecticides and fungicides (Fischer et al., 2011)	**Verapamil** (Dean et al., 2001)
BSEP	**Bile salts** (Dean et al., 2001)	**Taxol** (Wang et al., 2003)	**Rifampicin** (Wang et al., 2003)
	Estradiol glucuronide (Wang et al., 2003)	**Vinblastine** (Wang et al., 2003)	**Taurocholate** (Fischer et al., 2011)
	Phosphatidylcholine (Glavinas et al., 2004)		**Troglitazone** (Glavinas et al., 2004)
MRP2	**Bile salts** (Chandra and Brouwer, 2004)	**Doxorubicin** (Chandra and Brouwer, 2004)	**MK571** (Fischer et al., 2011)
	Glucuronide, glutathione and sulfate conjugates (Chandra and Brouwer, 2004)	**Glucuronide, glutathione and sulfate conjugates** (Fischer et al., 2011)	**Probenecid** (Smeets et al., 2004)
	Hormones (Fischer et al., 2011)		**Glibenclamide** (Zhu et al., 2011)
	Organic anions (Glavinas et al., 2004)		
BCRP	**Bile salts** (Fischer et al., 2011)	**Doxorubicin** (Chandra and Brouwer, 2004)	**Fumitremorgin C** (Hegedus et al., 2009)
	Sulfate and glucuronide conjugates of estradiol (Szakacs et al., 2008)	**Methotrexate** (Sarkadi et al., 2004)	**Gefitinib** (Ni et al., 2010)
		Statins (Ni et al., 2010)	**Ko143** (Fischer et al., 2011)

Table 1.3: List of selected substrates and inhibitors of the canalicular efflux transporters MDR1, BSEP, MRP2 and BCRP. A wide range of diverse compounds both endogenous and exogenous are substrates of these ABC efflux transporters in mammalian species. Transporters are also susceptible to inhibition by a number of compounds, affecting their ability to eliminate xenobiotics, potentially resulting in the accumulation of co-transported compounds.

1.9.1 Multidrug resistance protein (MDR1)

MDR1, also referred to as P-glycoprotein (P-gp) is a member of the ABCB subfamily, the most variable of the seven transporter subfamilies (Glavinas et al., 2004). The trout abcb1 gene has been cloned and shown to have a wide tissue distribution with high levels of expression in the liver, brain, kidney and intestine (Zaja et al., 2008b, Loncar et al., 2010). MDR1 is known to play an important role in the disposition of pharmaceuticals in humans due to its significant substrate overlap with CYP3A (Kim, 2002, Ieiri, 2012). This relationship is thought to be maintained in fish species due to the similar pattern of expression and tissue distribution (Loncar et al., 2010).

The major function of the protein is in the direct removal of unmodified, often toxic xenobiotics from cells, and it possesses a high transport capacity and broad substrate specificity (believed to transport at least 1000 different substrates) (Glavinas et al., 2004, Zaja et al., 2008b). In the liver, MDR1 is highly expressed in the canalicular membrane of hepatocytes (Figure 1.3), key to its role in the excretion of xenobiotics into bile (Ieiri, 2012) and the protein has been identified in the apical membranes of hepatocytes in a wide variety of fish species (Fischer et al., 2011). The abcb1 sequence in rainbow trout has been partially sequenced with the fragment obtained exhibiting 63% homology with human abcb1 and 78% homology with zebrafish abcb1b (Zaja et al., 2008b, Caminada et al., 2008).

Studies using isolated fish hepatocytes and hepatocyte derived cell lines from rainbow trout, have demonstrated characteristic MDR1 mediated transport activities with known mammalian MDR1 substrates and the inhibition of transport using reported mammalian inhibitors (Caminada et al., 2008, Zaja et al., 2008b, Hildebrand et al., 2009, Fischer et al., 2011).

1.9.2 Bile salt export pump (BSEP)

The bile salt export pump is a member of the ABCB subfamily and the principal mediator of bile salt efflux in a wide variety of organisms, exhibiting a very different, more selective substrate specificity to the closely related MDR1. BSEP is almost exclusively expressed in the liver, at the apical membrane of hepatocytes and largely transports physiological substrates, especially bile salts, across the canalicular membrane (Dean et al., 2001, Wang et al., 2003, Noe et al., 2005). The ability of BSEP to transport some xenobiotic compounds such as vinblastine and taxol has also been shown, however, this transporter is not thought to be as significant as others with respect to the excretion of foreign compounds (Arrese and Ananthanarayanan, 2004). Deficiencies in BSEP function can result in cholestatic liver diseases due to the accumulation of bile causing hepatocyte necrosis (Wang et al., 2003, Arrese and Ananthanarayanan, 2004)

Like in mammalian species, BSEP is predominantly expressed in rainbow trout hepatocytes, with a 754 fold and 114 fold greater expression level than MDR1 and MRP2 respectively (Zaja et al., 2008b, Loncar et al., 2010). The rainbow trout abcb11 gene has been sequenced and is highly homologous to the human and zebrafish genes (69% and 72% respectively) (Zaja et al., 2008b).

1.9.3 Multidrug resistance-associated protein 2 (MRP2)

MRP2 is a member of the ABCC subfamily, which has 12 members and in mammalian species, MRPs 1–3 are the key mediators of xenobiotic efflux. These proteins have broad and overlapping substrate specificities but different tissue distributions and patterns of expression (Glavinas et al., 2004). MRP1 appears to be the most important member of the ABCC subfamily with respect to xenobiotic efflux in mammals, as it is ubiquitously expressed, whereas MRP2 and MRP3 have more limited tissue distributions (May et al., 2008, Fischer et al., 2010). In rainbow trout however, MRP1 expression is relatively low in the majority of tissues compared to other MRP subtypes, with significantly greater levels of MRP2 and MRP3 expression. MRP2 expression is greatest in the liver and kidney of trout (Loncar et al., 2010, Fischer et al., 2010).

Studies in mammalian systems have shown that MRP proteins have overlapping substrate spectra with MDR1 however, the preferred MRP substrates are phase I and phase II metabolites, especially glutathione, glucuronide and sulfate conjugates as well as endogenous compounds including hormones and organic anions (Table 1.3). MRP2 has also been associated with the transport of bile acids and conjugated bilirubin (Glavinas et al., 2004, Fischer et al., 2010, Fischer et al., 2011).

Unlike most other MRP type transporters, MRP2 is localised in the apical membrane of polarised cells and in the liver, it is a key mediator of xenobiotic efflux into the bile (May et al., 2008). This apical localisation in hepatocytes is yet to be confirmed in rainbow trout, however it has been shown that in killifish kidney, MRP2 is solely expressed in the apical membrane of the proximal tubules (Miller et al., 2007, Loncar et al., 2010). The full abcc2 gene sequence in

rainbow trout has been identified, exhibiting 75% and 58% sequence identity with zebrafish and human abcc2 respectively (Zaja et al., 2008b).

1.9.4 Breast cancer resistance protein (BCRP)

The breast cancer resistance protein plays a key role in xenobiotic efflux, involved in the regulation of both the intestinal absorption and biliary excretion of compounds (Zaja et al., 2008c). In mammals, the tissue distribution of BCRP shows similar patterns to that of MDR1 and there is considerable overlap in substrate specificity between BCRP, MDR1 and MRPs (Breedveld et al., 2006, Ni et al., 2010). As a result, BCRP transports a range of chemically unrelated compounds including products of phase I and phase II metabolism. Endogenous compounds including natural estrogens are also substrates for BCRP but only as sulfate conjugates and not in an unconjugated from (Breedveld et al., 2006, Hegedus et al., 2009, Ieiri, 2012). BCRP is also susceptible to inhibition by a wide range of compounds (Ni et al., 2010). The protein is a half transporter, possessing a single NBD and TMD, requiring dimerisation to enable transporter activity (Hegedus et al., 2009, Ieiri, 2012).

The full abcg2 gene sequence has been cloned in rainbow trout liver and exhibits a high sequence homology to the corresponding gene in humans (60%) and to zebrafish abcg2a (72%) (Zaja et al., 2008c). Expression of BCRP in the liver of rainbow trout was substantially higher than that of MRP2 (6 fold) and MDR1 (42 fold), suggesting that it is a highly influential mediator of the multi-xenobiotic defence in the apical membrane of hepatocytes (Sarkadi et al., 2004, Zaja et al., 2008c).

1.10 Use of *in vitro* techniques in fish ecotoxicology

The majority of metabolic studies for the assessment of xenobiotic persistence and toxicity in fish are conducted *in vivo*, however, in recent years, increased pressures within industry have prompted organisations to improve their observance of ethical considerations by reducing the number of animals used in regulatory testing. The development and optimisation of reliable and robust alternative testing strategies have become increasingly important, with great emphasis on the application of *in vitro* screening techniques (particularly cell culture) to evaluate risks to the environment from chemicals (Bickley et al., 2009, Mazzoleni et al., 2009). The supplementation of *in vivo* studies with studies conducted *in vitro* to aid screening prioritisation brings this form of regulatory testing in line with the 3Rs principle of reduction, refinement and replacement (Kretlow et al., 2010). This has become especially important since the introduction of regulations for the testing of chemical contaminants, for example REACH in Europe; resulting in the potential requirement of the assessment of more than 140,000 substances in Europe alone, a process that has been projected to require the use of 54 million vertebrate animals (Hartung and Rovida, 2009).

In fish, as in mammals, the liver is the principal organ of xenobiotic biotransformation, therefore, the primary emphasis of bioaccumulation studies has been on the use of various *in vitro* fish liver models including isolated perfused whole liver, liver slices, primary hepatocytes, cell lines, S9 fractions and microsomes, each with their own advantages and limitations (Schmieder et al., 2000, Brandon et al., 2003, Nabb et al., 2006, Han et al., 2009, Smith et al., 2010). These are summarised in Table 1.4. The use of primary hepatocytes in conventional style suspension or monolayer culture is currently commonly used in ecotoxicological studies.

Increasing Complexity

Technique	Advantages	Disadvantages
In vivo	– Exact representation	– Not compliant with 3Rs principle – Direct exposure to live organisms – Large number of animals used in replicates
Isolated perfused liver	– Most accurate *in vivo* representation – Bile collection possible through cannulation – Retention of non hepatocytes – Shown to exhibit similar hepatic clearance rates to those measured *in vitro*	– Limited functional integrity (≤ 3 hours) and time of incubation – Can only use one compound per animal
Liver tissue slices	– Maintenance of *in vivo* structural organisation – Long term storage possible – Technique does not require proteolytic digestion – Retention of non-parenchymal cells – Easy to compare data with slices from other organs – Histopathological analysis can be conducted in addition	– Impaired oxygen and nutrient diffusion within slices – Difficult to accurately measure viability – Short period of viability (only up to a few days) – Short lived CYP activity (< 24 hours)
Primary cell culture	**Hepatocytes in spheroid culture**	
	– Preserved morphology and ultrastructure of hepatocytes *in vivo* – Re-establish cell-cell and cell-ECM interactions – Maintained viability and differentiated status for extended periods – Retain structural polarity and functional bile canaliculi – Ease of sampling in suspension	– Problems with oxygenation and nutrient delivery > 150 µm – Lack vascular network of whole tissue – Lack of complete control over dimensions and organisation during development – Labour intensive
	Freshly isolated hepatocytes in suspension	
	– Ease of sampling – Rapid dispersion of test compounds within culture – Do not require additional time in culture	– CYP expression and viability short lived – Rapid loss of differentiated function – Lack of bile canaliculi due to loss of polarity

Table 1.4: The advantages and disadvantages of a range of hepatic *in vitro* systems used in ecotoxicology studies.

Systems are ordered by relatedness to *in vivo* complexity (continued on page 42).

Increasing Complexity

Technique	Advantages	Disadvantages
Primary cell culture	**Hepatocytes in monolayer culture**	
	– Well established – Numerous techniques available to maintain liver specific characteristics (e.g. matrix modification and co-culture) – Storage potential through cryopreservation – Studies of gene expression modulation possible – Precise control of conditions – Re-establishment of cell-cell contacts through monolayer formation	– Short term maintenance of differentiated status – Rapid decreases in CYP expression – Isolation technically difficult and time consuming – Cell damage and alterations in gene expression during isolation – Loss of normal cell polarity
Immortalised cell lines	– Standardisable and widely available – Low variability in results – Easy to culture compared to primary hepatocytes	– Incomplete expression of families involved in xenobiotic detoxification – Immortalisation and de-differentiation known to affect mRNA expression – Low levels of phase I and II enzyme expression
Subcellular fractions	**S9 fraction**	
	– Contains both cytosolic and microsomal fractions – Quick and less labour intensive preparation – Easily amenable to high throughput screening	– Requires supplementation with NADPH and phase II enzyme cofactors – Lower enzyme activity than microsomes and cytosol
	Microsomal fractions	
	– Low cost and ease of use – Studies of inhibitor effects possible – Can easily navigate problems with variability through pooling	– Lack of phase II metabolic enzymes (except for UGT) – Requires supplementation with NADPH – Not suitable for complete quantitative estimations of biotransformation
	Cytosolic fractions	
	– Presence of phase II enzymes (except UDPGT) at higher concentrations than S9 fractions	– Studies of CYP not possible – Requires supplementation with cofactors of phase II enzymes

Table 1.4: The advantages and disadvantages of a range of hepatic *in vitro* systems used in ecotoxicology studies.

Systems are ordered by relatedness to *in vivo* complexity (continued from page 41).

1.10.1 Subcellular fractions

Recently, the use of subcellular fractions, including 9000 *g* supernatants of liver homogenate (S9) and microsomes have become very popular for the estimations of metabolic potential in the measurement of the persistence of environmental compounds as part of the ERA of chemicals. The use of S9 has been advocated for such work and the system has been championed as an alternative to studies using whole cells as they are quick, cheap and relatively less labour intensive to prepare (Johanning et al., 2012). Liver S9 fractions are derived from homogenised tissue, centrifuged at 9,000 x *g* (an intermediate step of microsomal preparation) (Figure 1.4), resulting in an enzyme suspension containing both the microsomal and cytosolic fractions of cells (Brandon et al., 2003). As a result, S9 fractions can provide information on both phase I and phase II metabolic pathways, a feature lacking in microsomes and cytosol (Ryu et al., 2006). Other distinct advantages of subcellular fractions in comparison to other *in vitro* systems are their high storage stability through cryopreservation, adaptability for high throughput screening and commercial availability (Cervenkova et al., 2001, De Graaf et al., 2002). Despite these advantages, the lack of cellular complexity and organelles of whole intact cells that are retained in isolated hepatocytes is a key disadvantage (Skett, 1994, Cross and Bayliss, 2000). Subcellular fractions require supplementation with NADPH (either directly, or through the supply of an NADPH regenerating system) to supply the CYP energy demand and supplementation with cofactors of phase II metabolism is necessary to activate these pathways (De Graaf et al., 2002, Brandon et al., 2003). In the case of microsomal and cytosolic fractions, there is also an absence of some phase II metabolic enzymes. As a result, these systems do not provide as close a representation of the *in vivo* situation as other *in vitro* models, potentially providing inaccurate predictions of metabolic rates and profiles (De Graaf et al., 2002, Han et al.,

2009). Furthermore, in some instances of metabolic assessment using S9 fractions, incubations have not included co-factors for conjugation reactions (Cowan-Ellsberry et al., 2008).

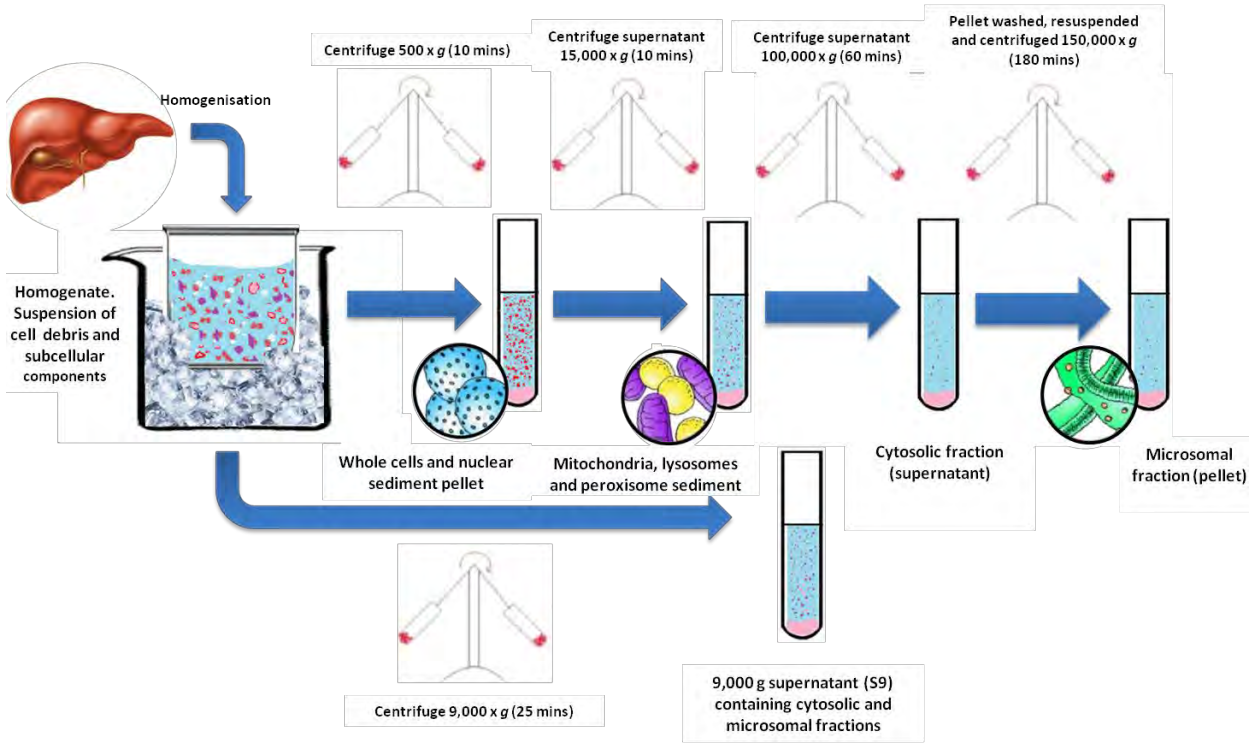


Figure 1.4: The preparation of hepatic subcellular fractions. The upper scheme represents the process of microsomal and cytosolic fraction preparation. Liver S9 fraction preparation (lower scheme) is an intermediate step of microsomal preparation, resulting in a supernatant containing both the microsomal and cytosolic fractions.

1.10.2 Isolated perfused liver and liver slices

Tissue and cell culture enable precisely controlled conditions to be maintained, providing a sensitive means by which toxicant-induced effects can be measured, in an environment considerably more representative of the *in vivo* internal structure of organs, in comparison to subcellular fractions. Simultaneous and repeated sampling with ease and over long time periods is afforded by these systems (Bickley et al., 2009, LeCluyse et al., 2012). The perfused trout liver model has been used to study a range of hepatic functions including xenobiotic biotransformation and biliary secretion and has been shown to exhibit hepatic clearance rates in agreement with estimates made using available *in vitro* data (Nichols et al., 2009). The isolated perfused liver model provides the closest representation to the *in vivo* situation, also having the advantage of allowing for the study of biliary excretion, which is not possible in subcellular fractions (Brandon et al., 2003, Ferrigno et al., 2012). Despite these advantages, the functional integrity and reproducibility of this system have been reported to be limited (up to 3 hours (Brandon et al., 2003)) and the system can only use one animal per experiment, meaning that the technique is not compliant with the 3Rs principle.

In comparison, the use of precision cut liver slices offers a more economical practice for the direct assessment of tissue through the cryopreservation potential of the technique, allowing efficient storage of excess material (Cervenkova et al., 2001). The structural organisation of the liver is maintained in slices and preparation is possible regardless of the amount of tissue available (Olinga et al., 1997). Histopathological inferences can also be made with tissue slices, allowing observations of necrosis and damage to the microarchitecture of the organ, which may occur as a result of compound toxicity. This system is limited however as activities and viability

are relatively short lived, due to impaired oxygen diffusion. It has been shown that in rat liver slices, the activity of a number of CYPs declined, below half of the initial levels within 24 hours (Hashemi et al., 2000, Brandon et al., 2003). Problems with the penetration of medium into the centre of slices have also been reported, slowing down the delivery of test compounds and limiting metabolism (De Graaf et al., 2002).

1.10.3 Primary hepatocyte culture

Primary hepatocyte cultures, directly derived from animal livers, provide a more ethically compliant system to whole tissue models and have proved an important and commonly used tool in the assessment of the potential of the metabolism and effects of environmental contaminants (Bickley et al., 2009, Wang et al., 2011b). The isolation of primary hepatocytes involves the disaggregation of cells from the liver through perfusion and subsequent purification by low-speed centrifugation, where hepatocytes and non-parenchymal cells are separated (Klaunig et al., 1985, LeCluyse et al., 2012). Primary hepatocytes have been shown to be highly representative of the *in vivo* system, retaining many of the liver specific functions of cells *in vivo* and possessing the complete complement of metabolic enzymes and cofactors, allowing integrated phase I and phase II studies to be conducted, without supplementation (unlike with subcellular fractions) (De Graaf et al., 2002, LeCluyse et al., 2012). Assays involving isolated hepatocytes are often more economical and faster to conduct than those requiring *in vivo* exposure and require smaller quantities of test compounds; an important feature for application in ERA, as testing occurs prior to commercial scale manufacture (Caminada et al., 2006).

Once isolated, primary hepatocytes can either be used immediately in suspension, or plated on dishes to allow attachment and formation of a confluent monolayer. The use of hepatocytes in suspension is advantageous as the cells are freshly isolated, no additional culture time is required and there is a relative ease of sampling and dispersion of compounds. However, as 'singlets', hepatocytes in suspension quickly lose the surface distribution of transporters and the polarised formation needed to sustain the biliary pole. As a result, they are unsuitable models for the study of the bile formation (Coleman and Roma, 2000). Freshly isolated hepatocytes have also been shown to be a short-lived *in vitro* system, maintaining viability and functionality for a maximum of 8–12 hours (Fariss et al., 1996, LeCluyse et al., 2012).

When plated, primary trout hepatocytes attach to cell culture surfaces, forming a monolayer in which cell-cell contacts are maintained and can remain intact and viable for at least 1 week (Hightower and Renfro, 1988). During this time, cells express all the major metabolising enzymes and transporter proteins, therefore retaining liver specific function (Klaunig et al., 1985). In monolayer culture however, the metabolic activities of cells is again limited, with a decline in viability, reduction in protein secretion and the rapid loss of CYP activity over time in culture evident, as a result of cellular de-differentiation (Elaut et al., 2006, Castell and Gomez-Lechon, 2009). This has also been linked to the poor maintenance of cell shape of hepatocytes in monolayer culture. Cell shape, polarity and inter-cellular communication are crucial features affecting cellular function and key to metabolic pathways and tissue specific phenotypes through the expression of transcription factors (Fukuda et al., 2006, LeCluyse et al., 2012). As a result, such *in vitro* studies are limited to assessing the acute effects of compounds in short term assays. Primary hepatocytes also show high variability in responses to exposure with compounds, which

are highly dependent on the quality of the isolation procedure, which itself has been shown to cause alterations in the expression of genes related to xenobiotic metabolism (Paine and Andreakos, 2004, Thibaut et al., 2009).

A number of additional techniques can be employed to circumvent these limitations, including supplementation with various types of culture medium, extracellular matrix (ECM) components and co-culture with non-parenchymal cells (Walker et al., 2000). ECM is known to be an important modulator of cell shape, polarity and function. Supplementation of monolayer culture dishes with ECM components or culturing on various types of substrata have been shown to increase the mRNA levels of some liver-specific genes, such as albumin (Ichihara, 1991, LeCluyse et al., 2012). The coating of culture plates with collagen, fibronectin and matrigel allows cells to adhere more tightly and quickly in 2D culture and maintain cell shape by limiting flattening, enhancing the maintenance of metabolic features (Castell and Gomez-Lechon, 2009). Despite this however, many liver-specific functions continue to be lost progressively (LeCluyse et al., 2012). The employment of sandwich cultures, in which monolayers are formed within layers of ECM has also been shown to offer enhanced cell shape in 2D culture, however in this form, hepatocytes take a markedly longer period to establish extensive cell–cell contacts compared to conventional monolayer culture (Castell and Gomez-Lechon, 2009).

Heterotypic interactions between hepatocytes and non-parenchymal cells have been shown to play a role in the function, differentiation and gene regulation of hepatocytes, through inter cellular signalling (Altin and Bygrave, 1988, Abu-Absi et al., 2004). It has been reported that co-culture systems maintaining parenchymal and non-parenchymal cells concurrently, stabilise the hepatocyte phenotype, showing improved morphological characteristics and

increased drug metabolism capabilities (Van Berkel and Van Tol, 1979, Padgham and Paine, 1993, Cross and Bayliss, 2000, Castell and Gomez-Lechon, 2009).

1.10.4 Hepatocyte cryopreservation and immortalised cell lines

Longevity of primary hepatocytes can be achieved through the use of cryopreserved cells, conferred through storage, with a reported ease of use and recovery of metabolic function (Mingoia et al., 2010). Recent advances in the cryopreservation of primary hepatocytes has enhanced their convenience, removing limitations requiring culture within hours of isolation and enabling repeat experimentation with cells from the same individual (LeCluyse et al., 2012). However, it is widely reported that after defrosting, their use is only effective in short term incubations, with viability in subsequent monolayer culture shown to be as low as 40% in the case of rat hepatocytes (Jackson et al., 1985). The continued deterioration of cellular function following thawing has also been highlighted in cryopreserved human hepatocytes, with a reported decrease in CYP activities by approximately 50% per day, stabilizing at 10–20% of the original activity (Li et al., 1999). As a result, like freshly isolated hepatocytes, cryopreserved cells are only effective in short term incubations (Jackson et al., 1985, Cross and Bayliss, 2000, Stephenne et al., 2010).

Permanent fish cell lines have become common model systems for determining ecotoxicological effects of pollutants as they offer a standardisable system that is easy to manipulate, with relatively low variability and more convenient and less laborious to prepare, without the requirement of fresh isolation (Thibaut et al., 2009). However, detailed

characterisation of the metabolic capabilities of most fish liver cell lines is lacking and information of the complete suite of xenobiotic detoxification characteristics is not comprehensive. For example, little is known about the cellular active transport mechanisms that control compound uptake and efflux (Fischer et al., 2011, Malhao et al., 2013). A number of permanent rainbow trout cell lines derived from various tissues are available and have been used in cytotoxicity assays with various compounds (Thibaut et al., 2009). The liver derived RTL-W1, R1 and liver hepatoma RTH-149 have recently been used in studies of drug transporter expression and activities, with a lack of functionality of BSEP and BCRP reported (Fischer et al., 2011). Despite their practical advantages, cell lines still suffer from loss of biotransformation activities, shown in human liver cell lines to be related to the maintenance of a less differentiated state than in associated primary cells (Rodriguez-Antona et al., 2002). In an assessment of a number of phase I and phase II metabolic activities in hepatocyte derived trout cell lines, much lower activities (up to 17.5×10^3 times) were measured in comparison to those reported in primary cultures of fish hepatocytes (Thibaut et al., 2009). As biotransformation is a key determinant in bioaccumulation and xenobiotic toxicity, the lower metabolic capacity of cell lines may result in study outcomes that greatly differ to those seen in primary hepatocytes.

1.10.5 Spheroid Culture

Traditional monolayer culture of primary hepatocytes is a technique best suited to short term use in acute exposure studies, as the advantageous features of the system are limited and associated with the loss of differentiated characteristics with time (Walker et al., 2000, Brandon et al., 2003, Brophy et al., 2009). The use of hepatocytes in spheroid culture, could potentially circumvent this limitation. These are primary cells which are allowed to aggregate in a rotational culture, producing 3D multicellular aggregates, which have been shown to sustain viability and liver specific activity for up to 60 days in the case of rat liver cells (Tong et al., 1990). The 3D environment created is more representative of the environment in which cells exist *in vivo*, surrounded by other interacting cells, maintaining native cell shape and polarity; features necessary for many of the specialised functions of hepatocytes and thought to be responsible for the conserved differentiation status, gene expression and longevity (Abu-Absi et al., 2002, Fukuda et al., 2006). It is also thought that the enhanced secretion of extracellular matrix compounds by hepatocytes in aggregate culture is involved in the enhanced survival and functionality of spheroids (Landry et al., 1985). A major benefit of the use of long term cultures for primary isolated hepatocytes is that an extended stabilisation period is provided, during which metabolic activity, levels of gene expression and other features influencing cellular phenotype can be recovered following the cell isolation process (Wright and Paine, 1992, LeCluyse et al., 2012). Rat hepatocyte spheroids have been reported to undergo an initial period of biochemical and functional turbulence as they mature in early culture and after about 6 days, functional status is said to recover and stabilise (Ma et al., 2003).

A number of physiological characteristics are reformed and maintained in spheroid culture including cell-cell contacts, tight junctions, abundant cytoplasmic organelles and bile canaliculi which reform as an enclosed vacuole into which bile may be secreted (Walker and Woodrooffe, 2001, Ma et al., 2003, Fukuda et al., 2006). These features make the system ideal for use in a broad range of studies, and 3D culture is well established in studies of mammalian systems. The retention of liver specific functions including albumin secretion, urea synthesis, drug metabolism and bile acid secretion have been shown in a number of studies with rat cells (Abu-Absi et al., 2004, Pampaloni et al., 2007, Brophy et al., 2009).

Differences in the cellular responses of hepatocytes in spheroid culture have also been described in comparison to those seen in conventional 2D culture. The toxic potential of cadmium and silver nanoparticles was reported to be significantly reduced in spheroid culture of HepG2 cells when compared to data obtained from conventional cell culture (Lee et al., 2009). Higher resistance to anticancer drugs in 3D tumour spheroids in comparison to the same cells in 2D culture has also been reported (Stevens, 2009). The responses measured in cells in spheroid culture have been shown to be more reflective of those exhibited *in vivo*, enhancing the predictive power of *in vitro* toxicity testing and use of the system in drug screening has been strongly promoted (Kunz-Schughart et al., 2000, Mueller-Klieser, 2000, Pampaloni et al., 2007). Indeed, spheroids are now routinely used as *in vitro* models in cancer and pharmaceutical testing (Stevens, 2009).

Despite the many advantages of the system, problems with immobilisation and oxygen depletion in spheroid culture have been encountered and the lack of a vascular network can hinder oxygen and nutrient delivery. However, reports in rat hepatocyte spheroids have suggested

that the diffusion of oxygen to individual cells is not the rate limiting factor in their formation and that central hepatocytes do not become hypoxic when spheroid diameter is in the region of 100–150 μm, with viability shown to remain high throughout structures (Abu-Absi et al., 2004, Glicklis et al., 2004, Stevens, 2009). Other disadvantages include, the fact that aggregates require a longer time period to form relative to monolayer cultures and that long term maintenance is considerably more laborious and resource intensive.

Very few studies on the use of fish hepatocytes in spheroid culture exist in the literature (Flouriot et al., 1993, Flouriot et al., 1995, Baron et al., 2012). The limited information on trout hepatocytes in spheroid culture includes evidence that vitellogenin mRNA expression and secretion is maintained for up to a month in culture and the system has been shown to be responsive to the effects of classical modulators of gene expression (Flouriot et al., 1993). However, such cultures have not been studied with respect to drug metabolism despite the enhanced xenobiotic capabilities reported in hepatocyte spheroids isolated from mammalian species. Maintenance of ethoxyresorufin-*O*-deethylation (EROD) activity and the expression of a range of genes important to hepatocyte function in rat hepatocyte spheroids have been demonstrated (Sakai et al., 2010a, Sakai et al., 2010b). An enhanced level of UGT, CYP1A2 and CYP3A4 activities in human hepatocyte cell lines cultured as spheroids compared to those of cells in monolayer culture have also been measured (Elkayam et al., 2006). Not only are these activities greater in comparison to conventional cultures, but they are also maintained for extensive periods with viability in spheroid culture (Brophy et al., 2009).

As a result of the maintenance of cellular differentiation, the increased longevity of hepatocyte spheroids and potentially enhanced metabolic capabilities, the application of 3D

cultures to the bioaccumulation assessment of environmental compounds could prove extremely advantageous. Long term viability and activity is a potentially important feature that may allow for the *in vitro* study of the effects of chronic exposure and metabolism of highly persistent chemicals. This is a timely advantage of such systems for the investigation of environmental contaminants, due to the recent concerns over the impact of chronic pollutants (Hutchinson, 2008).

1.11 Aims and objectives

The overall objective of this project was to assess the potential advantages of 3-dimensional hepatocyte cultures for use in fish ecotoxicological studies and compare these features with those of currently used, conventional *in vitro* systems.

Specifically, using hepatocytes from the rainbow trout (*Oncorhynchus mykiss*), the aims were:

- To test the hypothesis that hepatocyte cultures are superior to subcellular fractions as currently used in the metabolic assessment of xenobiotic compounds, by assessing and comparing their metabolic activities both quantitatively and qualitatively, using fluorescent substrates of enzymes involved in xenobiotic metabolism and the model pharmaceutical ibuprofen.

- To optimise the culture of hepatocyte spheroids for further exploration of the hypothesis that their greater complexity, relatedness to *in vivo* hepatocyte structure and potential long term maintenance of viability and functionality, make them effective alternatives for assessment of xenobiotic metabolism when compared to hepatocytes in conventional monolayer culture.

- To investigate the potential for incorporation of measurements of transporter function in hepatocyte spheroid preparations, through assessment of levels and patterns of expression of the key efflux transporters expressed in rainbow trout liver and measurements of their functionality, using specific fluorescent substrates and inhibitors.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Corporation (Poole, UK) unless stated otherwise and of the highest purity available.

2.2 Fish and maintenance

Female, adolescent rainbow trout (*Oncorhynchus mykiss*), with a wet weight range of 300–500 g and between 1–1.5 years of age, were purchased from Arley Fish Farm Limited, UK and held at the University of Birmingham for a minimum 15 day acclimation period prior to first use. Fish were fed floating proprietary pellets (GP Pellets, Cheshire, UK) daily and kept in 180 L round tanks with a constant flow of non re-circulated, de-chlorinated and aerated water. The aquaculture conditions were at 15 ± 1°C; pH 7.5; hardness > 150mg/l $CaCO_3$ and > 80% oxygen saturation and these parameters were measured daily. A photoperiod of 12 hours light and 12 hours dark was employed and fish were fasted for 24 hours prior to sacrifice and production of liver preparations.

2.3 Euthanasia and dissection

Fish were euthanised following the Schedule I protocol of the Animals (Scientific Procedures) Act 1986 - rendered unconscious by a sharp blow to the head, with subsequent destruction of the brain via pithing. Sacrificed trout were weighed, surface sterilised with 70% ethanol and dissected to allow access to the liver. Sampling was always conducted at the same time of day (a.m.).

Dissection involved removal of the left lateral fillet. Using surgical scissors, a longitudinal cut through the body wall was made from the anus to the isthmus, followed by a lateral incision from the anus to the region approximately 5–10 mm below the lateral line. This incision was continued laterally towards the operculum, following the lateral line and then ventrally, following the opercular opening to include the pectoral fin. Extreme care was taken not to scar the liver during this process. The dissected fillet was removed exposing the viscera and heart, which remained beating, preventing blood coagulation. Displacement of the pyloric caceae and lower intestine exposed the liver and the hepatic portal vein.

2.4 Isolation of hepatocytes

Hepatocytes were isolated using a modified version of the collagenase perfusion technique (Klaunig et al., 1985, Nabb et al., 2006) in which trout livers were perfused *in situ*, through the hepatic portal vein, using a cannula consisting of a 0.8 mm needle joined to a section of polyethylene tubing (0.76 mm inside diameter; 1.22 mm outside diameter [Clarke, Coatbridge, UK] and connected to a peristaltic pump [Sci-Q 400, Watson Marlow, Cornwall, UK]) at a flow rate of 2 ml/min, firstly with calcium- and magnesium-free Hanks' balanced salt solution (HBSS) (supplemented with 2.3 mM ethylenediaminetetraacetic acid [EDTA] and 4.2 mM sodium bicarbonate [$NaHCO_3$]; pH 7.8), for approximately 20 minutes. During this period, the heart was severed at the ventral aorta using butterfly scissors to prevent the build-up of pressure in the liver. Digestion was then conducted using a dissociating solution containing 6.7 mM calcium chloride ($CaCl_2$), 3.15 mM potassium chloride (KCl), 0.3 mM sodium phosphate (Na_2HPO_4), 11.76 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 160 mM sodium chloride (NaCl),

4.2 mM $NaHCO_3$ and 260 mg/l collagenase (Roche Applied Science, 11088858001) for approximately 15 minutes. A final perfusion with Dulbecco's Modified Eagle's Medium (DMEM); pH 7.8, was then conducted for approximately 5 minutes to clear the collagenase from the liver. All solutions were pre-incubated at 15°C and flow rate remained constant throughout perfusion. Once digested, the liver was removed from the body cavity, the bile duct was sealed and the gall bladder removed. Livers were washed with DMEM, dabbed dry and weighed on a petri dish.

Hepatocytes were isolated from the organs by mechanical dispersion into DMEM and passed through a 100 µm nylon cell strainer (BD falcon, Massachusetts, USA). Cells were collected and washed 3 times in DMEM following 3 periods of low speed centrifugation (30 x *g*, 5 minutes, 15˚C), counted using a haemocytometer and viability (consistently ≥ 90%) was measured based on the exclusion of 0.04% trypan blue.

2.5 Cell culture

All cell culture work was conducted in sterile conditions provided by a class II tissue culture cabinet (Aura B4, Bio Air, Italy). Cell culture equipment, media and all other solutions used were either obtained sterile or sterilised by autoclaving.

2.5.1 Primary hepatocytes

Isolated primary hepatocytes were plated at a density of 1 x 10^6 cells/cm^2 on 24-well collagen type I coated microplates (Iwaki, Japan) in DMEM and stored in a 15°C ± 1°C stationary incubator. Hepatocytes used in suspension were purified to 1 x 10^6 cells/ml in centrifuge tubes, transferred to 50 ml conical flasks in DMEM and kept in a 15°C shaking incubator at 90 rpm (Infors-HT, Bottmingen, Switzerland).

2.5.2 Spheroid culture

Considerable work was undertaken over a period of 10 months to develop and optimise a method to consistently prepare high quality and viable spheroids that survived for extensive periods. More information is provided in Chapter 4 and the final culture conditions were as follows. Cells isolated following hepatocyte purification were resuspended in DMEM/nutrient mixture F-12 Ham supplemented with L-glutamine, 15 mM HEPES, $NaHCO_3$, serum replacement 3 (20 ml/L) and an antibiotic/antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B) at a concentration of 5 x 10^6 cells/ml. Cell suspensions were plated in sterile, non-treated 50 x 18 mm petri dishes (Sterilin, Newport, UK)

and placed on a gyratory shaker (Stuart Scientific, Stone, UK) to aggregate at 50 rpm and 17°C ± 1°C. Cell culture medium was changed after every 2 days and spheroids were used for initial metabolic assessment when maturity was confirmed after 10 days (at this time there was little to no change in morphology, compared to earlier time points, visualised by light microscopy. In developmental gene expression assessments, spheroids were used from 5 days post isolation and up to 25 days post isolation in transporter assessments.

2.5.3 Liver S9 preparation

S9 fractions (9000 g supernatants) were prepared from a subset of the same digested liver preparations as used for hepatocyte isolation and taken prior to mechanical isolation. Samples retained on ice were transferred to centrifuge tubes (BD falcon) and homogenised in 4 volumes of a buffer containing 42 mM Tris, 150 mM KCl, 2 mM EDTA and 1 mM dithiothreitol (DTT), pH 7.8, using an electronic homogeniser (Ultra turrax T8; IKA Werke, Staufen, Germany) with a series of 30 second pulses and cell degradation was confirmed by light microscopy. The homogenate was centrifuged (9000 x g, 25 minutes, 4°C) and the supernatant was retained and pooled. This total S9 was vortexed, aliquoted into 500 µl microfuge tubes (Eppendorf) and stored at -80°C until use.

2.6 Flow cytometry

Changes in spheroid shape and size during development were assessed using the forward scatter properties of flow cytometry. Spheroids were washed twice and either resuspended in 500 µl of phosphate buffered saline (PBS) and used immediately in flow cytometry assessment, or fixed (1% v/v paraformaldehyde in PBS) and stored at 4°C in the dark until required. Spheroids were loaded into flow cytometry tubes and analysed using CytoCount fluorescent microspheres (Dako, Stockport, UK) as a reference population, on a BD FACScalibur™ flow cytometer, using Cell QuestPro software (Becton Dickinson, Oxford, UK).

2.7 Protein assays

Protein concentrations of cultured hepatocyte preparations and S9 fractions were measured using the Bradford Assay, using BSA (1–20 µg) as a standard (Bradford, 1976). Primary cultured cells were removed from wells using cell scrapers (Corning), transferred to 1.5 ml microfuge tubes and sonicated for 3 periods of 15 seconds, with 35 second intervals (100 watt, 20% amplitude; SONICS Vibra Cell, Connecticut, USA), on ice. Hepatocytes in spheroid culture and in suspension were sonicated in the same manner. Aliquots of the sonicated hepatocyte samples and S9 fractions were assayed using a 1:5 diluted Bradford reagent stock (Bio-Rad, Munich, Germany).

To allow for the direct comparison of measured reaction rates in S9 fractions with those measured in preparations of isolated hepatocytes, cellular protein content was adjusted to produce an S9 equivalent protein content using a conversion factor (1.549 mg cellular protein ≈ 1 mg S9

protein) representing the scaled difference in the protein content of 1 x 10^6 hepatocytes measured using the Bradford assay before and after S9 preparation.

2.8 Ethoxyresorufin-*O*-deethylase (EROD) assay

Hepatocytes were treated at various time points (6 hours, 24 hours, 48 hours) post cell isolation or freshly isolated in suspension and as spheroids, with 7-ethoxyresorufin (8 µM final concentration in dimethyl sulfoxide [DMSO]) as described by (Jones et al., 2010), in 24-well plates. 7-ethoxyresorufin has been shown to be metabolised by CYP1A in rainbow trout (Carlsson and Part, 2001, Jonsson et al., 2006, Lennquist et al., 2011). Dicoumarol (10 µM final concentration in DMSO) was added based on its ability to inhibit DT diaphorase (as demonstrated in mammalian systems (Hosoda et al., 1974, Chen et al., 1999), to prevent metabolism of the resorufin product by DT Diaphorase. Plated cells in phenol red free DMEM were used in triplicate and incubated for up to 6 hours with the substrate (7 time points per assay) at 15^{o}C in a final volume of 1 ml. The final concentration of DMSO in the reaction mixture was 0.2% (v/v). After incubation, cells were sonicated and centrifuged (100 x *g*, 5 minutes). Supernatant (750 µl) was then added to either 250 µl of 100 mM sodium acetate buffer (pH 4.5) or 250 µl of a β-glucuronidase (150 Fishman units)/aryl sulfatase (1200 Roy units) extract (Roche Applied Science, Mannheim, Germany) diluted 666 fold in 100 mM sodium acetate buffer. This was used to cleave glucuronic acid or sulfate conjugates produced, giving a measure of the total resorufin production and indirectly informing on conjugative activity. Supernatants were incubated for 2 hours at 37°C, after which 1 ml of 100% ethanol was added to the incubated samples in a fluorescence cuvette and sample fluorescence was measured using a fluorimeter at

λ_{ex} 530 nm and λ_{em} 590 nm (LS50B, Perkin Elmer, Boston, USA). The amount of resorufin produced per 1 x 10^6 cells at each time point was calculated from a standard curve produced by measuring the fluorescence of resorufin prepared in media at a number of concentrations (0–500 pmol). A negative control of cells in phenol red-free DMEM alone was used in all investigations.

S9 fractions at a final concentration of 1 mg/ml protein were incubated with 100 mM sodium phosphate buffer (pH 7.8) and an NADPH-regenerating system (10 mM glucose 6-phosphate, 0.1 mM EDTA, 15 mM magnesium chloride ($MgCl_2$), 0.53 mM nicotinamide adenine dinucleotide phosphate (NADP) and 2 units/ml glucose 6-phosphate dehydrogenase), with 10 μM dicoumarol at 15°C for use in EROD assays. For metabolic assessment in the presence of cofactors for phase II enzymes, uridine diphosphate glucuronic acid (UDPGA) and 3-phosphoadenosine-5'-phosphosulfate (PAPS) were added, providing a final concentration of 2 mM each. Assays were conducted as previously described, in a final volume of 1 ml and assessment of conjugated product was made using the same β-glucuronidase/aryl sulfatase mixture. Heat inactivated S9 (100°C, 30 minutes) was used as a negative control in S9 assays.

2.9 Ethoxycoumarin-O-deethylase (ECOD) assay

Primary hepatocytes in monolayer culture (24 hours post isolation), were exposed to 7-ethoxycoumarin (100 μM final concentration in DMSO) in a final volume of 1 ml made up with phenol red free DMEM, for up to 6 hours (6 time points per assay). The final solvent concentration was 0.1% (DMSO). Following incubation, cells were sonicated and centrifuged as described previously and fluorescence was measured at λ_{ex} 368 nm and λ_{em} 456 nm. The amount of hydroxycoumarin was also calculated in a similar manner, using a hydroxycoumarin standard curve (0–1000 pmol).

2.10 4-Methylumbelliferone (4-MU) glucuronidation assay

Cell samples (suspension and monolayer cultures) and S9 fractions (both supplemented and un-supplemented with UDPGA) were exposed to the fluorescent, non-specific UGT substrate 4-methylumbelliferone (4-MU). Incubations of 4-MU (50 μM final concentration) with cells in phenol red free media and S9 fractions (1 mg/ml protein) in 100 mM sodium phosphate buffer (final incubation volumes of 1 ml) were conducted at 15°C, in the dark, for up to 60 minutes (cells) and 120 minutes (S9). The final solvent concentration used in this assay was 0.02% methanol. Following incubation, cellular samples were sonicated and centrifuged as described previously and these, along with the exposed S9 fractions, were measured for sample fluorescence using a Bio-Tek FL600 fluorescence plate reader (Bio-Tek Instruments Inc., Vermont, USA) at λ_{ex} 370 nm and λ_{em} 480 nm. To ensure that any change in fluorescence was in fact due to glucuronidation, a subset of samples were also treated with β-glucuronidase to cleave glucuronic acid conjugates, or a sodium acetate buffer control as described previously.

2.11 Lactate Dehydrogenase (LDH) leakage assay

Cell viability following EROD assays (1, 5 and 6 hour incubations) or individual treatment with 7-ethoxyresorufin or dicoumarol (maximum solvent concentration 0.2% DMSO) was assessed using the LDH leakage assay. Following substrate incubation, cells were removed from wells and centrifuged (200 x *g*, 5 minutes). Potassium phosphate (100 mM, pH 7.0), sodium pyruvate (23 mM), reduced nicotinamide adenine dinucleotide (NADH, 12 mM) and cell culture supernatant (200 µl) were added to a silica cuvette, which was then placed into a spectrophotometer set to measure absorbance at 340 nm and the decline in absorbance was measured for 1 minute. A positive control of cells lysed with 10% triton X 100 for 30 minutes on ice and a negative control of cells incubated for the same time periods as in the EROD assay but in the absence of 7-ethoxyresorufin, dicoumarol and DMSO were also measured for LDH leakage. The decline in A_{340} was plotted against time, the gradients of the lines generated were measured and compared to that of total enzyme leakage (triton X 100 treated) to obtain % viability.

2.12 Reactive oxygen species (ROS) assay

Hepatocytes in monolayer culture (24 hours post isolation) were exposed to 7-ethoxyresorufin and dicoumarol as described previously. After incubation, cells were removed from wells, washed in PBS and resuspended in phenol red free media. The compound 2', 7'-dichlorodihydrofluorescein-diacetate (10 μM final concentration) was added to the media and cells were incubated for a further 30 minutes. Cells were subsequently washed and dichlorofluorescein fluorescence was measured at λ_{ex} 485 nm and λ_{em} 530 nm on a fluorescence plate reader. Hydrogen peroxide (H_2O_2; VWR BDH Prolabo, Lutterworth, UK) was used as a positive control for ROS production.

2.13 Ibuprofen exposures

Primary trout hepatocytes and trout liver S9 fractions (under different conditions of co-factor supplementation) were exposed to 10 μM ibuprofen sodium salt (CAS No. 31121-93-4) or an identical concentration of ibuprofen containing ^{14}C labelled ibuprofen (donated by Drug Metabolism and Pharmacokinetics IM, AstraZeneca, Alderley Park) over a range of time periods (5, 10, 30, 60, 120 minutes) in serum free cell culture media or sodium phosphate buffer (100 mM) respectively at 15^{o}C. S9 fraction exposures were conducted in a shaking incubator at 90 rpm. All exposures were conducted in final volumes of 1 ml. At the end of the required incubation period, cells were removed and sonicated and cellular protein was precipitated by addition of an equal volume of ice cold acetone. An equal volume of ice cold acetone was also added directly to S9 fractions following incubation. Precipitated proteins were removed by centrifugation (2,500 x g, 5 minutes) and the retained supernatants from cell and S9 incubates

were stored at -80°C and later analysed for the presence of ibuprofen metabolites by liquid chromatography-mass spectrometry (LC-MS), using analytical standards of ibuprofen sodium salt and 1-hydroxy-ibuprofen.

The metabolism of ibuprofen by rat S9 fractions was also assessed to allow inter-species comparisons to be made. Rat livers were obtained from the Biomedical Services Unit, University of Birmingham Medical School and perfused *ex situ*. S9 fractions were prepared from these livers as described previously and incubations with ibuprofen were conducted at 37°C and 90 rpm.

2.14 Assessment of CYP involvement in ibuprofen metabolism

To assess the influence of particular cytochrome P450 isoforms on the metabolism of ibuprofen in S9 fractions, known inhibitors of various isoenzymes were added prior to incubation (see Table 2.1). S9 fractions supplemented with phase II cofactors and an NADPH regenerating system were preincubated with inhibitors (10 μM final concentrations) for 5 minutes at 15 °C and 90 rpm, followed by the addition of 10 μM ibuprofen containing the ^{14}C radiolabel and further 20 minute incubation. Protein precipitation and preparation for LC-MS analysis were conducted as described previously.

Inhibitor	Major CYPs known to be inhibited (Species)	References
Fluconazole	CYP2C9, CYP2C19 (Human)	(Huang et al., 2007, Vestergren et al., 2012)
Ketoconazole	CYP1A, CYP3A (Atlantic Salmon, Atlantic Cod) CYP3A family (Human)	(Hegelund et al., 2004, Hasselberg et al., 2005, Vestergren et al., 2012)
Quercetin	CYP2C8 (Human, Rat)	(Projean et al., 2003, Lu et al., 2010)
SKF525A	Non-selective	(Savi et al., 1994, Yoon et al., 2007)

Table 2.1: Cytochrome P450 inhibitors used in ibuprofen metabolism assessment and the isoforms which they most specifically inhibit.

2.15 LC-MS analysis of ibuprofen metabolites

Metabolites and parent compound in primary cell homogenates and S9 fractions exposed to ibuprofen were separated by liquid chromatography under optimised conditions and quantified using a linear ion trap mass spectrometer (Thermo Fisher Scientific). The *m/z* for parent ibuprofen (205) and its metabolites (221: hydroxy-ibuprofen) were detected in negative ion mode and with reference to the well documented mammalian metabolism of ibuprofen (Kepp et al., 1997). Accurate mass of ions and MS-MS fragmentation were studied for the parent compound and potential metabolites detected in exposed samples. Liquid chromatography and mass spectrometry operating conditions and parameters are outlined in Table 2.2.

Column	Halo C_{18} 2.7 µm, 150 x 4.6 mm or Waters Xbridge $C_{18,}$ 5 µm, 50 × 2.1 mm
Oven Temperature	40 ± 0.1 °C
Injection Volume	10 µl
Elutant A Elutant B	10 mM Ammonium Acetate Acetonitrile
Gradient (A:B)	Time 0 min: 90%:10% Time 2 min: 90%:10% Time 10 min: 5%:95% Time 12 min: 5%:95% Time 12.1 min: 90%:10% Time: 14 min: 90%:10%
Flow Rate	500 µl/min
Autosampler	CTC Analytics HTC Pal
Source Voltage	3.0 kV
Source Current	100 µA
Sheath Gas	32 units
Sweep Gas	21 units
Capillary Temperature	350°C

Table 2.2: Liquid chromatography-mass spectrometry conditions and parameters for the analysis of ibuprofen exposed cellular and subcellular fraction samples.

2.16 Scintillation counting

Fractions collected from the liquid chromatography of S9 samples exposed to ^{14}C ibuprofen in CYP inhibition assays were collected at 1 minute intervals for 25 minutes. These fractions were mixed with scintillation fluid (Optihasehisafe 3; Fisher Scientific, Loughborough, UK) in a 1:10 ratio in glass scintillation vials. The amount of ^{14}C recovered in each fraction was counted using three, 1 minute counts on a Packard 40 Tricarb 1600 TA Liquid Scintillation Analyser (Packard Instruments; PerkinElmer). Counts were recorded as disintegrations per minute (dpm) and fractions in which radiolabel was detected were analysed by mass spectrometry using the same parameters as mentioned previously.

2.17 Nuclear magnetic resonance (NMR) spectroscopy

Sample preparation:

S9 fractions exposed to ibuprofen were diluted in 100 mM sodium phosphate buffer (pH 7.0) to a final volume of 540 µl and mixed with 60 µl of the internal reference 10 mM sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TMSP, Cambridge Isotope Laboratories) in D_2O (GOSS Scientific Instruments Ltd.). The final mixture (600 µl) was loaded into 10 cm NMR tubes and transferred to the magnet.

Measurement:

1D ^1H NOESY spectra were acquired at 25°C, using a 500 MHz Bruker spectrometer equipped with a cryogenically cooled probe. 1D spectra were acquired with a long relaxation delay (d1) of 4 seconds to guarantee near complete longitudinal relaxation. The spectral width of

the acquired spectra was 12 ppm, with 32,768 increments. The transmitter frequency offset was 4.696 ppm to suppress the water signal by pre-saturation. For exponential multiplication (EM) a window function was used and a zero filling to 32,768 points was applied. Measurements were carried out with deuterium frequency locking after shimming. Spectra were obtained within 8.5 minutes using 64 scans. During data processing spline baseline (Ludwig and Gunther, 2011) was applied to spectra using the MetaboLab software (Gunther et al., 2000).

Data analysis:

NMR data were processed using NMRLab in the MATLAB programming environment (Gunther et al., 2000, Ludwig and Gunther, 2011). NMR resonances of ibuprofen were assigned using the Chenomx software and the Human Metabolite Database (HMDB). A 1-hydroxy-ibuprofen standard in 100 mM phosphate buffer (ph 7.0) was used to obtain its NMR resonances. Assignment of other ibuprofen metabolites was based on the available literature (Vanderhoeven et al., 2006, Holmes et al., 2007, Johnson et al., 2007).

2.18 RNA extraction

Whole liver sections (100 mg) excised during the hepatocyte isolation process, freshly isolated hepatocytes, hepatocytes in monolayer culture and spheroids were collected, washed with PBS (cells) and stored in RNA later at -20°C.

Total RNA was extracted from stored samples using the trizol, chloroform, glycogen extraction method. Briefly, RNA later was completely removed from samples and whole tissues or cells were homogenised in trizol solution (1 ml) in RNase free microcentrifuge tubes (Axygen, California, USA). Following a 5 minute incubation at room temperature, chloroform (200 µl) was added and after vigorous shaking, the mixture was centrifuged (12,000 x *g*, 15 minutes, 4°C). The upper aqueous phase (500 µl) of the bi-phasic solution was transferred to a fresh microcentrifuge tube and glycogen (10 µg, Fermentas, UK) was added. The aqueous phase was mixed with 70% isopropanol (500 µl), incubated at room temperature for 10 minutes and centrifuged (12,000 x *g*, 10 minutes, 4°C). The resulting pellet was washed with 75% ethanol (Fisher Scientific, Loughborough, UK), air dried for 10 minutes, resuspended in RNase-free water (50 µl, Qiagen, Crawley, UK) and incubated at 60°C for 15 minutes. RNA quality was assessed by agarose gel electrophoresis and quantified by spectrophotometry using a Nanodrop ND1000 (Thermo Scientific, LabTech, East Sussex, UK) and DNA contamination was removed by treatment with a genomic DNA-free treatment kit (Ambion, Austin, U.S.A.). Storage of RNA was at -80°C.

2.19 Ethanol/Acetate precipitation of RNA

RNA extracted from cells producing an A_{260}:A_{230} ratio below 1.5 (as measured by nanodrop), were subjected to ethanol/acetate precipitation. Ethanol (100%, 2 volumes, -20°C) and sodium acetate (3 M, pH 5.2) were added to RNA samples and incubated at -80°C for 1 hour. Following centrifugation (15,000 x *g*, 4°C, 30 minutes) and removal of supernatant, the pellet was washed in ethanol (70%), centrifuged (15,000 x *g*, 4°C, 5 minutes) and air dried for 30 minutes. The dried final RNA pellet was re-suspended and stored at -80°C, until required.

2.20 cDNA synthesis

Total RNA isolated as described above was used for first-strand cDNA synthesis using the SuperScript II Reverse Transcriptase kit (Invitrogen, Paisley, U.K.). A mixture of RNA (500 ng) and 1 µl random hexamers (50 ng; Bioline, London, U.K.), made up to a final volume of 15 µl with sterile dH_2O, was incubated at 65°C for 5 minutes. DTT (2 µl, 0.1 mM), first strand buffer (5 µl), superscript II reverse transcriptase (0.5 µl), a 25 mM dNTP mix (0.4 µl) and sterile dH_2O (2.1 µl) were subsequently added and cDNA synthesis conducted by incubation at 25°C for 10 minutes, 42°C for 90 minutes and 70°C for 15 minutes in a thermocycler (Eppendorf Mastercycler Gradient; Eppendorf, Cambridge, UK). The cDNA produced was quantified by spectrophotometry and stored at -20°C.

2.21 Polymerase chain reaction (PCR)

cDNA was used as a template for PCR and used to validate the sequence specific primers (Alta Bioscience, Birmingham, U.K) designed for target genes using Primer 3 software or as described in the literature (Table 2.3). Stock solutions (10 μM) of each primer were prepared and 50 μl reactions made using components of the DreamTaq DNA Polymerase PCR kit (Fermentas, U.K.), in microcentrifuge tubes. DNA polymerase (1.25 units), forward and reverse primers (10 pmol each), 2 mM dNTP mix (0.2 mM each), 10 x DreamTaq buffer (5 μl), template DNA (100 ng) and dH_2O were combined to a final volume of 50 μl. The PCR programme consisted of an initial 5 minute denaturation period at 95°C, 35 cycles of denaturation at 95°C for 1 minute, 1 minute annealing periods at the required primer annealing temperature and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 5 minutes in a thermocycler. The resulting products were analysed by DNA gel electrophoresis and comparisons of fragment size were made using a 100 base pair ladder (New England Biolabs, Ipswich, UK).

Gene	Primer Sequence	Product Size (bp)	References
CYP1A	**F:** 5’-GAT GTC AGT GGC AGC TTT GA-3’ **R:** 5’-TCC TGG TCA TCA TGG CTG TA-3’	104	N/A
CYP2K1	**F:** 5'- CTC ACA CCA CCA GCC GAG AT-3' **R:** 5'- CTT GAC AAA TCC TCC CTG CTC AT-3'	164	(Matsuo et al., 2008)
CYP2M1	**F:** 5'-GCT GTA TAT CAC ACT CAC CTG CTT TG-3' **R:** 5'-CCC CTA AGT GCT TTG CAT GTA TAG AT-3'	195	(Matsuo et al., 2008)
CYP3A27	**F:** 5’-GAC GGT GGA GAT CAA CG-3’ **R:** 5’-GAG GAT CTC GAC CAT GG-3’	240	(Randelli et al., 2011)
UGT	**F:** 5’-ATA AGG ACC GTC CCA TCG AG-3’ **R:** 5’-ATC CAG TTG AGG TCG TGA GC-3’	113	N/A
ABCB1	**F:** 5'-GGA ACT GTC CTC ACC GTG TT-3' **R:** 5'-GGG GTT TAT TGT CGG TGA TG-3'	136	N/A
ABCC2	**F:** 5'-CCA TTC TGT TCG CTG TCT CA-3' **R:** 5'-CTC GTA GCA GGG TCT GGA AG-3'	150	N/A
ABCG2	**F:** 5'-AGG CCT GCT GGT GAA CCT G-3' **R:** 5'-ACT CAT TAA TTT GGA GAG CTG TTA GTC C-3'	101	(Fischer et al., 2011)
ABCB11	**F:** 5’-CCG ACC AGG GCA AAG TGA TT-3’ **R:** 5’-CAG AAT GGG CTC CTG GGA TAC-3’	101	N/A
18S rRNA	**F:** 5-TGG AGC CTG CGG CTT AAT TT-3’ **R:** 5’-ATG CCG GAG TTT CGT TCG TT-3’	170	(Fischer et al., 2011)

Table 2.3: PCR primers and product sizes.

2.22 Agarose gel electrophoresis

Gel electrophoresis was used to separate DNA molecules and to analyse RNA quality following extraction, with gel percentage varied according to use and fragment size. Powdered agarose (Bioline, London, UK) was dissolved in 50 ml 1 x TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM Na_2EDTA, pH 8) supplemented with ethidium bromide (0.5 µg/ml), by heating. The gel was set in a gel tank at room temperature and submerged in 1 x TBE buffer containing ethidium bromide (0.5 µg/ml). Nucleic acid samples were combined with 1 µl 6 x gel loading dye (New England Biolabs, Hertfordshire, U.K.) and loaded into sample wells alongside the appropriate DNA molecular weight ladder (New England Biolabs, Hertfordshire, U.K.). Samples were subjected to electrophoresis at 80 V for 40 minutes and the gel visualised by UV illumination using a bio imaging unit (Geneflow, Staffordshire, UK).

2.23 DNA sample purification and sequencing

PCR products were purified using QIAquick spin columns (Qiagen Ltd, West Sussex, UK), according to the manufacturer's protocol. Briefly, 0.1 volumes of sodium acetate (3 M, pH 5.2) and 5 volumes of PB buffer were added to 1 volume of the remaining PCR product. The samples were mixed and transferred to QIAquick spin columns and centrifuged (5,900 x *g*, 1 minute). The flow through was discarded and buffer PE (750 µl) was added to the columns followed by centrifugation as in the previous step. The flow through was again discarded and columns centrifuged again under the same conditions. Columns were placed in 1.5 ml microcentrifuge tubes and 50 µl of an elution buffer added to the columns. DNA was eluted by centrifugation (5,900 x *g*, 1 minute) and quantified by spectrophotometry.

Purified DNA samples were then sequenced using an ABI3730 DNA analyser (Applied Biosystems, UK) by the Functional Genomics and Proteomics Unit, University of Birmingham, Birmingham, UK. Reactions contained 3 ng of purified DNA and 0.3 µl of either the forward or reverse primer (10 pmol) in a final volume of 10 µl made up with sterile water. The sequencing results of the amplified DNA fragments obtained were used in BLAST searches (www.ncbi.nlm.nih.gov/blast) to confirm that the primers amplified the selected genes of interest.

2.24 Real-time PCR

Once it was confirmed that the primer sequences amplified the correct target, real-time PCR was conducted using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). cDNA prepared from RNA extracted from various tissue and cell samples were used as the template for reactions with the SensiFast SYBR Hi-Rox kit (Bioline, UK). Five biological replicates were used for each of the target genes, with each individual assessed in triplicate. Samples were run in 96 well plates, each sample containing cDNA (90 ng), 2 x sensiFast SYBR (10 µl), forward and reverse primers (at various concentrations - a range of 1–5 pmol- dependant on primer efficiency values calculated during optimisation runs) and nuclease free water in a final volume of 20 µl. PCR cycle parameters were: 95ºC, 30 seconds for denaturation; and 60ºC, 30 seconds for combined annealing and extension. No template controls were also run using sterile dH_2O.

Melt curves for all samples were plotted and analysed using the ABI Prism 7000 SDS software to ensure only a single product was amplified and primer dimers were not formed. PCR primer efficiencies were calculated using absolute fluorescence values measured in each well and

the LinRegPCR software as described by (Ramakers et al., 2003). Threshold cycle (C_t) values were recorded for each sample in the linear phase of amplification and the data quantified using the Pfaffl method of relative quantification (Pfaffl, 2001). Differences in C_t values were assessed by one way ANOVA (SPSS v16.0) and when significant, values were normalised to the internal reference gene (18S rRNA).

2.25 Assays of uptake and elimination of fluorescent probes

Uptake assays were conducted using fluorescent compounds known to be substrates of particular drug efflux transporters (see Table 2.4). Spheroids were washed twice in PBS and re-suspended in phenol red free DMEM or PBS. The appropriate volume of fluorescent dye was added to achieve the required concentration and cells were incubated at 17°C, in the dark and on a gyratory shaker at 50 rpm at a range of time points (0, 5, 15, 30, 45, 60, 90, 120 and 180 minutes). After incubation, cells were kept on ice, washed three times in PBS, re-suspended and lysed in 0.1% Triton X-100 in PBS using an ultrasonic water bath (Camlab Transsonic T460, Cambridge, UK) in the dark. Lysate was loaded onto fluorescence 96 well plates (BD Falcon) and fluorescence measured at the relevant wavelength for each substrate (Table 2.4) using a Bio-Tek FL600 fluorescence plate reader.

To assess the role of transporters through the use of inhibitors, assays of the fate of fluorescent probes were repeated to include specific transporter inhibitors. Substrates and inhibitors were dissolved in various solvents as noted and appropriate vehicle controls were used for each. Hepatocyte spheroids were washed twice in PBS and re-suspended in phenol red-free

DMEM or PBS. Inhibitors at a range of concentrations (detailed in Table 2.5) were added to cultures and incubated for 10 minutes on a gyratory shaker at 50 rpm, following which, the appropriate volume of fluorescent substrate was added to reach the desired concentration. Total solvent concentrations did not exceed 0.5% (v/v) and appropriate solvent controls were always used. Spheroids were incubated in the dark, at 17°C, on a gyratory platform at 50 rpm, for 60 minutes. Following incubation, spheroids were washed 3 times in PBS and lysed as described above. Lysate was loaded into fluorescence 96 well plates and fluorescence measured at the relevant wavelength for each substrate. Fluorescent dye accumulation was measured and compared to control cells exposed to the relevant fluorescent substrate in the absence of the specific inhibitor.

Fluorescent Compound	Efflux Transporter	Concentration (µM)	Solvent (maximum concentration)	Fluorescence Wavelengths
Calcein AM	MRP2	10	DMSO (0.4%)	λex: 494 nm; λem 517 nm
Cholyl-lysyl-fluorescein	BSEP	5	Sterile dH_2O	λex: 485 nm; λem 520 nm
2',7'-Dichlorodihydrofluorescein diacetate	BSEP	10	DMSO (0.05%)	λex: 495 nm; λem 530 nm
Hoechst 33342	BCRP	10	Sterile dH_2O	λex: 340 nm; λem 510 nm
Rhodamine 123	MDR1	10	DMSO (0.05%)	λex: 511 nm; λem 534 nm

Table 2.4: Fluorescent compounds used in spheroid uptake and elimination studies

References: (Wang et al., 2003, Zaja et al., 2007, Caminada et al., 2008, Zaja et al., 2008a, Zaja et al., 2008b, Fischer et al., 2011)

Efflux Transporter	Inhibitor	Concentration Range (µM)	Solvent (maximum concentration)
MDR1	Cyclosporine A	0.01–20	DMSO (0.1%)
MRP2	Probenecid	0.5–1000	NaOH (0.01%)
BCRP	Sodium taurocholate	1–1500	Sterile dH_2O
BSEP	Ko 143	0.01–20	DMSO (0.4%)

Table 2.5: Specific inhibitors used in efflux transporter inhibition studies

References: (Noe et al., 2002, Zaja et al., 2007, Caminada et al., 2008, Zaja et al., 2008a, Zaja et al., 2008b, Fischer et al., 2011)

2.26 Confocal microscopy

Cells exposed to various fluorescent compounds were washed twice in PBS and re-suspended in phenol red free PBS. Suspended cells were pipetted into glass bottom culture dishes (MatTek Corporation, Massachusetts, USA) for imaging. Images were acquired using a Leica TCS SP2 confocal microscope (Leica Microsystems, Milton Keynes, UK) with a 63x oil immersion objective lens. Fluorochromes were excited using an argon laser at the wavelengths denoted in Table 2.4.

2.27 Statistical Analysis

All statistical analyses were performed using SPSS version 16 software. Normal distribution and homogeneity of variance of all data sets was assessed by Shapiro-Wilk and Levene's tests respectively. Data which were normally distributed and displayed homogeneity of variance were analysed by one-way ANOVA with a Tukey post-hoc test, or by independent samples *t*-test (parametric tests). Data which were not normally distributed, or did not display homogeneity of variance were analysed by non-parametric statistics, using Kruskal-Wallis, or Mann-Whitney *U* test. *p* values are indicated by typographical symbols as described in the legends of figures.

Chapter 3

The metabolic capabilities of cellular and subcellular systems for screening of bioaccumulation potential

3.1 Introduction

Persistence, bioaccumulation and toxicity (PBT) are key parameters of the tiered testing strategy implicated in the Environmental Risk Assessment (ERA) of pharmaceuticals (Hutchinson, 2008). PBT is also a feature of the screening and classification of potentially hazardous chemicals based on the environmental cut-off criteria of EU REACH regulations (Ehrlich et al., 2011, Oberg and Iqbal, 2012). In aquatic species, calculations and measurements of potential uptake, accumulation, metabolism and elimination are undertaken as part of ERA; however, this work can involve considerable numbers of animals and can be technically difficult. Alternatives to live animal tests are gaining substantial consideration for application in regulatory and environmental settings for a number of reasons, including the ethical and legal protection given to fish and the principles of reduction, refinement and replacement (the 3Rs) (Halder et al., 2010). *In vitro* techniques are a useful tool in understanding cell- and organ- specific mechanisms of toxicity and metabolic fate and their use in such studies is often a cheaper alternative to those requiring the *in vivo* exposure of animals (Eisenbrand et al., 2002, Laville et al., 2004, Caminada et al., 2006). Moreover, *in vitro* studies can be high throughput and can help to prioritise chemicals for more detailed analyses. Currently, investigations into the use of *in* vitro techniques using fish cells or subcellular preparations as models for screening compound toxicity and bioaccumulation potential are increasing, with the aim of developing a number of high throughput screens for use in regulatory processes.

A key question posed in regulatory research is how best to predict compound fate and effects in aquatic biota. With respect to bioaccumulation, measurements of metabolic potential in fish are currently being used to predict bioaccumulative potential and to refine estimates of

bioaccumulation factors for *in silico* models (Cowan-Ellsberry et al., 2008). The principle considers that there is an inverse relationship between degree of metabolism and the bioaccumulative potential of a compound. An essential component of the refinement of the ERA strategy is the selection of the most accurate *in vitro* systems for the generation of these datasets.

A number of *in vitro* systems are widely used in ecotoxicological studies (see Section 1.10). Subcellular fractions, including 9000 *g* supernatants of liver homogenate (S9) and microsomes are commonly employed to estimate the persistence of chemicals that may enter the environment. The use of S9 has recently been advocated for such work and the system has been promoted as an alternative to studies using whole cells as they are quick, cheap and relatively less labour intensive to prepare (Johanning et al., 2012). However, such incubations often do not include co-factors for conjugation reactions and, frequently, incubations persist for extensive time periods, often longer than one hour, thus beyond initial reaction rates (Cowan-Ellsberry et al., 2008, Han et al., 2009, Gomez et al., 2011). This can be particularly problematic for the assessment of highly persistent chemicals with low rates of metabolism. Furthermore, these *in vitro* systems lack the cellular complexity and organelles of whole intact cells that are retained in isolated hepatocytes (Skett, 1994, Cross and Bayliss, 2000). As a result, S9 fractions are unlikely to provide as close a representation of the *in vivo* situation as whole cells, potentially providing inaccurate predictions of metabolic rates and profiles.

The aim of this investigation was to test the hypothesis that hepatocyte cultures are superior to S9 both quantitatively and qualitatively in the prediction of bioaccumulation potential due to the aforementioned limitations of subcellular fractions, through comparative studies and to determine the utility of hepatocytes cultured under different conditions. The metabolism of the

non-steroidal anti-inflammatory drug, ibuprofen [2-(4- isobutylphenyl) propionic acid], a prominent example of a pharmaceutical product detected in the environment, was assessed to determine potential differences in the biotransformation profile of this compound by S9 fractions under conditions of varied phase II cofactor status and in isolated primary hepatocytes. Comparisons of enzyme activities were made using 7-ethoxyresorufin and 7-ethoxycoumarin which have been shown to be substrates for several CYP enzymes in mammals, including CYP1A1 and 2E1 in rat for EROD and CYP1A2, 2B6 and 2C for ECOD (Kern et al., 1997, Moorthy et al., 2000, Khan et al., 2002). It has been shown that the former is metabolised at least by CYP1A in rainbow trout (Carlsson and Part, 2001, Jonsson et al., 2006, Lennquist et al., 2011). Glucuronidation activities were assessed using the non-specific UGT fluorescent substrate 4-methylumbelliferone (Wang et al., 2011a).

3.2 Results

3.2.1 Metabolic activity of primary hepatocytes

EROD reaction rates were calculated from measurements of both conjugated and unconjugated resorufin product over various incubation periods for hepatocytes in suspension and hepatocytes in monolayer culture (6, 24 and 48 hours post isolation). Following preliminary assessments at various incubation time points, comparisons between different cellular preparations were made after 30 minutes of incubation with 7-ethoxyresorufin. When adjusted for protein concentration, it was clear that oxidative metabolic activity in 30 minute incubates was greatest in suspension hepatocytes (immediately after isolation) and decreased 6 hours after plating. Oxidative activity appeared to be partially recovered at 24 hours and 48 hours after plating (although this was not statistically significant) (Figure 3.1).

Despite oxidative metabolic capacity being greatest in suspension hepatocytes, the same trend was not evident for the proportion of resorufin that was conjugated. This was significantly higher in cells assessed in culture 24 hours and 48 hours post isolation, compared to hepatocytes in suspension and in culture 6 hours post isolation ($p < 0.05$, Independent samples *t*-test) (Table 3.1 and Figure 3.2).

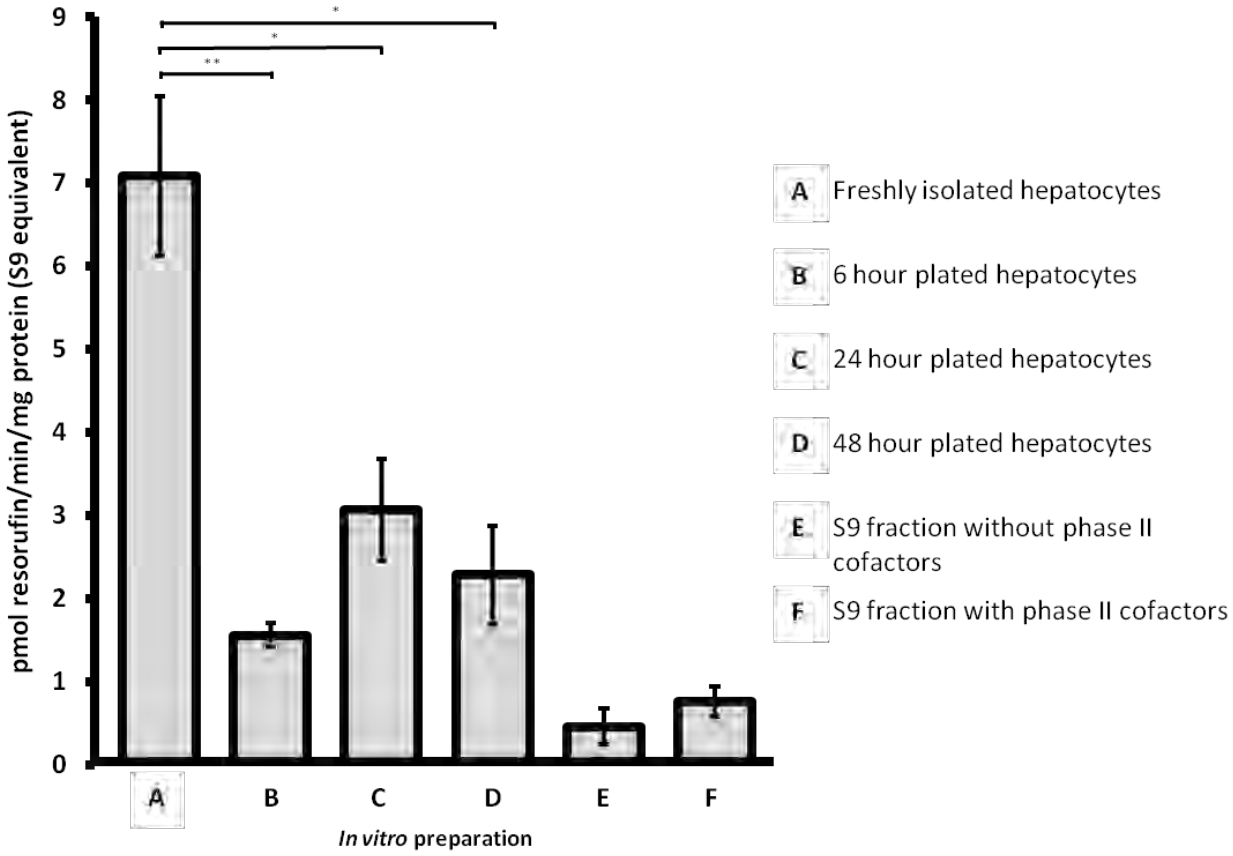


Figure 3.1: Comparative rates of total resorufin production by different hepatocyte preparations (30 minute incubates) and S9 fractions (10 minute incubates) under different phase II cofactor supplemented conditions. Cellular protein content was adjusted for an S9 protein concentration equivalent, using a conversion representing the difference in protein content of 1 x 10^6 hepatocytes before and after S9 preparation. EROD activity was considerably greater in whole cells at all cultured time points than in S9 fractions. Mean values ± SEM; Hepatocyte preparations- 30 minute incubates, S9 fractions- 10 minute incubates; $^{*}p < 0.05$, $^{**}p < 0.01$ Mann-Whitney *U* test (n = 8 fish).

Cellular preparation	% oxidised product as conjugates
Freshly isolated hepatocytes in suspension	9.9 ± 2.3
6 hour plated	11.1 ± 5.2
24 hour plated	33.9** ± 9.5
48 hour plated	28.6* ± 8.9

Table 3.1: Proportion of resorufin conjugated in different hepatocyte preparations. Mean values; n = 8 fish; ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$ Independent samples *t*-test.

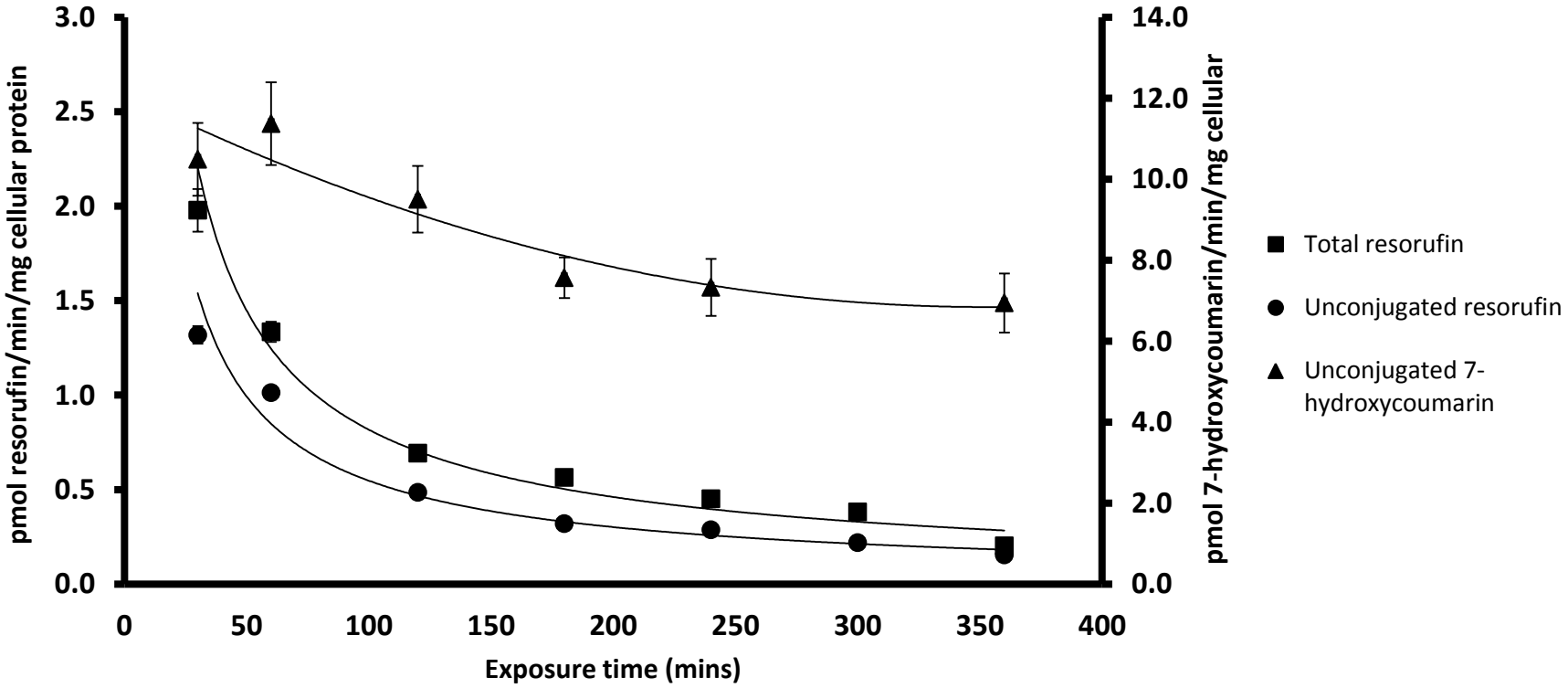


Figure 3.2: EROD and ECOD activities of primary cultured hepatocytes (24 hours post isolation). Average rate of resorufin/7-hydroxycoumarin production in hepatocytes; n = 8 fish ± SEM EROD; n = 5 fish ± SEM ECOD.

When incubations with 7-ethoxyresorufin were extended beyond 30 minutes, a marked decline in EROD activity over exposure time was seen in primary hepatocyte cultures. For example, in monolayer cultures after maintenance for 24 hours, the amount of total resorufin produced per unit time of subsequent incubation with 7-ethoxyresorufin, declined from 1.98 ± 0.32 pmol/min/mg cellular protein at 30 minutes after substrate addition, to 0.38 ± 0.08 pmol/min/mg cellular protein at 300 minutes after substrate addition (Figure 3.2).

3.2.2 Studies to help explain the decline of EROD activity in cells with time

To determine whether this decline in activity over incubation time was due to compound-induced cytotoxic effects, cell viability was assessed using the LDH cytotoxicity assay. Hepatocyte viability remained relatively constant (> 90%) in all cell isolations throughout these incubation times when compared to control cells of the same culture time points (Figure 3.3).

To test if inherent CYP oxidative activity was declining simply as a feature of time of culture, supplementary EROD assays were conducted with primary hepatocytes, following pre-incubation with or without solvent (a maximum of 0.2% DMSO) for 360 minutes. No significant differences in EROD reaction rates measured during 30 minute incubations over a total pre-incubation time period of 360 minutes, in the presence of vehicle without substrate, were observed for cells previously cultured for 24 hours (Figure 3.4); 2.01 ± 0.5 pmol/min/mg cellular protein in samples pre-treated with 0.2% DMSO for 360 minutes, compared to 1.93 ± 0.6 pmol/min/mg cellular protein in control cells. This suggests that the decline in activity seen above (Figure 3.2) is dependent on the presence of the 7-ethoxyresorufin substrate, though not necessarily caused by this compound directly.

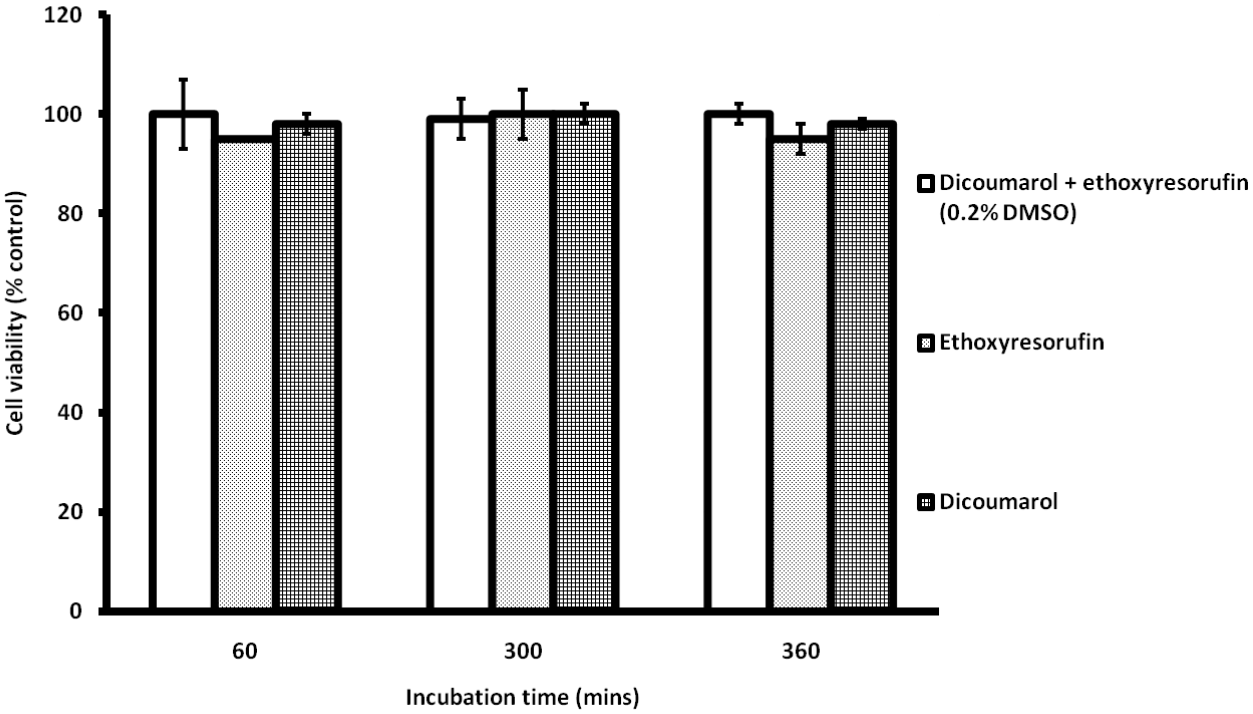


Figure 3.3: Viability of cultured hepatocytes 24 hours post isolation following incubation with various combinations of EROD assay substrates (7-ethoxyresorufin and dicoumarol) assessed using the LDH leakage assay. Data are displayed as percentage of untreated control at the same culture time point ± SEM for hepatocyte preparations from 3 different trout livers. No significant difference in LDH leakage was observed between control and treated cells; Kruskal-Wallis test.

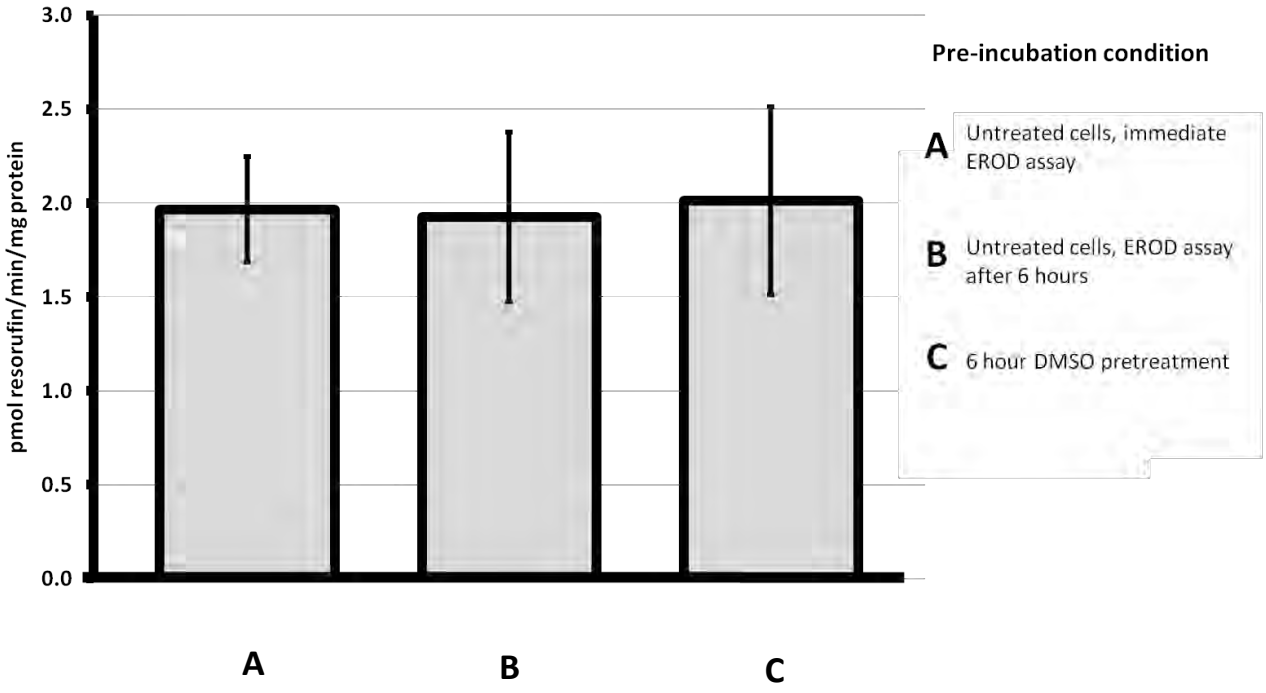


Figure 3.4: Rates of resorufin production (after 30 minute 7-ethoxyresorufin incubation) in primary cultured hepatocytes 24 hours post isolation, pre-treated with 0.2% DMSO for 360 minutes or control cells unexposed to solvent. Rates adjusted for cellular protein content ± SEM for hepatocyte preparations from 3 different trout livers.

In contrast to the results with EROD, when primary cultured hepatocytes, 24 hours post isolation, were incubated for the same time course with the broad CYP2 substrate 7-ethoxycoumarin, there was only a 1.5 fold decline in activity after 360 minutes of incubation, whereas in the same time period with EROD, a 10 fold reduction in activity was evident (Figure 3.2). Overall these results suggest that the relatively rapid decline in EROD rate with incubation time appeared to be related to the presence of the substrate 7-ethoxyresorufin.

A possible hypothesis for this substrate specific decline in activity is the potential production of reactive oxygen species (ROS), in the presence of 7-ethoxyresorufin. This hypothesis was briefly tested to assess the possibility, using the 2',7'-dichlorodihydrofluoroscein diacetate fluorescence assay to measure ROS. The mean percentage fluorescence intensity relative to untreated controls was greater following exposure of primary hepatocytes in monolayer culture to 7-ethoxyresorufin. This increase was statistically significant at all time points except at 240 minutes (Figure 3.5). Thus ROS production offers a possible explanation for loss of CYP activity.

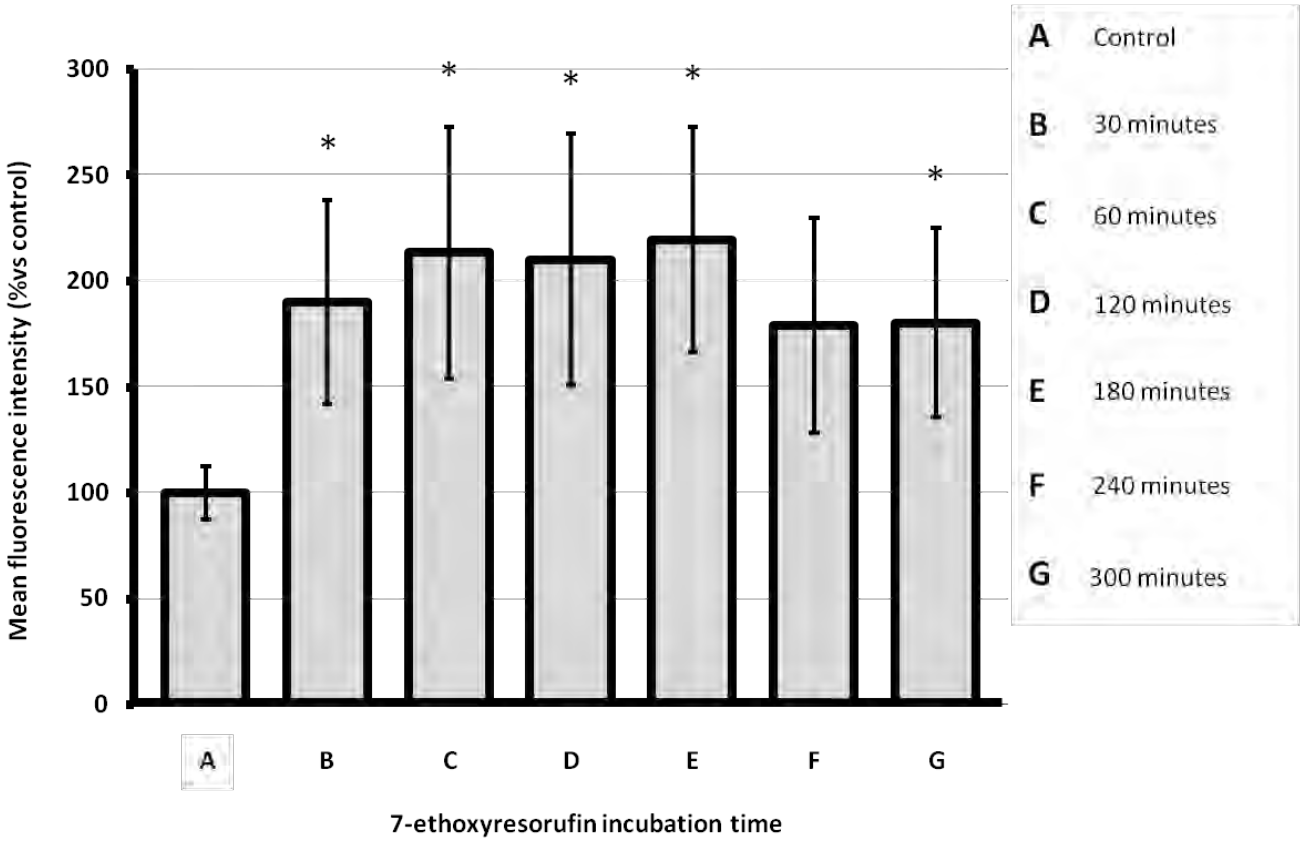


Figure 3.5: ROS levels in primary cultured hepatocytes 24 hours post isolation, following incubation with 7-ethoxyresorufin over a range of time points (30–300 minutes). Data are displayed as average fluorescence intensity as a percentage of that of an untreated control ±SEM for hepatocyte preparations from 3 different trout livers. $^{*}p < 0.05$ (significantly different from control), Mann-Whitney *U* test.

3.2.3 Comparative metabolic activity of S9 fractions

To allow for the direct comparison of EROD reaction rates measured in S9 fractions with those measured in preparations of isolated hepatocytes, cellular protein content was adjusted to produce an S9 equivalent protein content using a conversion factor (1.549 mg cellular protein ≈ 1 mg S9 protein) representing the scaled difference in the protein content of 1 x 10^6 hepatocytes experimentally measured using the Bradford assay before and after S9 preparation.

Importantly, EROD reaction rates measured for 10 minutes in S9 preparations were significantly lower ($p < 0.05$, Mann-Whitney *U* test) than those obtained in cellular assays, and significantly lower ($p < 0.05$, Kruskal-Wallis test) even when S9 was supplemented with phase II cofactors (Figure 3.1). As with hepatocytes, the activity declined rapidly with time of incubation beyond 10 minutes despite the fact that the substrate remained in excess. For example, the amount of total resorufin produced per unit time declined from 0.46 ± 0.04 pmol/min/mg S9 protein after a 10 minute 7-ethoxyresorufin incubation, to 0.21 ± 0.03 pmol/min/mg S9 protein after 60 minutes (from n = 8 fish) (Figure 3.6). Even recognising that liver S9 will include protein from the relatively small proportion of non-hepatocytes (which make up approximately 20% of liver volume), the oxidative activity was markedly lower than as found in isolated hepatocytes. Pre-incubations of S9 fractions (in the absence of phase II cofactors) were conducted in a similar fashion to those described for primary cultured hepatocytes, to assess whether the decline in activity was due to the depletion of cofactors or was compound induced. No significant difference ($p = 0.908$, independent samples *t*-test) was seen between the EROD rates (10 minute incubations) of S9 fractions pre-incubated for 60 minutes in comparison to fractions incubated without solvent pre-treatment (Figure 3.7).

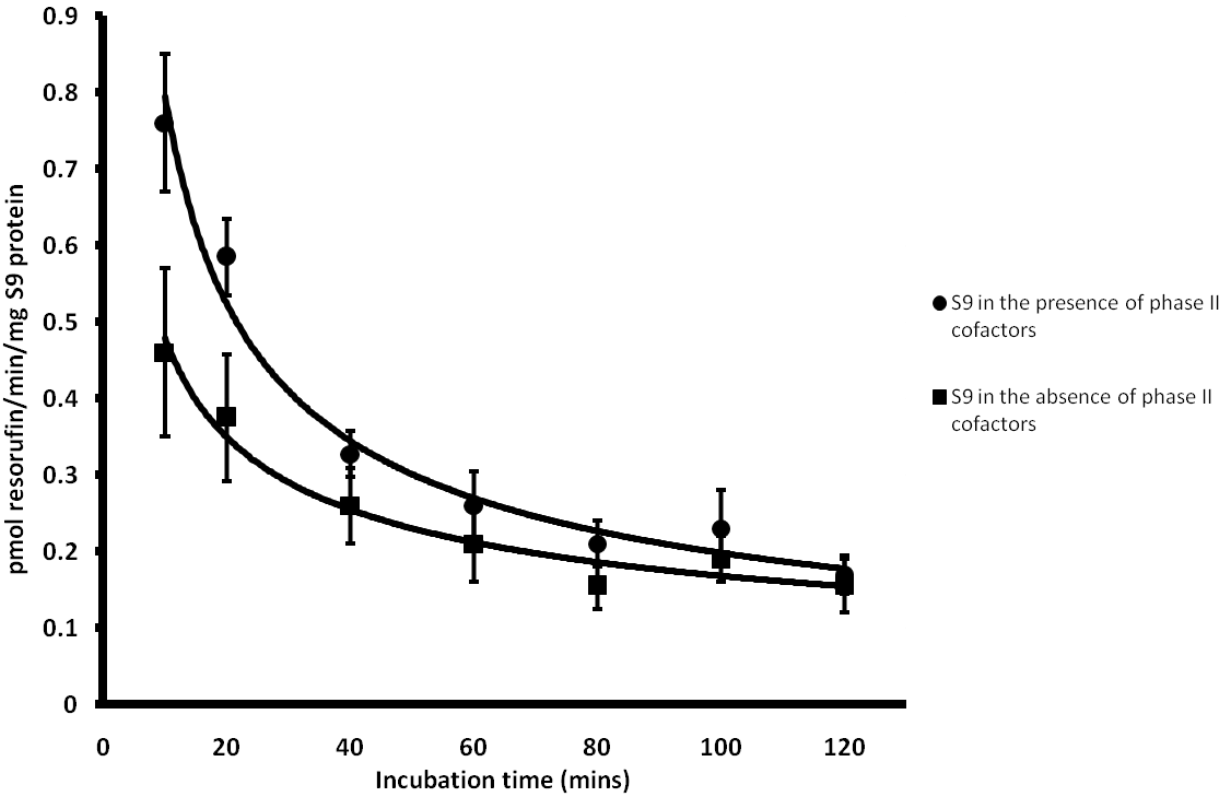


Figure 3.6: EROD activity of liver S9 fractions under different phase II cofactor conditions. Average rates adjusted for S9 protein content ± SEM for S9 fractions prepared from 8 different trout livers.

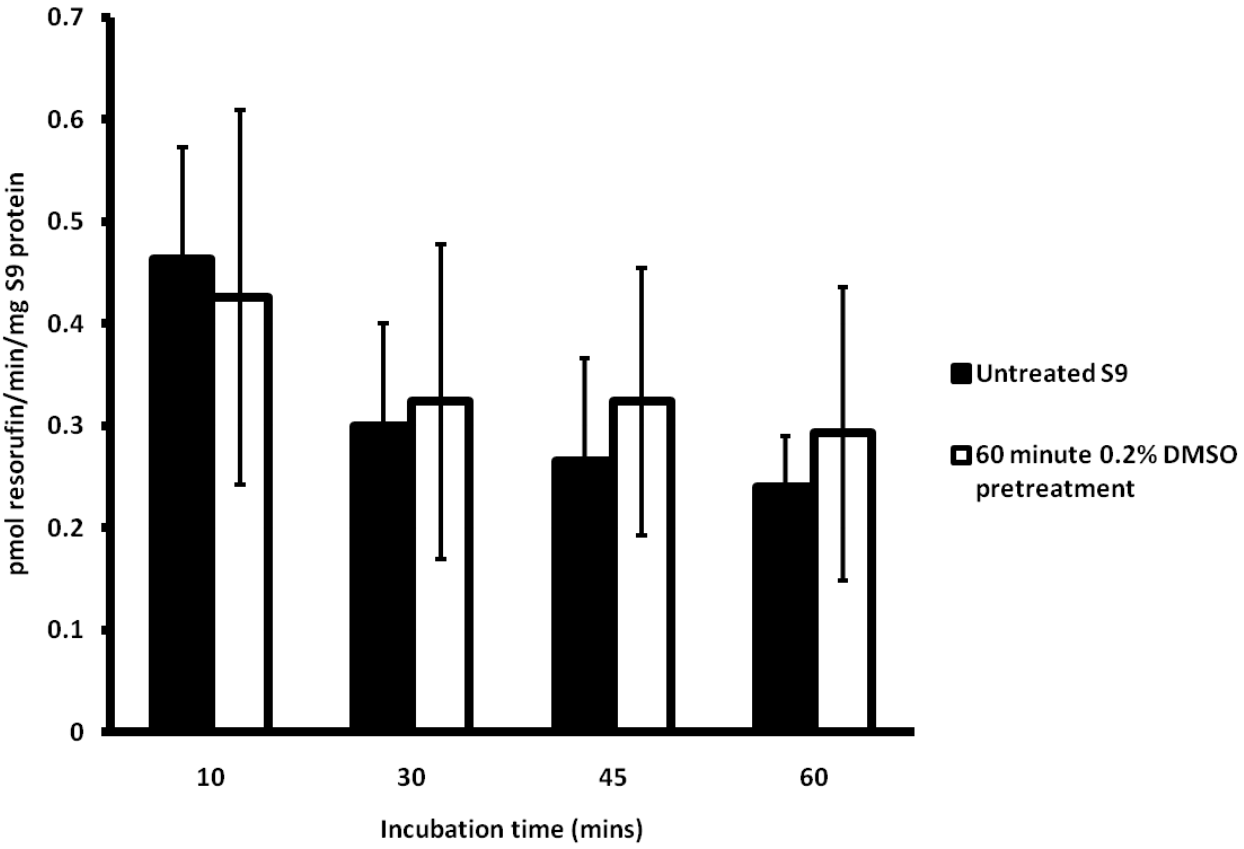


Figure 3.7: Rates of resorufin production (after 7-ethoxyresorufin incubation at various time points) in S9 fractions (un-supplemented with phase II cofactors) pre-treated with 0.2% DMSO for 60 minutes or control fractions unexposed to solvent. Rates are adjusted for S9 protein content ± SEM for S9 fractions prepared from 8 different trout livers.

ROS production by S9 fractions exposed to 7-ethoxyresorufin was also measured using the same fluorescence assay as mentioned previously. The mean fluorescence intensity measured as a function of untreated controls was consistently lower than as measured in hepatocytes, potentially due to a relatively low esterase activity in S9 fractions. Nevertheless, the assay was sufficiently sensitive to assess formation of ROS as evidenced by significantly elevated fluorescence in S9 fractions treated with hydrogen peroxide as a positive control (188.4 ± 20% of control). In 7-ethoxyresorufin treated S9 the level of ROS production relative to controls was found to be significantly elevated at time points of 7-ethoxyresorufin incubation beyond 20 minutes (Figure 3.8). This small but significant elevation is despite the apparent insensitivity of the assay using S9 and is still in accord with a potential role of ROS in the decline of CYP activity.

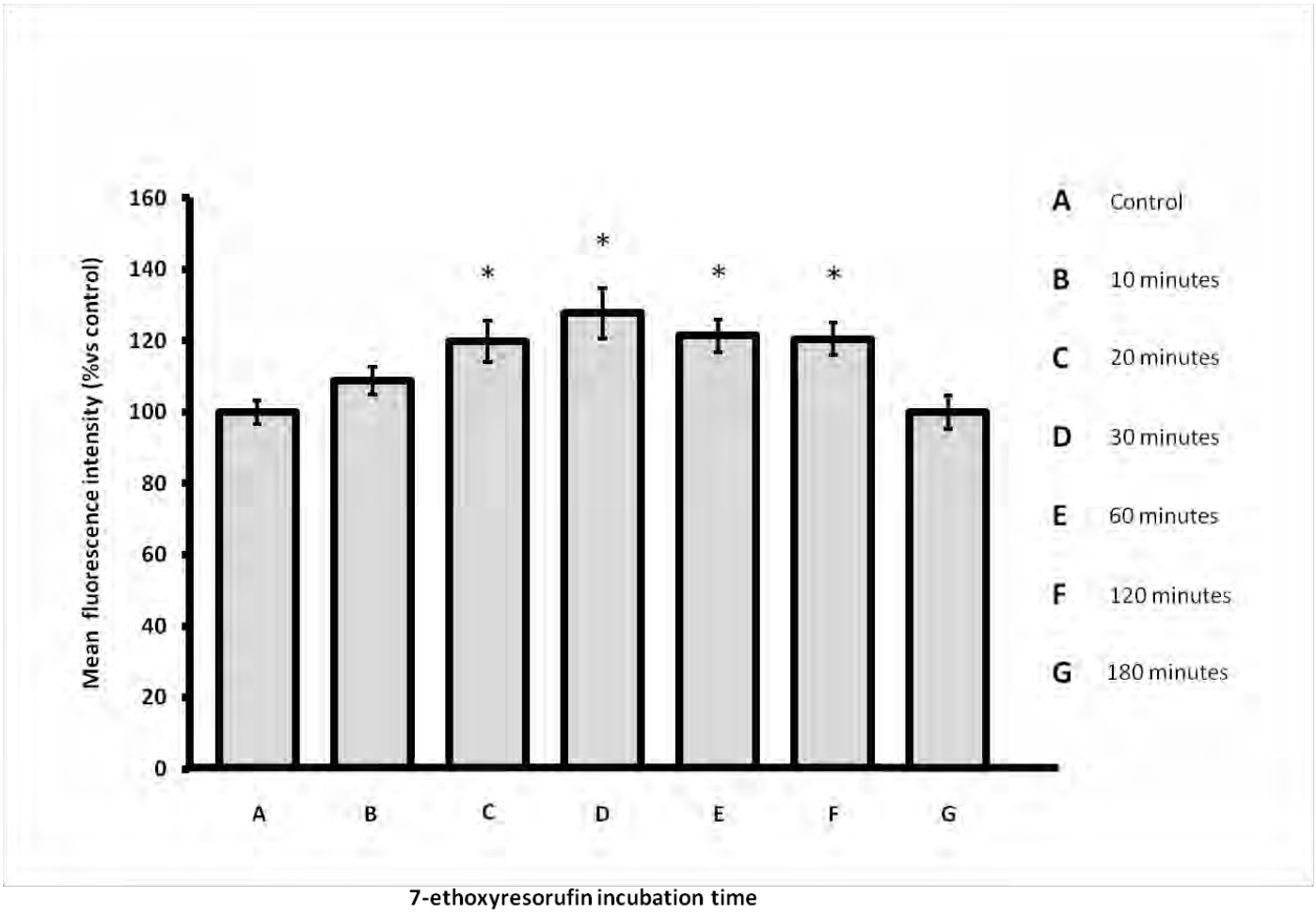


Figure 3.8: ROS levels measured in trout liver S9 fractions, following incubation with 7-ethoxyresorufin over a range of time points (10–180 minutes). Data are displayed as average fluorescence intensity as a percentage of that of an untreated control ± SEM for S9 fractions from 8 different trout livers. $^{*}p < 0.05$ (significantly different from control), Mann-Whitney *U* test.

As expected, no conjugative activity was detected in S9 fractions not supplemented with phase II cofactors, seen by a negligible difference in resorufin production with and without β-glucuronidase/aryl sulfatase treatment (Figure 3.9). When phase II cofactors were added to liver S9 fractions, an increase in EROD rate was detected. At the same 10 minute time point, average EROD rate in fractions supplemented with cofactors (0.77 pmol/min/mg S9 protein) was 1.65 times greater than in cofactor free fractions (0.46 pmol/min/mg S9 protein) (Figures 3.1 and 3.6). However, even in the presence of phase II cofactors, the EROD rate in S9 was almost ten-fold lower than in freshly isolated primary cells in suspension (c.f. S9 protein, Figure 3.1).

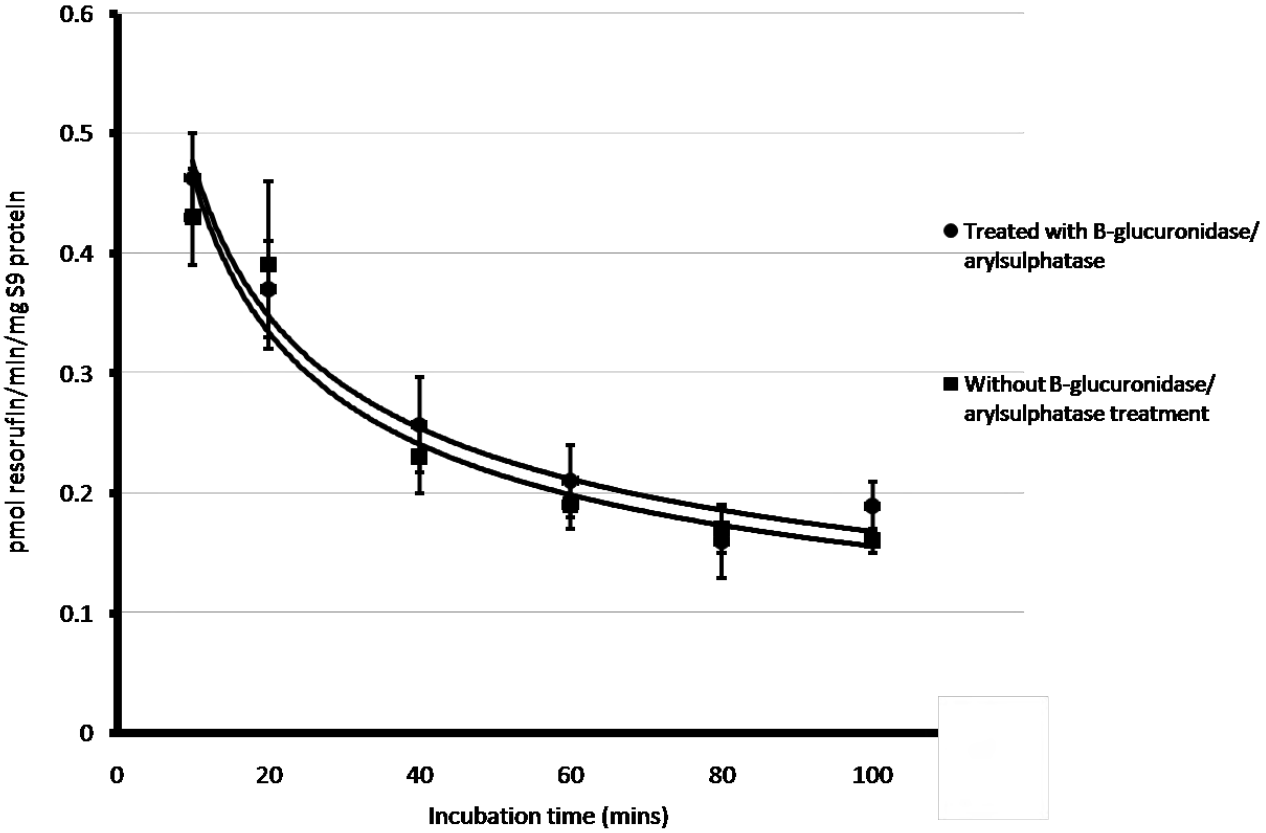


Figure 3.9: EROD activity of liver S9 fractions in the absence of phase II cofactors. Fractions were treated at each time point with either a β-glucuronidase/arylsulfatase mixture or a sodium acetate buffer control. Average rate of resorufin production in S9 fractions from the livers of 8 different fish ± SEM.

3.2.4 Comparative ibuprofen metabolism by *in vitro* preparations

LC-MS analyses of ibuprofen treated hepatocytes in monolayer culture (24 hours post isolation) and liver S9 samples revealed the presence of both ibuprofen and ibuprofen metabolites, verifying the metabolic activity of these *in vitro* systems towards this pharmaceutical. In blank samples of spiked buffer alone, only the parent compound was recovered, confirming that there was no non-enzymatic production of metabolites. Fragment ions of parent ibuprofen were detected by an MS-MS transition of *m/z* 205 → 161 and hydroxy-ibuprofen metabolite fragments were detected by an MS-MS transition of *m/z* 221 → 177).

Following exposure of primary hepatocytes 24 hours post isolation, to 10 μM ibuprofen, two hydroxy-ibuprofen isomers (retention times 5.31 and 5.95 minutes) were detected in samples. Using qualitative analysis, of their relative abundance, there was a time dependent increase in the peak areas of the hydroxy-metabolites (Figure 3.10 A2 compared to B2) and a concurrent decrease in the peak area of the parent ion (Figure 3.10 A1 compared to B1) during a 180 minute incubation (Figure 3.10).

The parent ibuprofen ion (*m/z* 205) and a single hydroxy-metabolite (*m/z* 221) were also detected in liver S9 fractions exposed to ibuprofen after 10 minutes of incubation (Figure 3.11). Using the mass fragmentation pattern produced, this hydroxy-metabolite was confirmed to be the 1-hydroxy-ibuprofen ion based on the fragments detected (m/z 69, 71, 105, 107, 134, 135, 147, 149, 159, 177) (Figure 3.12). No metabolites other than hydroxy-metabolites were detected.

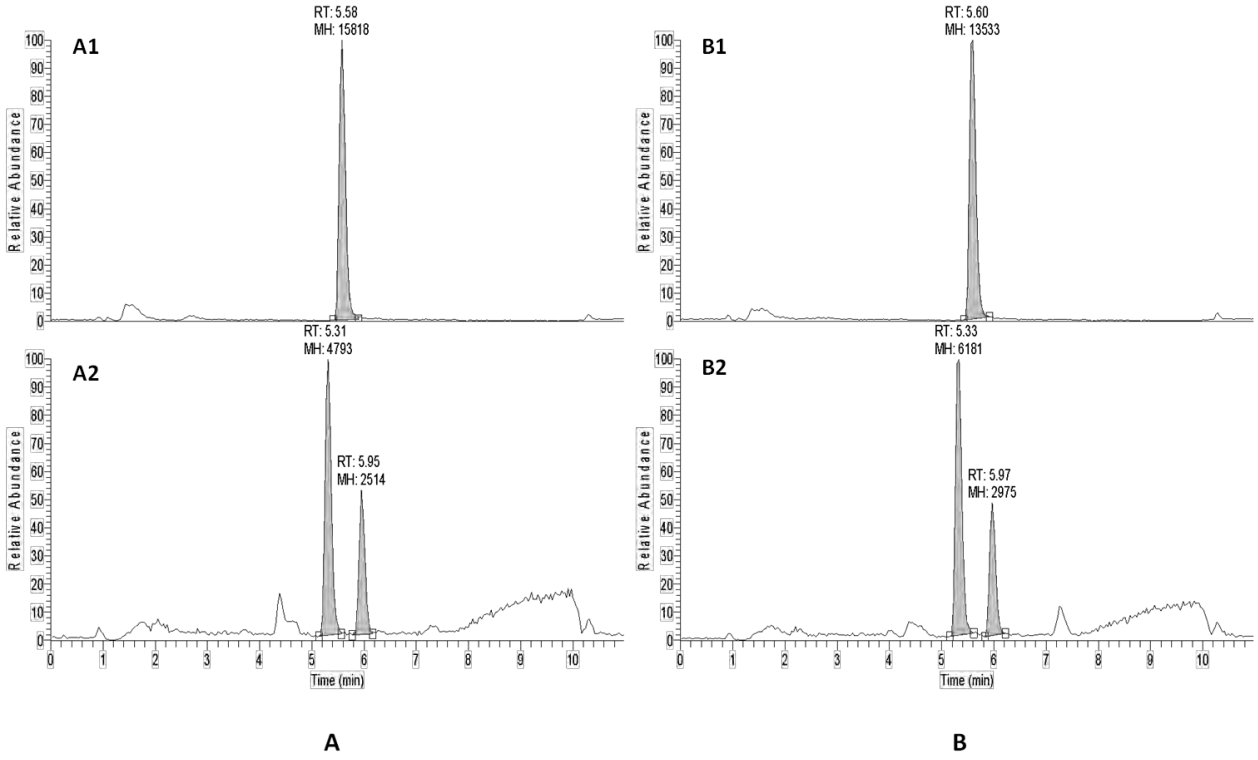


Figure 3.10: The metabolism of ibuprofen by primary cultured hepatocytes (24 hours post isolation) assessed by LC-MS. Cells were exposed to ibuprofen (10 µM) for 5 minutes (A) and 180 minutes (B). The upper panels (A1 and B1) show the identification of the ibuprofen parent ion (*m/z* 205) and the lower panels (A2 and B2) show hydroxy-ibuprofen ions (*m/z* 221). Qualitative estimates show a relative decrease in the amount of parent and an increase in the amount of both metabolites identified over the time course of incubation. This is indicated by the changes in MH values, which is a relative quantification based on the integrated peak areas. RT represents the retention time of parent or metabolites on the column.

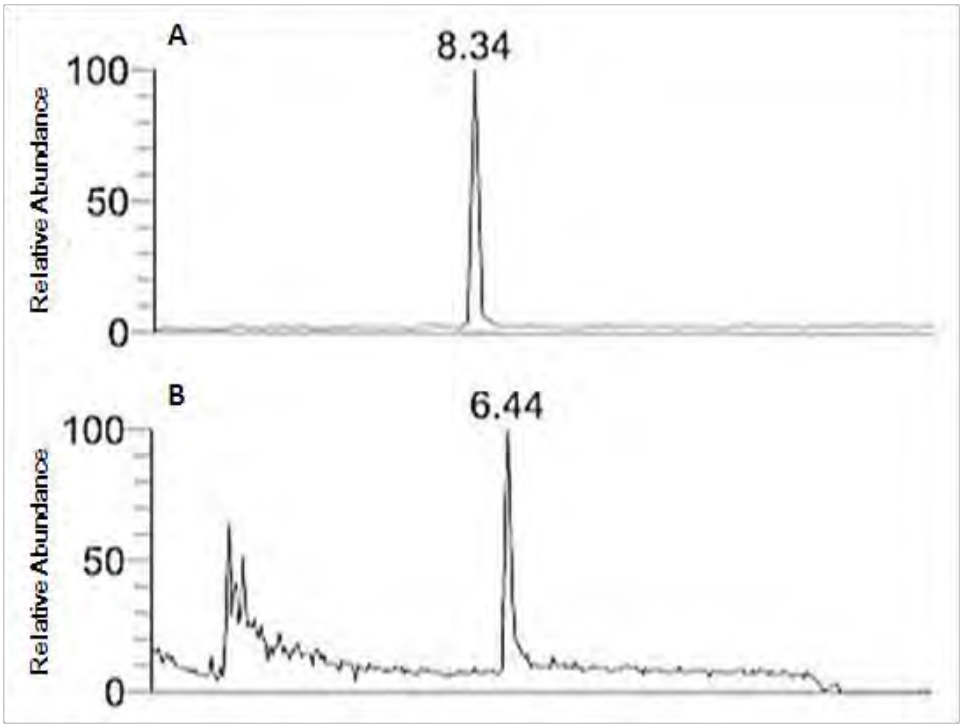


Figure 3.11: The metabolism of ibuprofen by hepatic S9 fractions assessed by LC-MS. S9 fractions were exposed to ibuprofen (10 μM) for 10, 30 and 60 minutes. Panel A shows the identification of ibuprofen parent (retention time = 8.34 minutes) and Panel B shows the single hydroxy-ibuprofen metabolite detected in S9 fractions (retention time = 6.44) after 60 minutes of incubation.

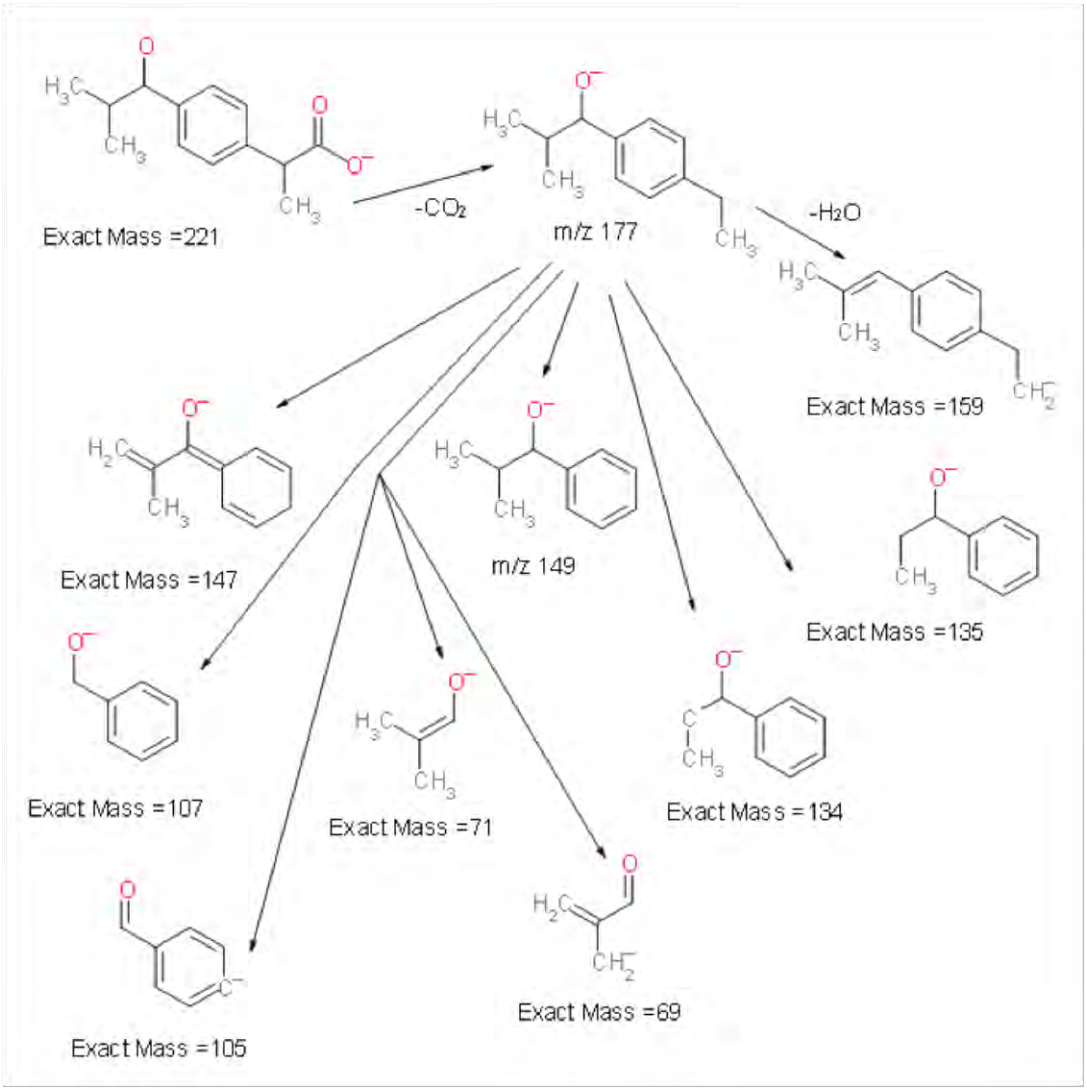


Figure 3.12: Mass fragmentation pattern of the sole hydroxy-metabolite detected in trout liver S9 fractions. The mass-mass fragmentation pattern confirms that the hydroxy-ibuprofen ion detected was the 1-hydroxy-ibuprofen derivative.

3.2.5 Assessment of CYP involvement in ibuprofen metabolism by trout liver S9 fractions

Assessment of the metabolism of ^{14}C ibuprofen in trout liver S9 fractions was performed to evaluate CYP involvement using specific inhibitors (see Section 2.14). Liquid scintillation counting of LC eluant fractions derived from incubations of ^{14}C ibuprofen with S9, both in the presence and absence of CYP inhibitors was conducted. In the LC fractions collected, considerable levels of radioactivity were detected relating to fractions in which ibuprofen (12–13 minutes) and hydroxy-ibuprofen (10–11 minutes) standards co-eluted. Hydroxy-ibuprofen derivatives appeared to be present in all samples analysed, however at considerably greater amounts in uninhibited trout S9 fractions, compared to incubations with CYP inhibitors (Figure 3.13). The largest amount of hydroxylated metabolite was detected in uninhibited rat S9 fractions and this was expected as enzyme activities in mammalian species are known to be considerably greater than in fish. The amount of ^{14}C recovered in the trout S9 fraction pre-treated with SKF525A was smaller than that recovered in fractions treated with other CYP inhibitors. This suggests that SKF525A provided the greatest level of inhibition of ibuprofen metabolism in comparison to the other CYP inhibitors used.

In the LC fraction corresponding to parent ibuprofen (12–13 minutes), a greater recovery of ^{14}C was also seen in trout S9 fractions pre-treated with CYP inhibitors, therefore in these samples, a larger amount of ibuprofen remained un-metabolised (Figure 3.14). This amount of un-metabolised ibuprofen was greater in trout S9 fractions pre-treated with SKF525A, than when pre-treated with other CYP inhibitors, again suggesting that SKF525A provided the greatest level of inhibition of ibuprofen metabolism.

The recovery of ^{14}C in the polar 3–4 minute fraction (Figure 3.13) suggests the possible detection of glucuronic acid conjugates in incubates with S9. Again, as expected, the largest amount of ^{14}C was detected in rat S9 fractions, and it is known that ibuprofen and its

metabolites are heavily glucuronidated in mammalian species. Interestingly, the potential production of glucuronic acid conjugates in trout liver S9 fractions appeared to be greater in the presence of CYP inhibitors, suggesting that glucuronidation may occur as an alternative metabolic pathway for ibuprofen in trout liver when CYP is inhibited. Although the identity of the glucuronides and hydroxy-metabolites in the LC eluates are yet to be confirmed by mass spectrometry (studies on-going), support of their identity comes from NMR spectroscopy analyses of S9 incubates as follows.

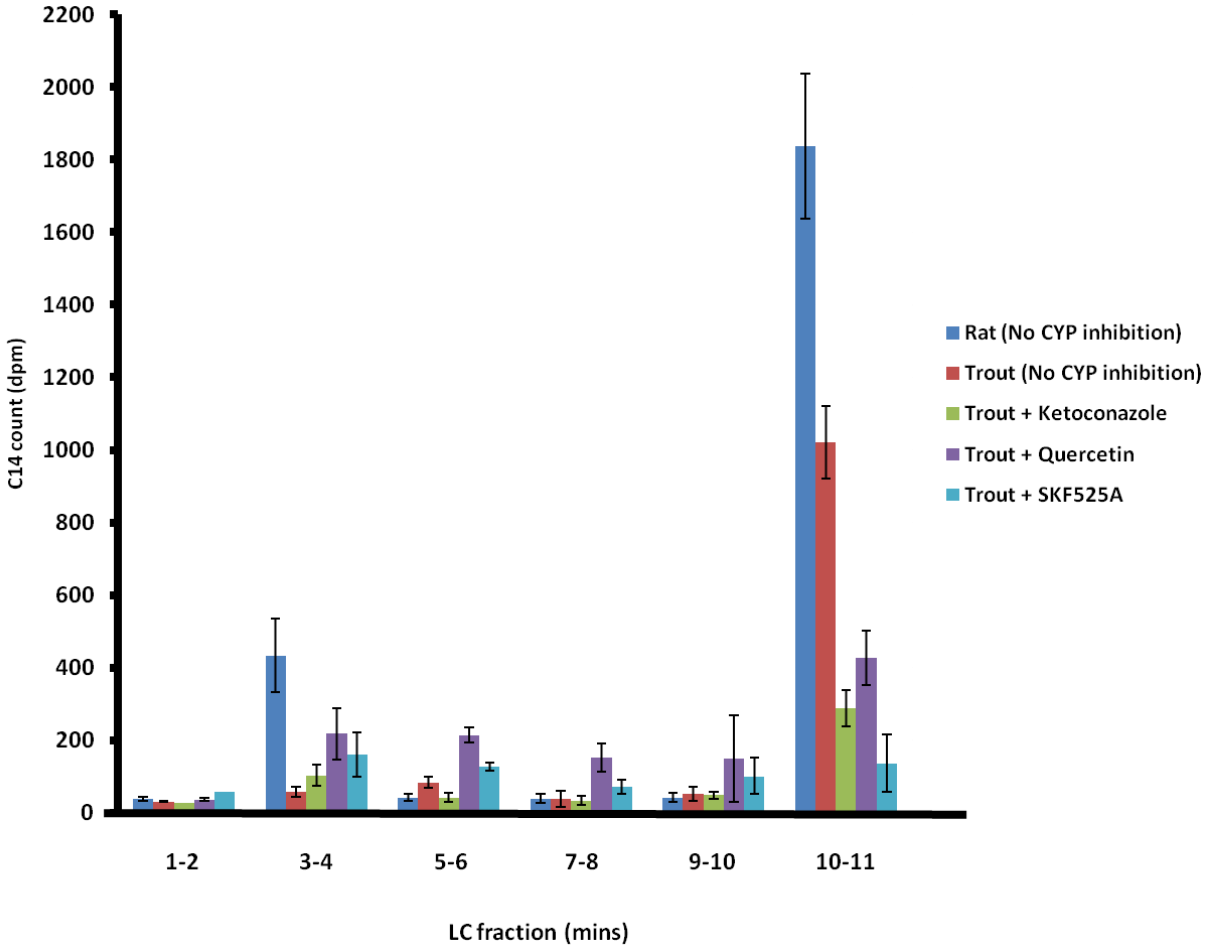


Figure 3.13: Amount of ^{14}C radiolabel (dpm) measured in liquid chromatography fractions corresponding to ibuprofen-metabolites. Recovery of metabolites was detected in fractions collected between 1–11 minutes. Hydroxy-ibuprofen derivatives (10–11 minutes) appeared to be present in all samples, however at considerably greater amounts in uninhibited rat and trout S9 fractions. Samples pre-treated with CYP inhibitors produced a much smaller amount of hydroxy-ibuprofen. The recovery of ^{14}C in the 3–4 minute fraction suggests the production of glucuronic acid conjugates in S9 fractions. Again the largest amount of ^{14}C was detected in rat S9 fractions. Interestingly, the production of potential glucuronic acid conjugates in trout liver S9 fractions appeared to be greater in the presence of CYP inhibitors, suggesting that glucuronidation occurs as an alternative metabolic pathway for ibuprofen in trout when CYP is inhibited. n = 8 livers (trout); 3 livers (rat). Parent compound (not shown) elutes at 12–13 minutes (see Figure 3.14).

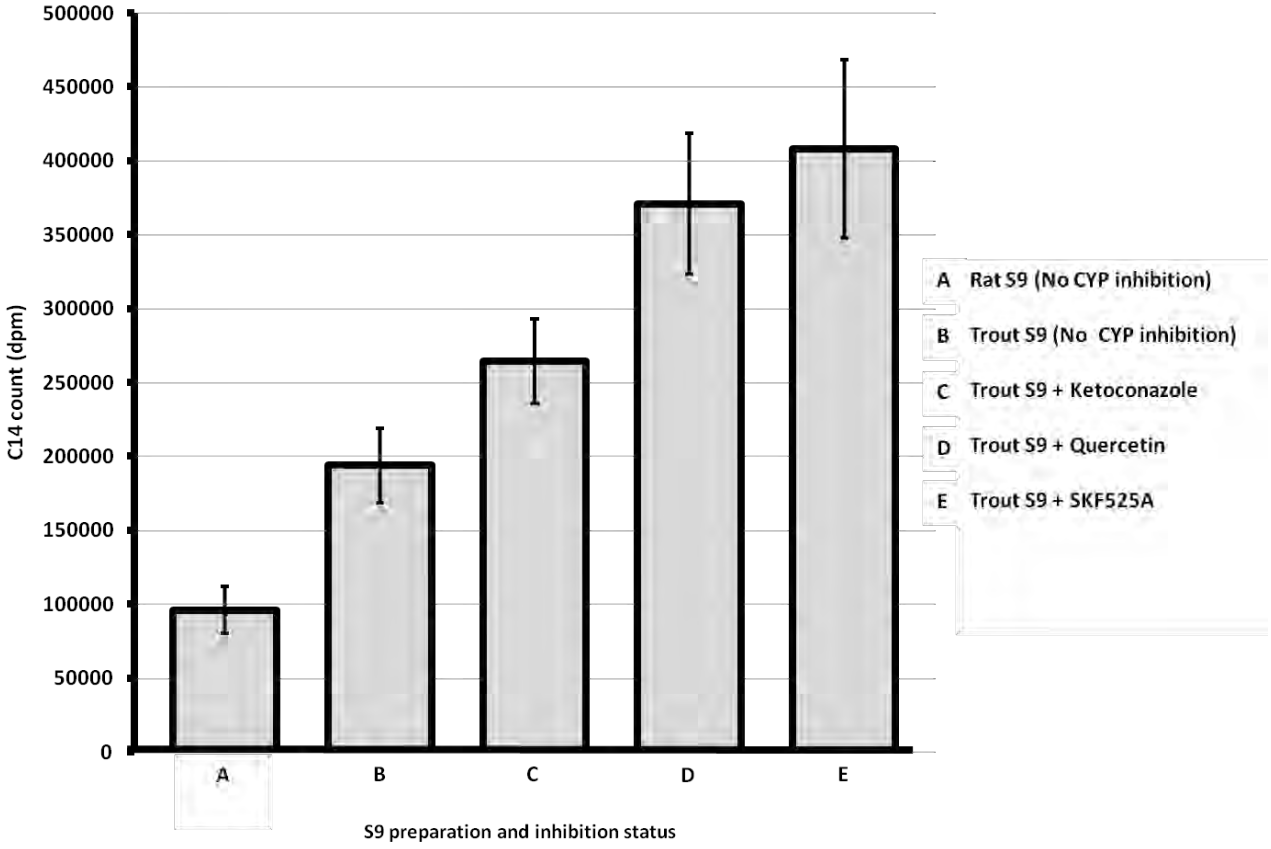


Figure 3.14: Amount of ^{14}C radiolabel (dpm) measured in liquid chromatography fractions corresponding to ibuprofen. Comparisons of ^{14}C recovery in liquid chromatography fractions collected at 12–13 minutes (corresponding to ibuprofen) correlate to the amount of parent compound remaining in S9 incubations. The largest amount of ibuprofen remained un-metabolised in trout S9 fractions pre-treated with SKF525A, than when pre-treated with other CYP inhibitors. The smallest proportion of un-metabolised ibuprofen was measured in rat liver S9 fractions suggesting that ibuprofen metabolism proceeds at a greater rate in rat liver. n = 8 livers (trout); 3 livers (rat).

NMR spectroscopy analyses of S9 incubates was also conducted. The proton NMR spectrum of a 1-hydroxy-ibuprofen standard solution was acquired in order to obtain its resonances (Figure 3.15A). This was compared to the spectra of S9 fractions exposed to ibuprofen. The spectrum of 1-hydroxy-ibuprofen consisted of seven signals, five of which were not visible in S9 samples due to the strong presence of background peaks from other components. The remaining two signals in acquired spectra however overlapped with those present in trout liver S9 exposed to ibuprofen, confirming the presence of 1-hydroxy-ibuprofen. The first of these overlapping doublet signals was in the aliphatic region at 0.73 ppm, corresponding to protons at position 1 (Figure 3.15C), with the second present in the aromatic part of the spectrum at 7.33 ppm, corresponding to protons at positions 5/6 (Figure 3.15D).

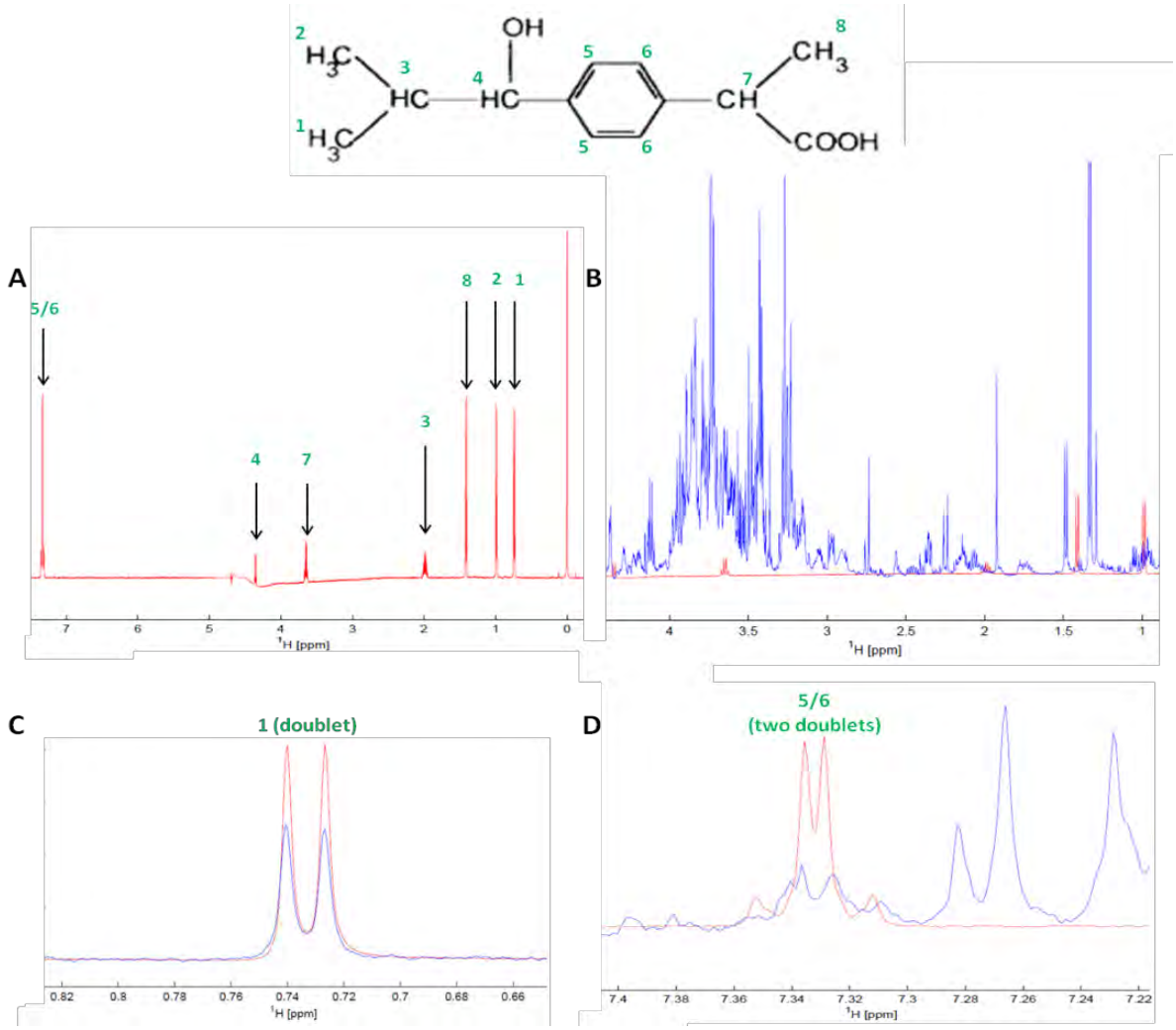


Figure 3.15: ^{1}H NMR spectra of 1-hydroxy-ibuprofen and trout liver S9 fractions exposed to ibuprofen. The spectrum of 1-hydroxy-ibuprofen contains seven signals. The signals correspond to the protons labelled on the structure above. The singlet at 0 ppm frequency relates to the TMSP internal reference (A). Spectra of the trout liver S9 fraction (blue lines) exposed to ibuprofen were overlaid and compared to the 1-hydroxy-ibuprofen standard (red line). Five signals of 1-hydroxy-ibuprofen in the main part of the spectrum were not clearly visible in the S9 sample due to the high background from other components (B). The doublet at 0.73 ppm (protons at position 1) was visible in the S9 fraction and the signal obtained overlapped that of the 1-hydroxy-ibuprofen standard (C). An overlapping doublet at 7.33 ppm in the aromatic region of the spectrum (protons at positions 5/6) was also visible in the trout S9 fraction when compared to the 1-hydroxy-ibuprofen standard (D).

1D proton NMR spectra of trout S9 fractions pre-treated with the four selected CYP inhibitors (fluconazole, ketoconazole, quercetin and SKF525A) were acquired and scaled to the intensity of the internal reference (TMSP at 0 ppm) for quantitative comparisons (Figure 3.16A). In these samples, none of the peaks attributed to 1-hydroxy-ibuprofen that were present in the trout S9 fractions exposed to ibuprofen without CYP inhibitors (0.74 ppm and 7.33 ppm), were detected (Figure 3.16). This indicates the absence of the 1-hydroxy-ibuprofen metabolite (or at least that very low concentrations were present), providing evidence of effective inhibition by the selected CYP inhibitors (see Figure 3.16). The 1-hydroxy-ibuprofen derivative was also absent in uninhibited rat S9 fractions as reported previously in the literature (Yang et al., 2000a).

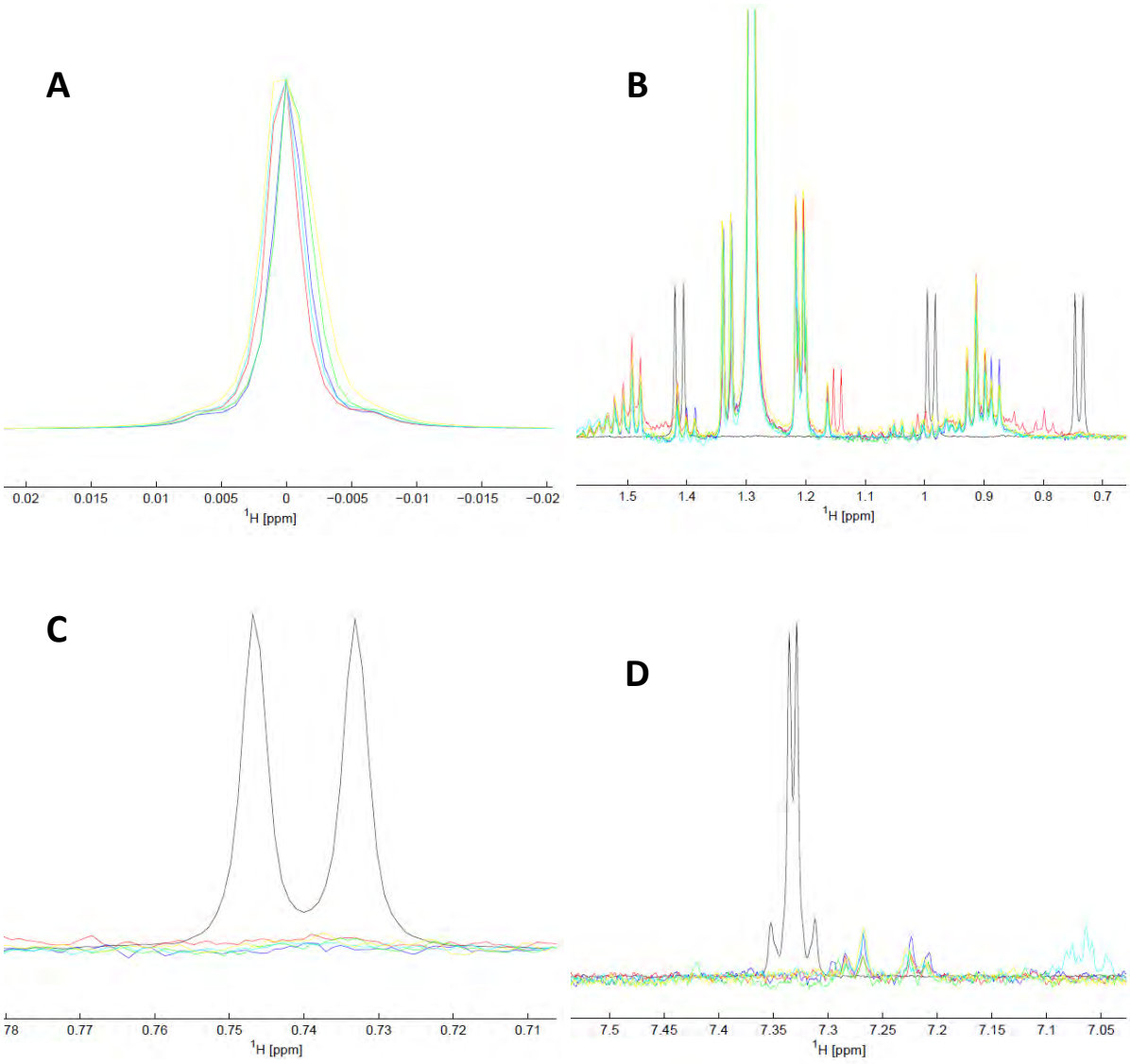


Figure 3.16: ^{1}H NMR spectra of trout S9 fractions pre-treated with four selected CYP inhibitors and uninhibited rat S9 fractions exposed to ibuprofen. Colours indicate spectra of fractions with different inhibitor treatments. Key- Fluconazole: cyan; Ketoconazole: yellow; Quercetin: green; SKF52A: blue; Rat uninhibited S9: red; 1-hydroxy-ibuprofen standard: black. Spectra were scaled to the intensity of the TMSP internal reference in order to allow quantitative comparisons between samples to be made (A). Spectra of all fractions were overlaid and compared to the 1-hydroxy-ibuprofen standard (B). Neither the doublet at 0.73 ppm - protons at position 1 (see Figure 3.15) - (C), nor the doublet at 7.33 ppm - protons at positions 5/6 (see Figure 3.15) - (D) relating to 1-hydroxy-ibuprofen were present in trout liver S9 fractions treated with inhibitors and uninhibited rat liver S9 fractions exposed to ibuprofen.

Comparisons of the heights of the doublet relating to parent ibuprofen (0.88 ppm - from the equivalent protons at positions 1 and 2. See Figure 3.17) give an indication of signal intensity, thus can be used as a measure of compound concentration. The S9 sample pre-treated with SKF525A produced the highest ibuprofen parent signal, indicating the largest remaining parent concentration and therefore, the greatest level of inhibition of ibuprofen metabolism in comparison to other CYP inhibitors used (Figure 3.17A). The same conclusion was reached in the radiolabel study.

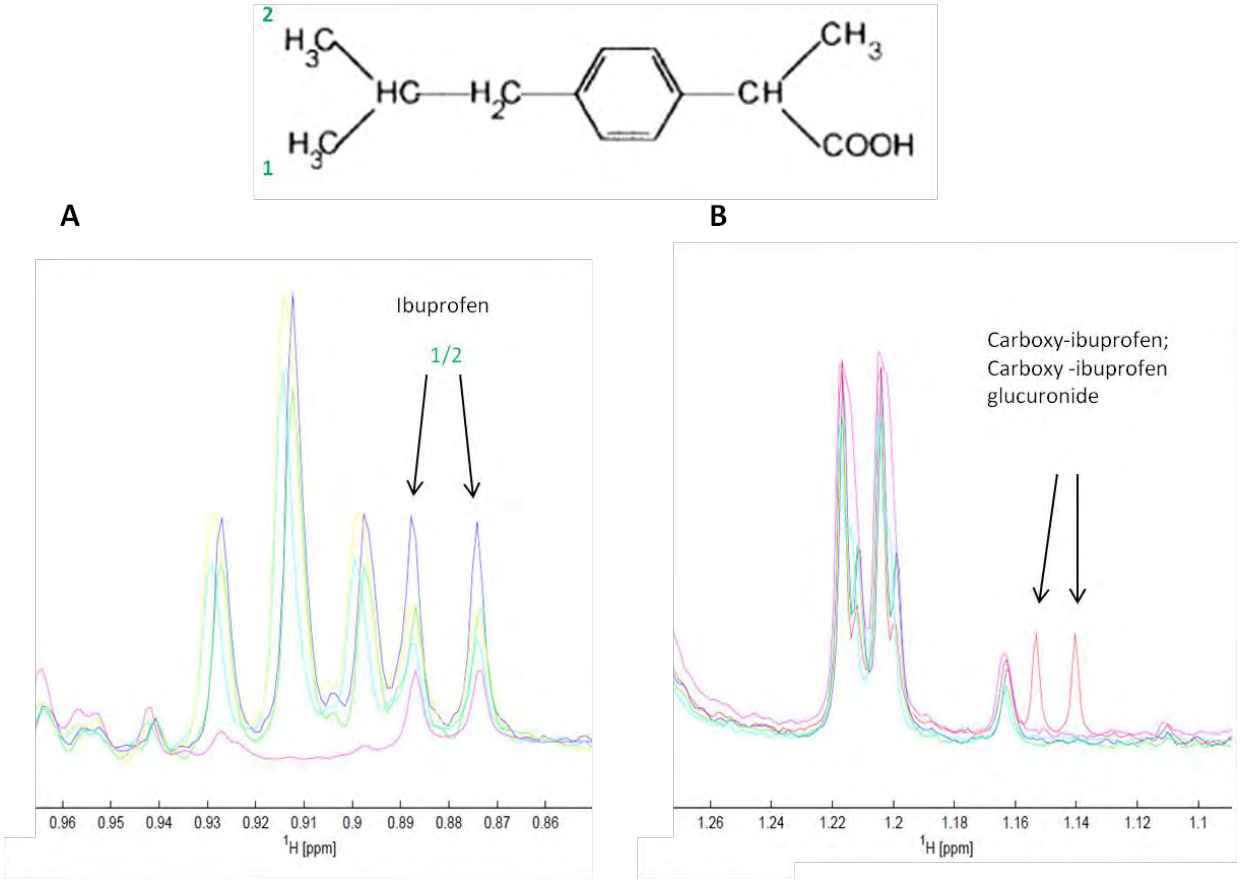


Figure 3.17: ^{1}H NMR spectra of trout liver S9 fractions pre-treated with CYP inhibitors and a rat liver S9 fraction (without inhibition), exposed to ibuprofen. Colours indicate spectra of fractions with different inhibitor treatments. Key- Fluconazole: cyan; Ketoconazole: yellow; Quercetin: green; SKF52A: blue; Trout S9 without CYP inhibition: magenta; Rat S9 without CYP inhibition: red; 1-hydroxy-ibuprofen standard: black. The signals correspond to the protons labelled on the structure above. Comparisons of the intensity of the doublet relating to ibuprofen parent (0.88 ppm - from the equivalent protons at positions 1 and 2), show that a larger proportion of ibuprofen remained un-metabolised when pre-treated with SKF525A, than when pre-treated with other CYP inhibitors (A). An additional doublet (1.145 ppm) present in rat liver S9 fractions (not subjected to CYP inhibition), was not present in trout S9 fractions (B). This peak corresponds to carboxy-ibuprofen and carboxy-ibuprofen glucuronide, confirming that one or both of these metabolites are produced in rat liver S9 fractions (as identified in published studies).

The metabolic profile of ibuprofen in rat liver differed from that of trout. As previously described in rat urine (Yang et al., 2000a) and in the present study, peaks relating to the presence of 1-hydroxy-ibuprofen were not present in rat S9 fractions. Additional signals not present in trout fractions were identified (Figure 3.17B - red line). The doublet at 1.145 ppm corresponds to carboxy-ibuprofen and carboxy-ibuprofen glucuronide, indicating that as seen *in vivo* one or both of these metabolites are produced in rat S9. Glucuronic acid conjugates of the parent compound were also present in rat S9 fractions exposed to ibuprofen, confirmed by the presence of doublets at 1.54, 1.55, 5.62 and 5.64 ppm (Figure 3.18).

Interestingly, in all trout S9 fractions pre-treated with CYP inhibitors prior to ibuprofen exposure, peaks relating to ibuprofen-glucuronic acid conjugates were identified (Figure 3.18). Glucuronides of both ibuprofen stereoisomers were detected with peaks at 1.51 ppm ((R)-ibuprofen glucuronide) and 1.52 ppm ((S)-ibuprofen glucuronide) and two doublets at 5.62 ppm and 5.64 ppm. Peaks of glucuronic acid conjugates were only present at trace levels in control trout liver S9 fractions (without inhibitor pre-treatment) (Figure 3.18). These results suggest that in trout, when CYP metabolic pathways are inhibited, 1-hydroxy-ibuprofen is not produced and parent ibuprofen is directly conjugated with glucuronic acid as an alternative pathway of biotransformation. The same conclusions were reached in the radiolabel study, although confirmation of the identity of this proposed ester glucuronide by mass spectrometry is on-going. A summary of the species differences of ibuprofen metabolism is shown in Table 3.2.

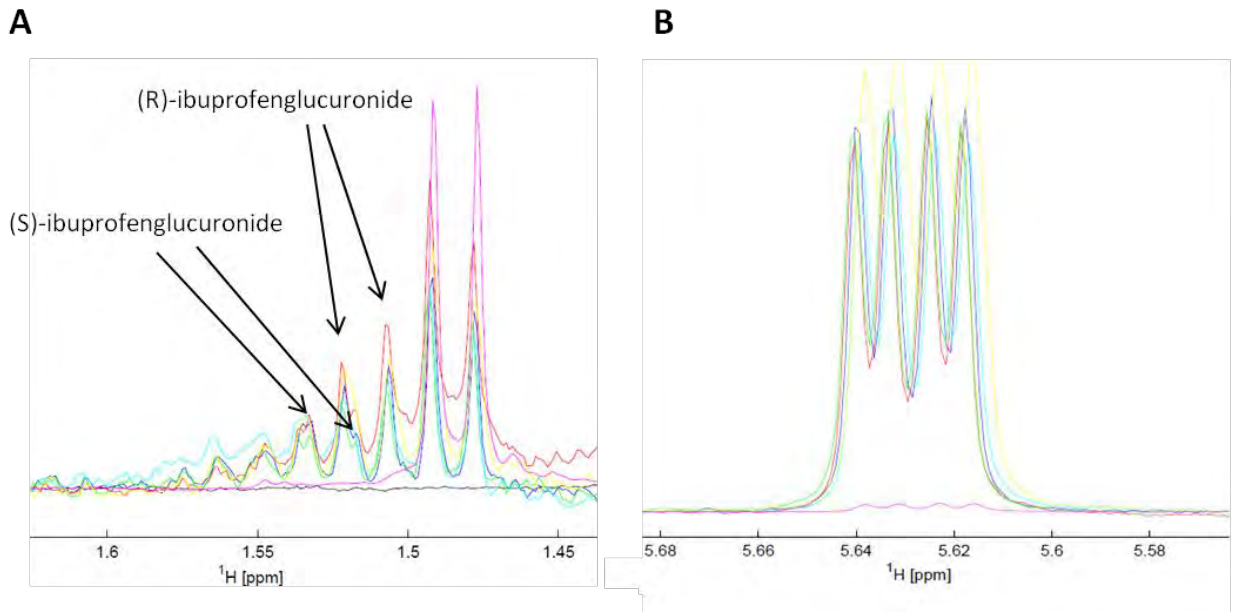


Figure 3.18: 1H NMR spectra of trout liver S9 fractions pre-treated with CYP inhibitors, control trout liver S9 (without CYP inhibtion) and rat liver S9 fraction (without inhibtion) following ibuprofen exposure. Colours indicate spectra of fractions with different inhibitor treatments. Key- Fluconazole: cyan; Ketoconazole: yellow; Quercetin: green; SKF52A: blue; Trout S9 without CYP inhibition: magenta; Rat S9 without CYP inhibition: red; 1-hydroxy-ibuprofen standard: black. Spectra of all fractions were overlaid and compared to a 1-hydroxy-ibuprofen standard. The production of ibuprofen glucuronides in trout liver S9 fractions pre-treated with CYP inhibitors was confirmed by the presence of a doublet corresponding to (R)-ibuprofen glucuronide (1.51 ppm) and a doublet corresponding to (S)-ibuprofen glucuronide (1.52 ppm) (A). The presence of two doublets at 5.62 and 5.64 ppm substantiates the presence of ibuprofen glucuronic acid conjugates in trout liver S9 fractions pre-treated with CYP inhibtors (B). These peaks were present only at trace levels in trout S9 fractions without pre-treatment with inhibitors (magenta line), suggesting that the parent compound is glucuronidated as an alternative pathway to CYP metabolism. These ibuprofen glucuronides are also produced in rat S9 fractions exposed to ibuprofen without CYP inhibitor pre-treatment.

X Major human metabolite X Minor human metabolite X Detected at trace levels

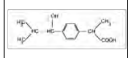
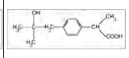
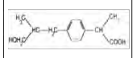
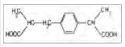
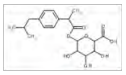
	System	1-OH 	2-OH 	3-OH 	Unknown Hydroxy	Carboxy-Ibuprofen 	Glucuronide 
Human	*In vivo*	X	X	X		X	X
Baboon	*In vivo*	X	X			X	X
Rat	*In vivo*		X			X	X
Rainbow Trout	*In vitro*	X			X		X
Zebrafish	*In vivo*				X X		

Table 3.2: Interspecies differences in ibuprofen metabolism. The major human metabolites of ibuprofen are the 2-hydroxy- and carboxy-ibuprofen derivatives as well as glucuronic acid conjugates of both the parent compound and hydroxylated metabolites. The 1-hydroxy-ibuprofen derivative is a minor human metabolite not detected in rat *in vivo* but which was identified in rainbow trout liver S9 fractions in this study. An additional hydroxy-ibuprofen derivative was identified in isolated trout hepatocytes, however its identity is yet to be confirmed. Glucuronic acid conjugation of parent and ibuprofen metabolites is a major metabolic pathway in mammalian species with respect to ibuprofen. This route seems less important in fish species as ibuprofen was largely eliminated as hydroxy-metabolites in zebrafish larvae (two hydroxylated isomers were detected). In the present study, only trace levels of ibuprofen-glucuronides were detected in trout liver S9 fractions, however, in the presence of CYP inhibitors, glucuronidation of the parent compound was enhanced (Mills et al., 1973, Kepp et al., 1997, Yang et al., 2000a, Jones et al., 2012).

3.2.6 Confirmation of glucuronic acid conjugate formation in *in vitro* preparations

As glucuronic acid conjugates of ibuprofen and hydroxy-ibuprofen are major metabolites detected in mammalian species, the lack of their identification in hepatocytes and the trace levels detected in subsequent S9 assays was unexpected. To confirm that these *in vitro* trout preparations were indeed able to produce glucuronides under un-inhibited CYP conditions, an assay using the fluorescent substrate 4-methylumbelliferone (4-MU) which has been shown to be glucuronidated by UGTs (without the requirement of prior oxidation) in a number of mammalian species (Kubota et al., 2007, Wang et al., 2011a), was conducted. 4-MU fluorescence can be measured at λ_{ex} 353 nm; λ_{em} 460 nm but when the molecule is glucuronidated, fluorescence intensity under these conditions decreases. Cleavage of the glucuronide can restore the fluorescence of the molecule.

In the presence of hepatocytes (freshly isolated in suspension and in monolayer culture) exposed to 4-MU, fluorescence declined during incubation and was significantly lower at both time points in comparison to the control sample (representing total 4-MU fluorescence). The fluorescence intensities measured at the different time points in all preparations (30 minutes to 60 minutes) were not significantly different from each other. However, the decline in fluorescence through incubation with cells was partially recovered by inclusion of the deconjugating enzyme, β-glucuronidase. This supports the occurrence of 4-MU glucuronidation in this system (Figure 3.19).

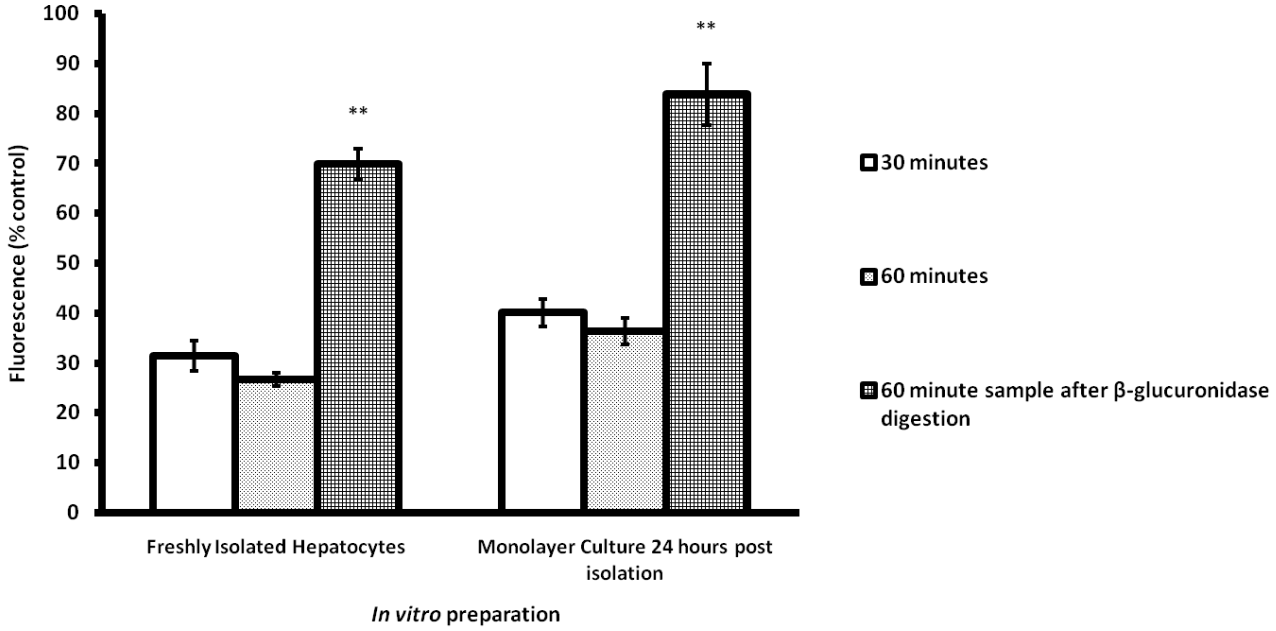


Figure 3.19: Glucuronidation of 4-MU by primary hepatocytes freshly isolated in suspension and in monolayer culture 24 hours post isolation. Data are displayed as mean fluorescence intensity as a percentage of the fluorescence of total 4-MU added to cells ± SEM; $^{**}p < 0.01$ (significantly different from 60 minute sample without β-glucuronidase treatment) (n = 3 fish).

When S9 fractions were supplemented with UDPGA and exposed to 4-MU, there was a significant decline in fluorescence intensity at all time points; also with an increase in fluorescence measurements recorded following β-glucuronidase treatment and this was significantly different from the intensities measured in undigested fractions (except at the 10 minute time point) (Figure 3.20). In the absence of UDPGA, there was no significant difference in the percentage of 4-MU fluorescence measured in S9 fractions before and after incubation, confirming the requirement of the cofactor for the activation of glucuronidation pathways.

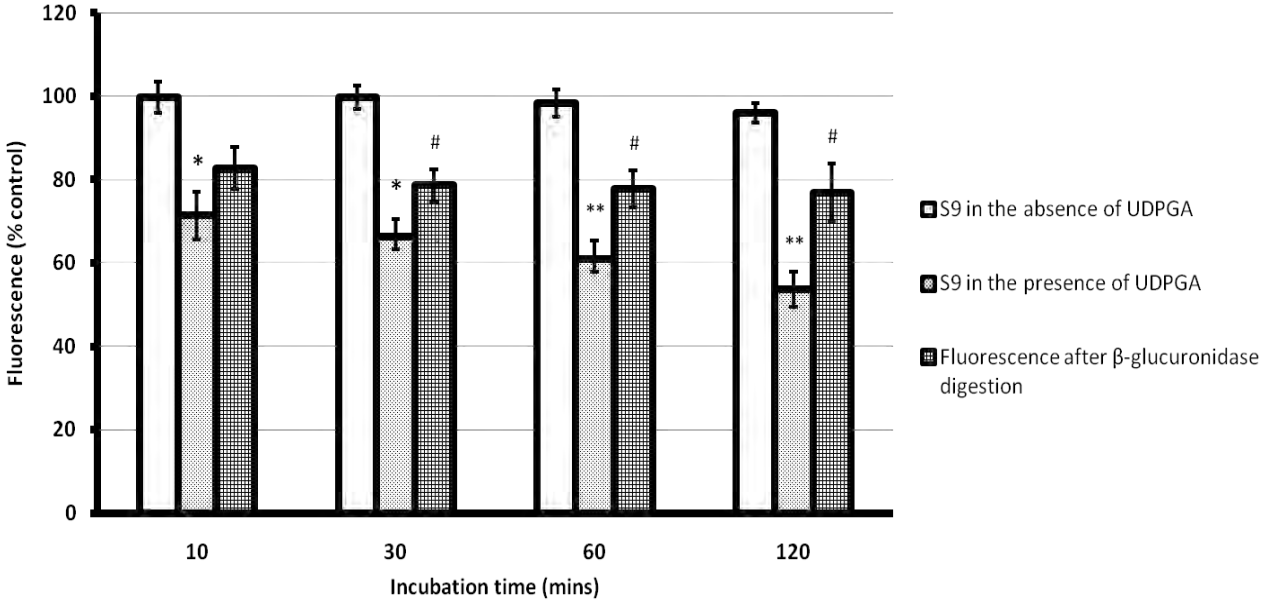


Figure 3.20: Glucuronidation of 4-MU by S9 fractions under different conditions of UDPGA supplementation. Data are displayed as mean fluorescence intensity as a percentage of the fluorescence of total 4-MU added to S9 fractions ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ (significantly different from control), $^{\#}p < 0.05$ (significantly different from S9 supplemented with UDPGA, with no β-glucuronidase treatment) Independent samples *t*-test (n = 5 fish).

3.3 Discussion

Current strategies in industry regarding the use of *in vitro* systems in ERA include the use of subcellular fractions to estimate the metabolism and potential bioaccumulation of chemicals in fish. Due to the economic and practical viability of the technique, the use of S9 fractions has widely been proposed for rapid assessments of bioaccumulation potential and screening prioritisation, a process that has become increasingly important since the introduction of EU REACH regulations in 2007. The principle considers that there is an inverse relationship between degree of metabolism and bioaccumulation potential. However, due to the fact that microsomal and S9 fractions lack a number of the physiological features important to metabolic processes, they do not represent the *in vivo* characteristics as accurately as cultured hepatocytes (Cross and Bayliss, 2000). These limitations, including the absence of cofactors for phase II enzymes, short-lived activity in incubations and the inherent assumption of cellular uptake of chemicals; could produce an inaccurate prediction of metabolic rates and altered metabolic profiles.

In this study, S9 fractions displayed a remarkably lower metabolic activity towards 7-ethoxyresorufin when compared to isolated hepatocytes in suspension and at different stages of monolayer culture. The EROD rates measured in S9 fractions obtained are comparable to those reported in a similar investigation of trout hepatic subcellular fractions (Han et al., 2009). The relatively low activity was observed even taking account of the statistically significant enhanced oxidative metabolic rate afforded by the presence of cofactors for phase II conjugation. As a result, S9 fractions would significantly under-predict the metabolic activity of intact cells and therefore overestimate bioaccumulation when compared to the use of primary cultured hepatocytes. This may well translate to an overestimation of *in vivo* bioaccumulation potential.

Whilst conjugative activity was detected in S9 fractions supplemented with phase II cofactors, both with 7-ethoxyresorufin and 4-MU, the requirement for this supplementation introduces another parameter that can result in changes in metabolic outcome. Altered concentrations and proportions of phase II cofactors in supplements may influence oxidative metabolic activity and the profile of metabolites produced. When supplemented with phase II cofactors, there was an apparent increase in EROD activity in S9 fractions. It is feasible that a lack of inclusion of conjugative cofactors in subcellular fractions, causes phase I product inhibition in the metabolic system. This is particularly likely in the case of glucuronidation since UGT is a microsomal enzyme physically linked to the CYP system. A previous study using rat liver microsomes, identified end-product inhibition as a mechanistic cause of the lower clearance determination obtained from microsomes compared with that of whole rat hepatocytes. Differences in clearance were attributed to the metabolite concentrations in the vicinity of the enzyme, which were higher in microsomes than in hepatocytes, increasing the likelihood of end-product inhibition in microsomes (Jones et al., 2005). This increased potential for product inhibition may also be an important feature of the S9 system, as it introduces another parameter through which persistence may be overestimated, as the absence of phase II cofactors, as occasionally used in early bioaccumulation assessments, appears to result in an underestimation of the metabolic potential of the system.

Whilst conjugative activity was detected in S9 fractions supplemented with phase II cofactors, the relative proportion of conjugation was significantly lower than that measured using cultured hepatocytes. This difference in phase II metabolic activity between systems may occur as this is experimentally controlled in subcellular fractions. It may be possible that altered concentrations and proportions of phase II cofactors in supplements may influence metabolic activity and the metabolites produced. However, an excess of known physiological

concentrations is used when subcellular fractions are supplemented with cofactors, to ensure maximal enzyme activity and in the case of UDPGA in this study, a 2 mM concentration was used as is common in industry (Johanning et al., 2012).

Despite having greater oxidative activities than the other cellular preparations investigated, the proportion of primary metabolites detected in a conjugated form was significantly lower in freshly isolated hepatocytes than in other cultures. However this does not necessarily indicate a similar rate of conjugation, since the proportion may be influenced by phase I product formation and potential deconjugation reactions. The use of whole cells may reflect *in vivo* phase II metabolism more accurately, supporting the hypothesis that S9 fractions may not be as suitable for use in metabolic assessment. The requirement of supplementing subcellular S9 fractions with cofactors of phase II enzymes increases the possibility of inter-laboratory variation at a level which may directly influence metabolic profiles. As a result, a degree of harmonisation is required for these types of study to confer uniformity to bioaccumulation predictions. This evidence for just one aspect examined here suggests that this could prove difficult to harmonise for more complex analyses. Indeed, recently concerns have been raised about the huge variability, both inter- and intra-laboratory, in results from studies of metabolic assessment using S9 fractions, due to differences in preparation methods and fraction supplementation (Owen, S.F., pers. comm.).

As seen in a similar study in rainbow trout, the conjugative activity of the *in vitro* systems investigated was relatively low in comparison to those seen in mammalian species; for example, in cultured trout hepatocytes, the measured glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene was significantly lower (97.2 pmol/min/mg protein), than that measured in rat hepatocytes (1400 pmol/min/mg protein (Nabb et al., 2006)). Cytosolic fractions derived from rainbow trout livers have also been shown to possess

sulfation activities towards the thyroid hormone triiodothyronine (T_3). The rate of sulfation measured, 14.0 pmol/min/mg (Finnson and Eales, 1998), was considerably lower than that measured in the cytosol of male Sprague-Dawley rat livers (530.9 pmol/min/mg protein (Gong et al., 1992)). This suggests that the involvement of phase II conjugative metabolic pathways of xenobiotic clearance in rainbow trout liver is of much less significance than in mammalian species; although the ability of trout to conjugate compounds has been extensively demonstrated both in this investigation and in the wider literature. In a study using trout liver S9 fractions, some glucuronide formation of ibuprofen metabolites, was suggested (Gomez et al., 2011). However, detailed information of its detection criteria were not given, UDPGA supplementation did not significantly enhance loss of parent and no glucuronides were detected following pre-treatment of fractions with β-naphthoflavone, a known inducer of UGTs (Di-Giulio and Hinton, 2008, Gomez et al., 2011). In the present study, there was evidence of trace levels of glucuronic acid conjugates based on the presence of ^{14}C derived from ^{14}C ibuprofen in polar fractions from LC analyses and NMR based evidence for glucuronic acid conjugates was obtained under conditions of CYP inhibition. Despite the potential for some glucuronic acid formation, this was at a very low level in trout liver S9 incubations without CYP inhibition. This low level of ibuprofen conjugation is in accord with observations in zebrafish larvae, in which ibuprofen was excreted largely as primary metabolites without conjugation (Jones et al., 2012). It is possible that fish, being in an aquatic environment, are less dependent on conjugation reactions in general. This is also highlighted by the increased presence of ibuprofen glucuronic acid conjugates in the presence of CYP inhibitors. Glucuronidation of the parent compound appears to be an alternative metabolic pathway for ibuprofen in trout when the dominant CYP system is inactive. Using the example of this alternative pathway, a lack of supplementation of S9 fractions with phase

II cofactors would alter the metabolic profile obtained, as ibuprofen glucuronides would not have been formed when CYP was inhibited. As a result, the alternative glucuronidation of the ibuprofen parent would not have been identified.

The results of this investigation on the limitations of S9 are in accord with other studies which have shown that subcellular fractions were much less effective than whole cells at predicting *in vivo* hepatic clearance (Ito and Houston, 2005, Brown et al., 2007). However, the use of hepatocytes to reflect *in vivo* metabolism is also not without limitations due to rapid de-differentiation and the ensuing loss of certain CYP activities in monolayer culture (LeCluyse, 2001). In this investigation, the rates of EROD were substantially higher in freshly isolated suspended cells in comparison to cells which were cultured as monolayers, declining further with time in culture up to 48 hours. This is likely to be due to the cellular de-differentiation of hepatocytes in culture and is in accord with a number of sources in the literature (Binda et al., 2003, Brandon et al., 2003, Elaut et al., 2006, Castell and Gomez-Lechon, 2009). It is also reported that alterations in CYP activity in isolated primary hepatocytes occur in two stages, during the isolation process and in the early stages (4–6 hours) of culture. This may explain the sharp decline in EROD activity after 6 hours in monolayer culture and the apparent partial recovery in 24 hour monolayer culture (Paine and Andreakos, 2004).

A rapid decline in EROD metabolic activity over incubation time was also seen when hepatocytes were exposed to 7-ethoxyresorufin. This appeared to be related to the presence of the substrate, rather than a decline of CYP during incubation time or loss of cell viability. Indeed, when cultured hepatocytes 24 hours post isolation were incubated with 7-ethoxycoumarin, the decline in activity measured over the same incubation time course was not as rapid, again suggesting the possibility that this was a substrate specific effect. This

decline in EROD activity over incubation time was also apparent in incubations with S9 fractions. This occurrence in subcellular fractions could be a result of the depletion of NADPH, however, NADPH re-generating systems as used in this investigation make this conclusion unlikely. Nevertheless, failure of this recycling system in S9 incubations could result in the cessation of the reduction of NADP and a decline in CYP catalytic activity. Another important consideration in this respect is the use of short incubation periods. Prolonged incubation times as often used in current metabolic assessment again has the potential to overestimate predicted persistence, due to the natural reduction in metabolic activity seen in the majority of *in vitro* systems.

It is also possible that the decline in activity with time of incubation may be related to the rapid depletion of the substrate through metabolism. When the amount of resorufin product produced was plotted against time, results were consistent with the substrate depletion theory, in that there appeared to be a plateau of product formed after a period of time. To further assess whether substrate depletion is in fact occurring, an increased substrate concentration could be used and the concentrations of 7-ethoxyresorufin and the resorufin product could also be measured analytically and monitored throughout the timecourse. However, substrate depletion appears unlikely based on the fact that a greater concentration of 7-ethoxyresorufin was used in this investigation, compared to a number of published studies and as provided routinely in commercial assays (Scholz and Segner, 1999, Nabb et al., 2006, Bartram et al., 2012). Furthermore, calculations of the amount of product formed compared to the amount of substrate added show a large excess of the latter. In theory, 7-ethoxyresorufin might be metabolised to other, non-fluorescent products by routes other than CYP1A metabolism which could contribute to substrate depletion, without enhanced fluorescence measurements. However, there are no reports of such pathways in the literature.

It is also possible that the production of reactive oxygen species (ROS) may play a role in the metabolic poisoning of CYP1A enzymes, resulting in reduced catalytic activity. Previous evidence of ROS production from 7-ethoxyresorufin in mammalian cellular systems (Green, R.M., pers. comm.), a product which could contribute to the rapid decline in EROD activity through CYP damage is available, forming the basis of the investigation into the involvement of a similar mechanism in trout hepatocytes. CYP isoforms heavily involved in xenobiotic metabolism are prone to catalytic cycle uncoupling, resulting in the formation of ROS. This process is highly dependent on the specific CYP isoform and substrate interaction and is reported to be common for CYP1 isoforms with promiscuous substrate specificity. Uncoupling occurs as a result of dissociation of the iron-oxygen bond, releasing a reactive superoxide radical that can interrupt the catalytic cycle (Harskamp et al., 2012). This can result in the loss of cellular oxidative homeostasis and damage to a number of cellular macromolecules, including the irreversible oxidation of the amino acid side chains of proteins and their subsequent inactivation (Stadtman and Levine, 2003, Birben et al., 2012). The data obtained in this study indicates the possibility that ROS induced CYP damage following 7-ethoxyresorufin incubation, resulting in metabolic inhibition may be feasible. Significant increases in the fluorescence intensity of the cellular indicator of ROS formation, DCF, were observed both in whole cells and in S9 fractions exposed to 7-ethoxyresorufin when compared to control cells and fractions not incubated with the substrate. Despite the apparent insensitivity of the assay with S9 fractions (potentially as a result of low esterase activity), the results indicate a potential role of ROS in the decline of CYP activity, alongside the possibility of substrate related inhibition. Whilst interesting, further investigations into these observations lie beyond the scope of this project but provide areas of further interest for future

pursuit. Problems with the EROD assay may be overcome by using alternative CYP1A substrates, for example phenacetin.

The detection of metabolites of ibuprofen in trout hepatic *in vitro* preparations was also demonstrated in this study. In primary trout hepatocytes, two hydroxylated metabolites of ibuprofen were produced in contrast to liver S9 fractions which produced a single hydroxy-metabolite (identified as 1-hydroxy-ibuprofen). This is in contrast to the proposed formation of the 2-hydroxy-derivative in a separate study (Gomez et al., 2011). Irrespective of the absolute identification of metabolites, the qualitative differences in results presented here again support intact cells as being advantageous to S9 for metabolic assessment. The results from this study are also in accord with a study assessing the ability of rat *in vitro* preparations to mimic *in vivo* drug metabolism, in which S9 fractions did not produce the same metabolites of a number of compounds as intact cells or *in vivo* systems and a discrepancy in phase II metabolism was also seen (De Graaf et al., 2002).

Ibuprofen metabolism is dependent on CYP2C8 and CYP2C9 isoforms in mammals, suggesting that trout also possess an analogous metabolic system, although it has also been suggested that CYP1A may be involved in ibuprofen metabolism by trout liver S9 (Gomez et al., 2011). The results of the CYP inhibition study confirm that hydroxy-ibuprofen formation is CYP dependent in trout and suggest the possibility that multiple isoforms may be involved, or that if a single isoform is involved, its activity is affected by compounds known to inhibit CYPs 2C8, 2C9, 2C19 and 3A in mammals (Projean et al., 2003, Huang et al., 2007, Lu et al., 2010, Vestergren et al., 2012). The non-selective CYP inhibitor SKF525A exhibited the greatest level of inhibition of oxidative ibuprofen metabolism. Along with 3-hydroxy-ibuprofen, the 1-hydroxy-ibuprofen derivative has been identified as a minor metabolite of human ibuprofen biotransformation (Kepp et al., 1997), but as evidenced in this study, is

potentially the major metabolite of rainbow trout ibuprofen metabolism. However, 1-hydroxy-ibuprofen was not identified in rat urine (Yang et al., 2000a) but has been identified as a major metabolite in white rot fungi (Marco-Urrea et al., 2009).

In conclusion, subcellular fractions as currently used have major limitations in reflecting hepatic xenobiotic metabolism and therefore are inferior when compared to the use of primary hepatocytes for the *in vitro* assessment of metabolism and bioaccumulation potential of chemicals. Whole cells provide a more complete and integrated metabolic profile, both quantitatively and qualitatively and appear to be a more accurate alternative for this type of metabolic assessment. The use of intact cells however is also not without limitation, with cellular de-differentiation with time in culture remaining a problem. It has also been suggested that the various cell isolation processes used in the preparation of primary hepatocytes, affect the expression of CYPs which may have an impact on their metabolic activities (Padgham and Paine, 1993, Paine and Andreakos, 2004). Numerous culturing techniques can be employed to improve the culture environment and aid the maintenance of differentiation in hepatocytes, with 3D aggregates receiving much attention in mammalian cell culture. In subsequent chapters, it is hypothesised that the use of hepatocytes in 3D aggregate culture which have been shown to maintain viability and differentiated characteristics for extended periods of time, may be even more advantageous than freshly isolated hepatocytes in this type of metabolic assessment, as freshly isolated hepatocytes are relatively short lived and liver specific gene expression and CYP function declines during the initial stages of cultivation (Brandon et al., 2003, Beigel et al., 2008, LeCluyse et al., 2012). A number of other features of cells in 3D aggregate culture may be advantageous to the use of fish hepatocytes in metabolic assessment and these are also assessed in the following chapters.

Chapter 4

The development and maintenance of xenobiotic metabolic function and stability of gene expression in trout hepatocyte spheroids

4.1 Introduction

Chapter 3 illustrated the degree of limitations of S9 fractions, as currently used in metabolic assessment and through comparative studies, determined the advantages (both quantitative and qualitative) of the utility of intact hepatocytes. Despite displaying greater metabolic activities in comparison to S9 fractions, as has been reported in a number of publications, primary hepatocytes in monolayer culture are prone to cellular de-differentiation and lose functionality and viability over time (LeCluyse, 2001, Castell and Gomez-Lechon, 2009).

As described in Chapter 1, a number of cell culture techniques can be employed to aid the maintenance of differentiation in hepatocytes in culture, including matrix modifications, cell co-culture and 3D culture strategies, each with different advantages (LeCluyse et al., 2012). In recent years, there has been an increase in focus on the benefits of 3D cell culture.

Due to their enhanced longevity, hepatocytes in spheroid culture also provide a recovery period during which any cellular damage, alterations in function or changes in gene expression induced during the isolation process, may be overcome. Evidence of alterations in CYP gene expression occurring during the hepatocyte isolation process in rats is available (Paine and Andreakos, 2004). It is reported that these alterations occur in two stages, during the isolation process and also in the early stages (4–6 hours of culture). As mentioned in the previous chapter, this could explain the sharp decline in metabolic activity after 6 hours in monolayer culture and the apparent partial recovery in 24 hour monolayer culture. Long term culture may provide an extended period during which metabolic activity can be further recovered. The loss of CYP activity and potential consequent changes in the profile of different CYP activities in rat hepatocyte culture has also been attributed to a loss of cell-cell contacts and the absence of extracellular matrix deposits (Wright and Paine, 1992). The use of

hepatocytes in spheroid culture may also circumvent this problem as cells maintain intercellular contacts and there is an enhanced secretion of extracellular matrix components by hepatocytes in aggregate culture (Landry et al., 1985).

Very few studies on the use of fish hepatocytes in spheroid culture exist in the literature (Flouriot et al., 1993, Flouriot et al., 1995, Baron et al., 2012) and their potential with respect to drug metabolism has not been investigated, despite the predicted maintenance of differentiation and the fact that the increased longevity of hepatocyte spheroids is a major advantage. This feature potentially allows for the study of the effects of chronic exposure and metabolism of highly persistent chemicals. This is a timely advantage for the investigation of environmental contaminants, due to the increase in focus on the impact of chronic environmental pollutants (Hutchinson, 2008). The use of spheroids in this type of assessment would hold significant advantages over the use of freshly isolated hepatocytes, as the latter is short-lived and would therefore prove impractical.

In this investigation, the hypothesis that, due to the aforementioned properties of 3D aggregate culture, primary trout hepatocytes cultured as spheroids may be superior to isolated hepatocytes in suspension, for use in environmental bioaccumulation studies, was tested. A method to routinely and consistently produce trout hepatocyte spheroids was devised and preparations assessed for their metabolic activities. The impact of the hepatocyte isolation process on the gene expression of 5 metabolic enzymes was investigated and compared to the expression in spheroids at different stages of development to see if this culture method confers a stable pattern (compared to that expressed in the whole liver) and level of expression.

4.2 Results

After a considerable amount of optimisation, an effective method was developed to consistently prepare hepatocyte spheroids of high quality which retained viability and functional integrity for extended periods. Details have not been shown for brevity but the main features determining optimal formation were efficiency of perfusion, concentration density of hepatocytes in suspensions, shaking speed, temperature during development and buffer supplementation. The optimised method is provided in Chapter 2.

4.2.1 Spheroid formation

Figure 4.1 illustrates the development of primary hepatocyte spheroids, from isolated cells in suspension, to maturity (8–10 days post isolation). Hepatocytes in suspension aggregated rapidly in rotational culture conditions and between 24–48 hours, many asymmetrical clusters of cells were visible when compared to the limited number of cell-cell contacts existing in original suspensions (4.1A). Between 2–3 days, the hepatocytes appeared to form a network of larger clusters, with fewer individual cells present in cultures (4.1B). By day 3, these loose cellular aggregates had enlarged, forming structures with a more identifiable spherical shape; however the outline of these structures was pitted and undulating, with individual neighbouring cells within clusters, visible (4.1C). At this point, these immature spheroids existed in a wide variety of shapes and sizes (with single cells from the original suspension still visible). Between days 5–7, spheroids became more uniform in shape, with larger diameters (4.1D) and by day 8, there was little to no change in their morphological arrangements when compared to earlier time points, with cells exhibiting a more homogeneous shape, a feature used to confirm morphological maturation in this study (4.1E).

Changes in size during spheroid formation and development were measured by flow cytometry, using comparisons of the forward scatter properties of microsphere standards, which provided a reference population (Figure 4.2). In preparations analysed 24 hours after isolation, there was a very large proportion of smaller particles (approximately 8–35 μm in diameter), representing individual hepatocytes in the suspension and slightly larger clusters of hepatocytes due to the rapid aggregation of individual isolated cells (as seen in Figure 4.1A). The reported diameter of individual primary trout hepatocytes is about 10–25 μm (Braunbeck et al., 1996). A shift in aggregate size, to the right of the histogram was evident over time in rotational culture, with a visible decline in the proportion of structures approximately 15–50 μm in size (representing loose aggregates of hepatocytes), with a concurrent increase in the proportion of larger structures approximately 50–80 μm in diameter (as a result of the joining of smaller aggregates, producing uniform spherical shapes). Spheroids began to reach a stable size (approximately 90–110 μm) from day 5 and from this point onwards, the size shift to the right of the histogram ceased, with a greater proportion of spheroids reaching this size range between days 5–7, with no major subsequent increase in diameter.

The spheroids produced in this project predominantly consisted of parenchymal cells, with the vast majority of non-hepatocytes, (which have been shown to contribute to about 15–20% of total liver volume (Segner, 1998), effectively excluded during the purification process. This provided a freshly isolated suspension consisting of ≥ 95% hepatocytes (measured by light microscopy, using a haemocytometer), therefore the spheroids produced were homotypic, 'hepato-spheroids', rather than heterotypic, multicellular structures. In this investigation, spheroids were used at 10 days post isolation for metabolic assays and from 5 days post isolation, up to 25 days post isolation in developmental, metabolic gene expression

assessments. However, spheroids from the cultures used in this investigation have been maintained viable and active for up to 40 days.

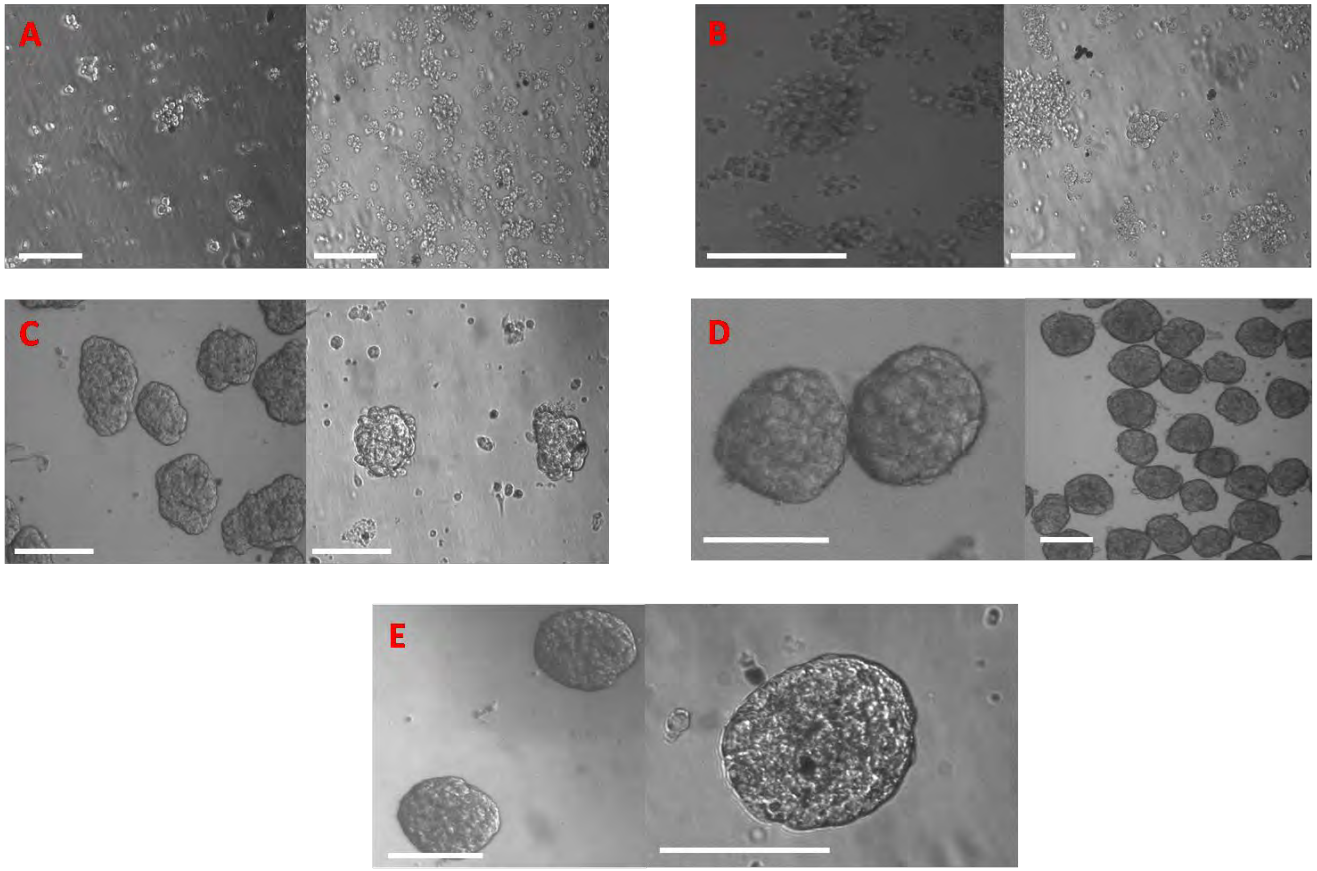


Figure 4.1: Stages of hepatocyte spheroid development. Light microscopy images of aggregating hepatocytes on a variety of days post isolation. Isolated hepatocytes in suspension began to aggregate after 24 hours, with many small, asymmetric clusters of cells present (A; 10x objective). Larger clusters form by day 3 (B; 40x and 10x objective), with unaggregated, individual cells still visible, but fewer in number. A more recognisable spherical shape was identifiable by day 3 but spheroids existed in a variety of shapes and sizes (C; 40x objective). Between days 5-7, spheroids became larger and more uniform in shape (D; 40x and 20x objective) and by day 8, displayed a more homogeneous shape, with little to no change in morphological arrangements (E; 40x objective). Scale bars indicate 100 µm.

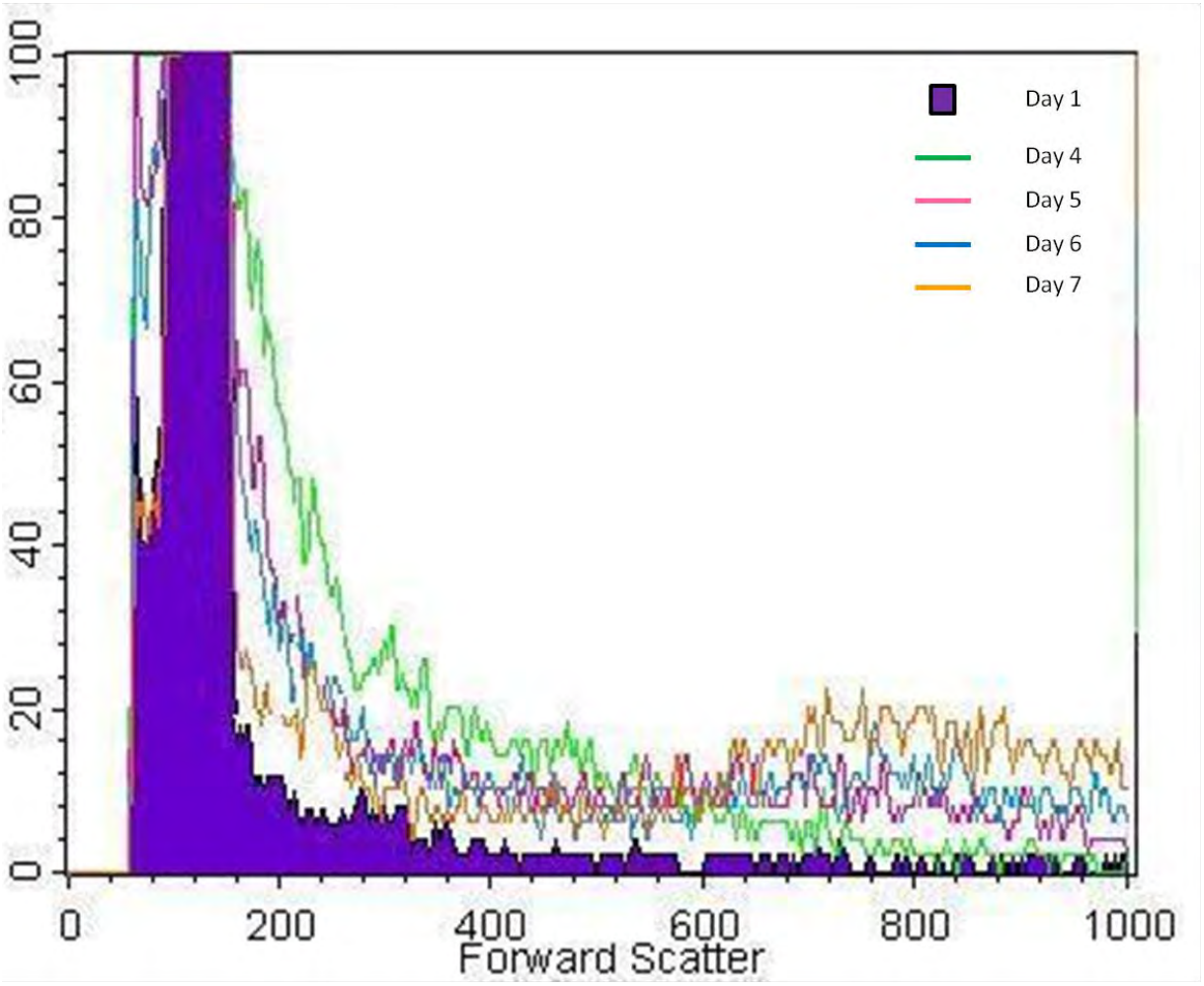


Figure 4.2: Histogram of the size distribution of hepatocyte spheroids during development. Changes in size during formation and development were measured by flow cytometry, using comparisons of the forward scatter properties of microsphere standards. A large proportion of individual cells and small clusters (approximately 8–35 µm in diameter) were visible 24 hours after isolation. A shift in cell size, to the right of the histogram was evident over time in rotational culture, with a decline in the proportion of smaller structures (approximately 15–50 µm in size) and a simultaneous increase in the proportion of larger structures (approximately 50–80 µm in diameter) Spheroids began to reach a stable size (approximately 90–110 µm) from day 5, with a greater proportion of spheroids reaching this size, up to day 7.

4.2.2 Metabolic activity of hepatocytes in spheroid culture

EROD reaction rates were calculated in an identical manner for hepatocytes in spheroid culture as for other hepatocyte preparations (see Chapter 3). Measurements were made after 30 minutes of incubation with 7-ethoxyresorufin, to allow a direct comparison to be made.

EROD activity in 10 day old spheroids (5.98 ± 0.54 pmol/min/mg S9 protein equivalent) was significantly greater than measured in S9 fractions both in the presence and absence of phase II cofactors (Figure 4.3). The level of activity measured in spheroids was not significantly different from that of freshly isolated hepatocytes in suspension, but was significantly greater than those measured in monolayer culture, reported in Chapter 3. Therefore, spheroid hepatocytes exhibit the greatest analogy of all hepatocyte preparations to the metabolic activity of freshly isolated hepatocytes with respect to this substrate. When phase II metabolic activity was assessed, once again, hepatocytes in spheroid culture exhibited similar activities to freshly isolated hepatocytes, as no significant difference was seen between the proportions of resorufin conjugated in these preparations (Table 4.1), whereas this proportion was significantly lower than recorded in monolayer culture. Therefore, the metabolic capabilities of hepatocytes in spheroid culture, 10 days post isolation, were most reflective of those measured in freshly isolated hepatocytes.

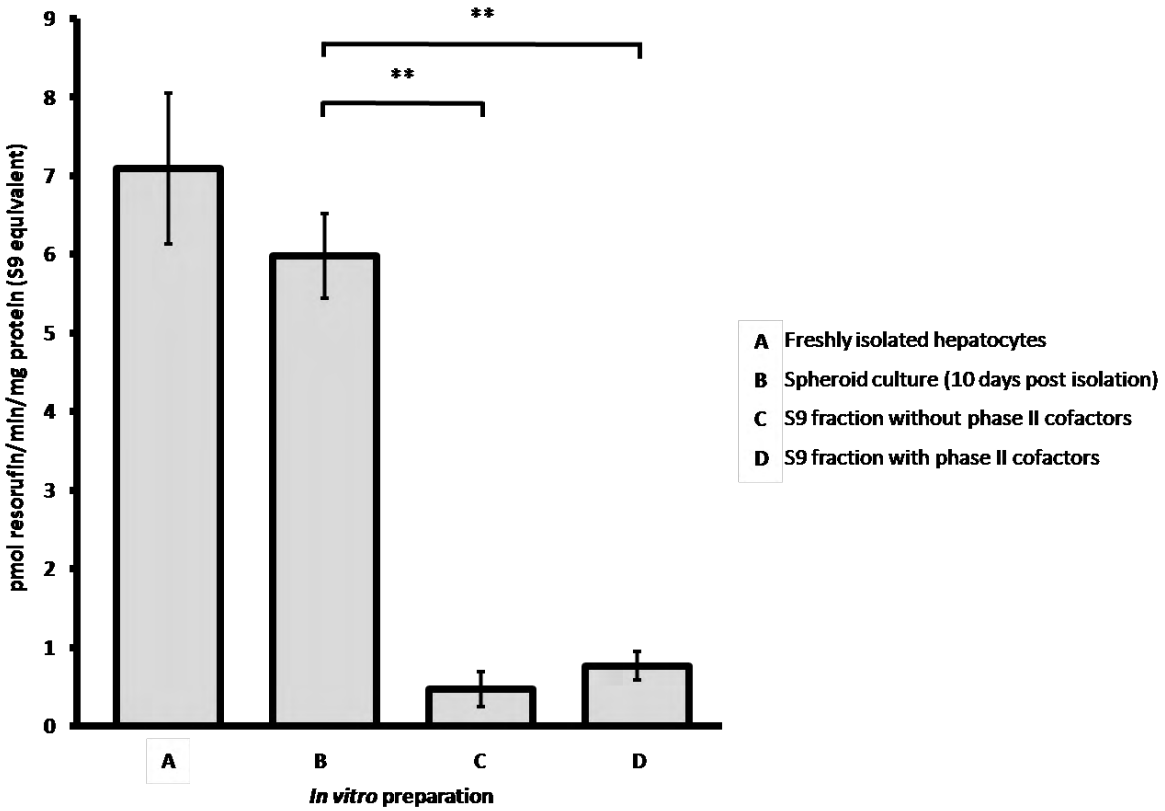


Figure 4.3: Comparative rates of total resorufin production by hepatocytes in spheroid culture, freshly isolated hepatocytes (30 minute incubates) and S9 fractions (10 minute incubates) under different phase II cofactor supplemented conditions. Mean values ± SEM; $^{**}p < 0.01$ Mann-Whitney *U* test (n= 8 fish).

Cellular preparation	% oxidised product as conjugates
Freshly isolated hepatocytes in suspension	9.9 ± 2.3
Hepatocytes in spheroid culture (10 days post isolation)	13.57 ± 5.7

Table 4.1: Comparison of the proportion of resorufin conjugated by freshly isolated hepatocytes and hepatocytes in spheroid culture. Mean values; n= 8 fish; ± SEM.

4.2.3 Changes in metabolic enzyme gene expression following trout hepatocyte isolation

To assess whether the expression levels of 5 different genes responsible for xenobiotic metabolism changed as a result of the collagenase digestion process, RNA was extracted from 100 mg sections of liver ligated and removed before and after hepatic perfusion. Extracted RNA was used to synthesise cDNA and gene expression levels were measured using real time PCR. A decline in the relative expression of all genes investigated was seen following the 3 stage collagenase digestion procedure, evident by an increase in Ct value measured. The changes in RNA content were not uniform however, with CYP3A27 and UGT exhibiting a relatively low fold repression (0.9 ± 0.18 and 0.88 ± 0.13 respectively; Figure 4.4). The expression of the CYP1A gene however, appeared to be more susceptible to alterations by the collagenase digestion process, with a 0.54 ± 0.17 fold change in expression. This decline was statistically significant from the untreated control ($p < 0.05$, Kruskal-Wallis test). CYP2K1 and CYP2M1 expression was repressed to a lesser extent (0.75 ± 0.19 and 0.69 ± 0.18 fold respectively), the reduction in expression level of these genes was not significant compared to the levels measured in undigested liver (Figure 4.4).

Despite inducing changes in gene expression, the collagenase digestion process did not alter the patterns of expression of the genes responsible for xenobiotic metabolism assessed in the respective individual fish (A, B and C). Whilst inter-individual differences in the levels of expression were seen between fish, with the exception of CYP1A and CYP3A27 in Fish A, the relative extent of each gene expression, remained unchanged following liver digestion (Figure 4.5). The expression of CYP3A27 in Fish A was the only replicate in which a greater level of expression of a gene was measured in a collagenase digested liver. This difference (an average change in Ct value of 0.58 ± 0.12) was statistically significant from the undigested control ($p < 0.05$, one way ANOVA).

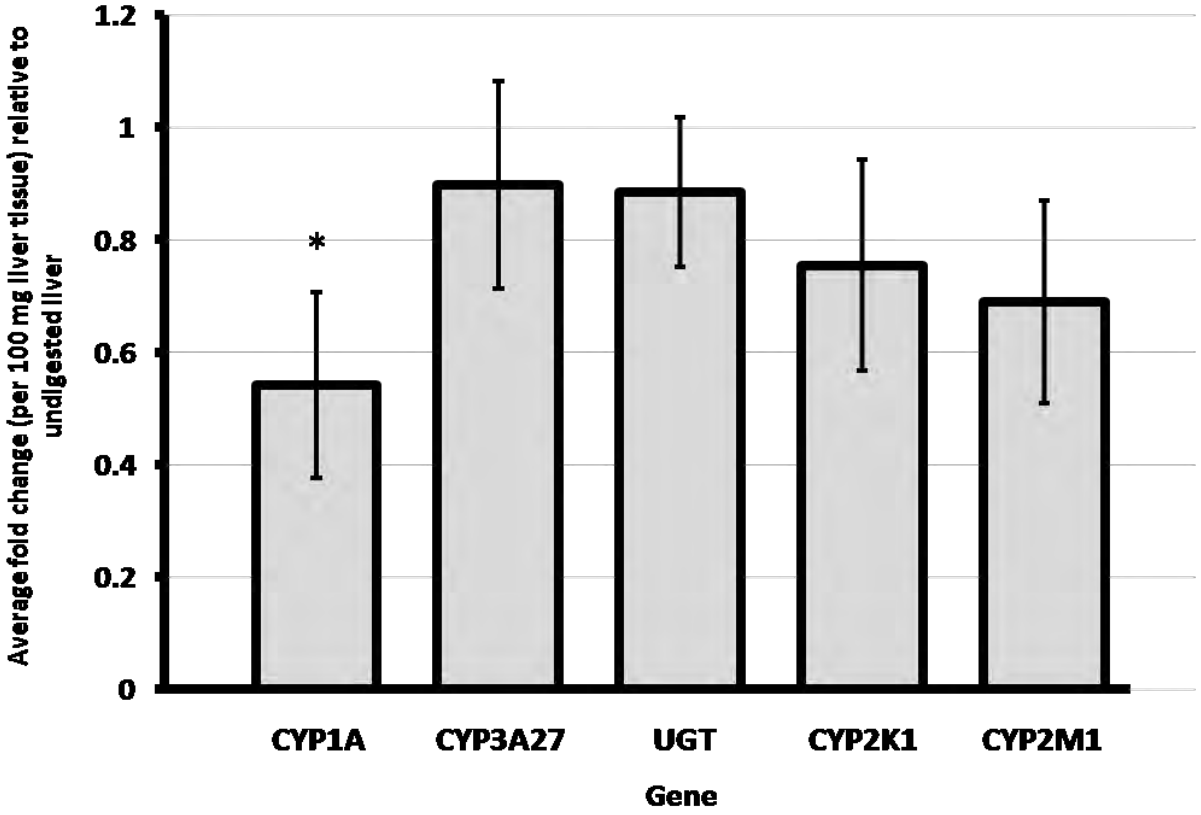


Figure 4.4: Relative fold change (measured by Pfaffl method) of CYP1A, CYP3A27, UGT, CYP2K1 and CYP2M1 genes expressed in whole livers of trout following collagenase digestion, compared to control livers. Bars represent the mean change in expression relative to the expression of 18S rRNA (1.0) in untreated livers ± SEM; * $p < 0.05$ Kruskal-Wallis test (n= 3 fish).

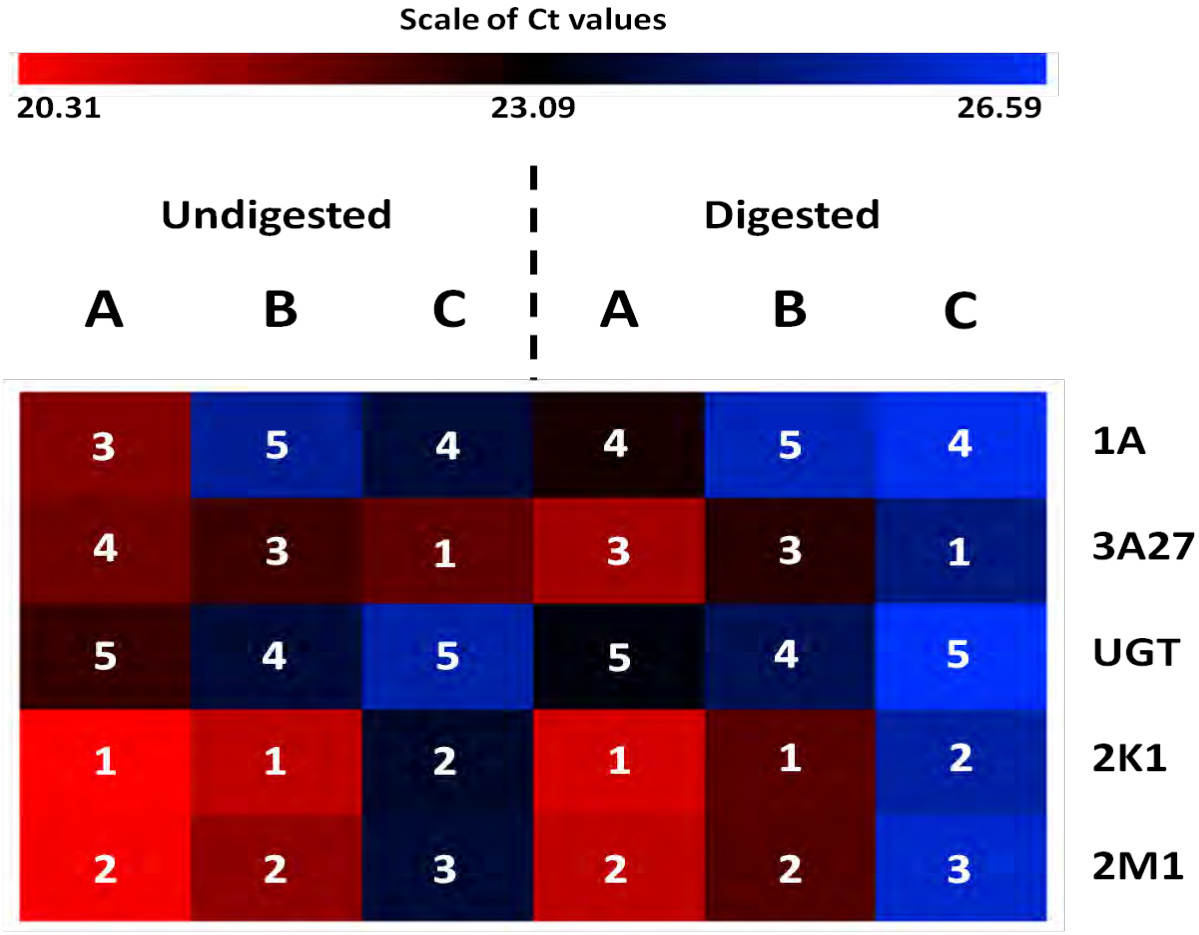


Figure 4.5: The levels and pattern of expression of CYP1A, CYP3A27, UGT, CYP2K1 and CYP2M1 in 100 mg samples of undigested and digested liver. Liver samples were isolated from 3 individual fish, A, B and C. Level of expression is indicated by colours representing deviation of Ct value from the median expression level of all genes measured. The bar above the heat map represents the scale of Ct values; red representing the lowest extreme Ct value measured (therefore a greater level of expression), blue representing the highest extreme Ct value measured (therefore the lowest level of expression) and black representing the median level of expression. Numbers indicate the pattern of expression in an ordered fashion; 1 representing the gene with the highest level of expression in an individual fish and 5 representing the gene with the lowest level of expression. The decline in the expression of all genes (with the exception of CYP3A27 in fish A) as a result of the digestion process can be seen by the changes in colour towards the highest extreme Ct value in the respective digested liver samples. With the exception of CYP3A27 and CYP1A in fish A, the pattern of expression remained unchanged, seen by the same numbering in respective digested liver samples.

4.2.4 Changes in the expression of genes involved in xenobiotic metabolism in different hepatocyte preparations

The transcript levels of CYP1A, CYP3A27, UGT, CYP2K1 and CYP2M1 were also assessed and compared in freshly isolated hepatocytes, hepatocytes in monolayer culture (24, 48 and 72 hours post isolation) and hepatocytes in spheroid culture (5, 7, 10, 15 and 25 days post isolation). A number of gene specific changes in expression levels in different hepatocyte preparations were seen (Figure 4.6). In general, there appeared to be a decline in gene expression levels in early monolayer culture, followed by a recovery in late monolayer culture and early stages in spheroid culture. Levels of expression of the genes of interest subsequently declined briefly in later stages of spheroid culture and then became relatively stable.

With the exception of CYP1A, the expression levels of all genes in monolayer culture 24 hours post isolation were less than, or equal to, those measured in freshly isolated hepatocytes. However, at subsequent stages of monolayer culture (48 and 72 hours post isolation), expression increased to a level greater than the expression in freshly isolated hepatocytes (with the exception of UGT expression, which was lower in both instances).

In most cases, expression levels measured in hepatocyte spheroids were greater than measured in freshly isolated hepatocytes and hepatocytes in monolayer culture. The expression of CYP1A was the exception to this observation. The level of expression of this gene, at all stages of spheroid development, was lower than measured in all monolayer cultures and less than, or equal to, the level recorded in freshly isolated hepatocytes. In spheroid culture, gene expression levels were generally greatest at 5 and 7 days post isolation, with a small decline at 10 and 15 days post isolation; with the exception of CYP1A and CYP3A27 at 10 days post isolation and CYP1A in spheroids 15 days post isolation. However, expression levels remained greater than measured in freshly isolated hepatocytes. Gene

expression later increased at 25 days post isolation (with the exception of CYP1A), to levels greater than measured at 10 days post isolation and less than but closer in comparison to those detected at 5 and 7 days post isolation.

Unlike with the collagenase digestion process, changes in the pattern of gene expression were seen in different hepatocyte preparations. Interestingly however, the relative extent of expression of each gene in spheroids at 10, 15 and 25 days post isolation was identical. This was also the case with hepatocytes in monolayer culture at all time points, however this pattern did not match with those in mature spheroids. Importantly, the difference in the pattern of expression of hepatocytes in monolayer culture included the observation that at all time points, a higher relative extent of CYP1A expression was measured, compared to all other hepatocyte preparations and liver samples. In all hepatocyte preparations, CYP3A27 exhibited the highest relative level of expression, in contrast to whole liver samples, where CYP2K1 expression was greatest. The expression level of UGT was lowest in all preparations (except in spheroids 7 days post isolation), matching the relative level of expression in whole liver samples.

Despite these differences, mature spheroids (10, 15 and 25 days post isolation) appeared to retain the most similar pattern of expression in comparison to that observed in the whole liver. However, caution must be exercised when making such comparisons as a smaller proportion of hepatocytes (in the region of 80%), contribute to the total liver protein in whole liver.

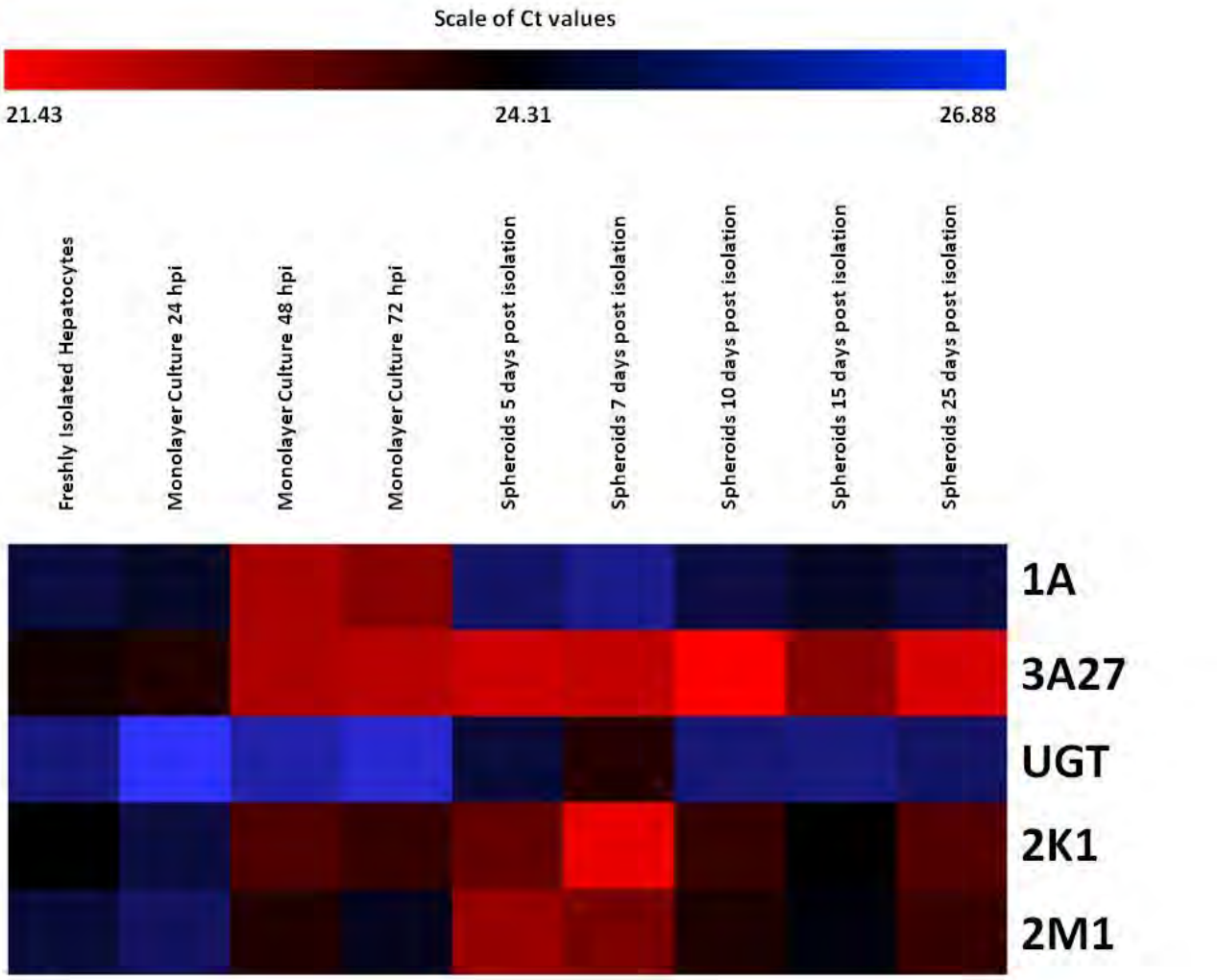


Figure 4.6: Expression levels of CYP1A, CYP3A27, UGT, CYP2K1 and CYP2M1 in freshly isolated hepatocytes, hepatocytes in monolayer culture and hepatocyte spheroids. Level of expression is indicated by colours representing deviation of Ct value from the median value of each of the individual genes measured at all conditions. The bar above the heat map represents this scale of Ct values; red representing the lowest extreme Ct value measured (therefore a greater level of expression), blue representing the highest extreme Ct value measured (therefore the lowest level of expression) and black representing the median level of expression. In general, a greater level of gene expression (with the exception of CYP1A) was seen in spheroid culture, evident by the higher distribution of red to the right of the map. Expression levels appeared to decline, recover and stabilise with time in spheroid culture. Despite this, expression levels for all genes in spheroid culture (with the exception of CYP1A) were greater than those measured in freshly isolated hepatocytes and greater than measured in monolayer culture 24 hours post isolation.

Principal component analysis was conducted on samples separated by method of preparation, to compare the expression profile of the different systems. Preparations were separated along the PC1 and PC2 axes, accounting for 69.8% and 20.9% of the observed variations respectively. Separation by these factors resulted in various distinctions between preparations (see Figure 4.7). Despite having the greatest similarity, a distinct difference in the expression profile of the genes investigated in undigested and digested whole liver samples was seen in the PCA scores plot. In comparison to these *in vivo* samples however, there was a clear grouping of hepatocytes in monolayer culture which displayed the most extreme degree of difference. Spheroids on the other hand, were positioned at the opposite extreme (closest in comparison to the *in vivo* profile of expression). Although there was variability of positioning depending on time in culture, in general, the expression profile of hepatocytes in spheroid culture appeared to be closer to the *in vivo* profile than that of monolayer culture. This was with the exception of the 10 and 15 day post isolation time points, which had a greater resemblance to the profile of freshly isolated hepatocytes, but appeared to be a transient difference due to the restored *in vivo* resemblance at 25 days post isolation. On the balance of evidence, the gene expression profile of hepatocytes in monolayer culture offered the poorest representation of the profile *in vivo*, followed by freshly isolated hepatocytes. Hepatocytes in spheroid culture offered the best analogy to the expression profile *in vivo*.

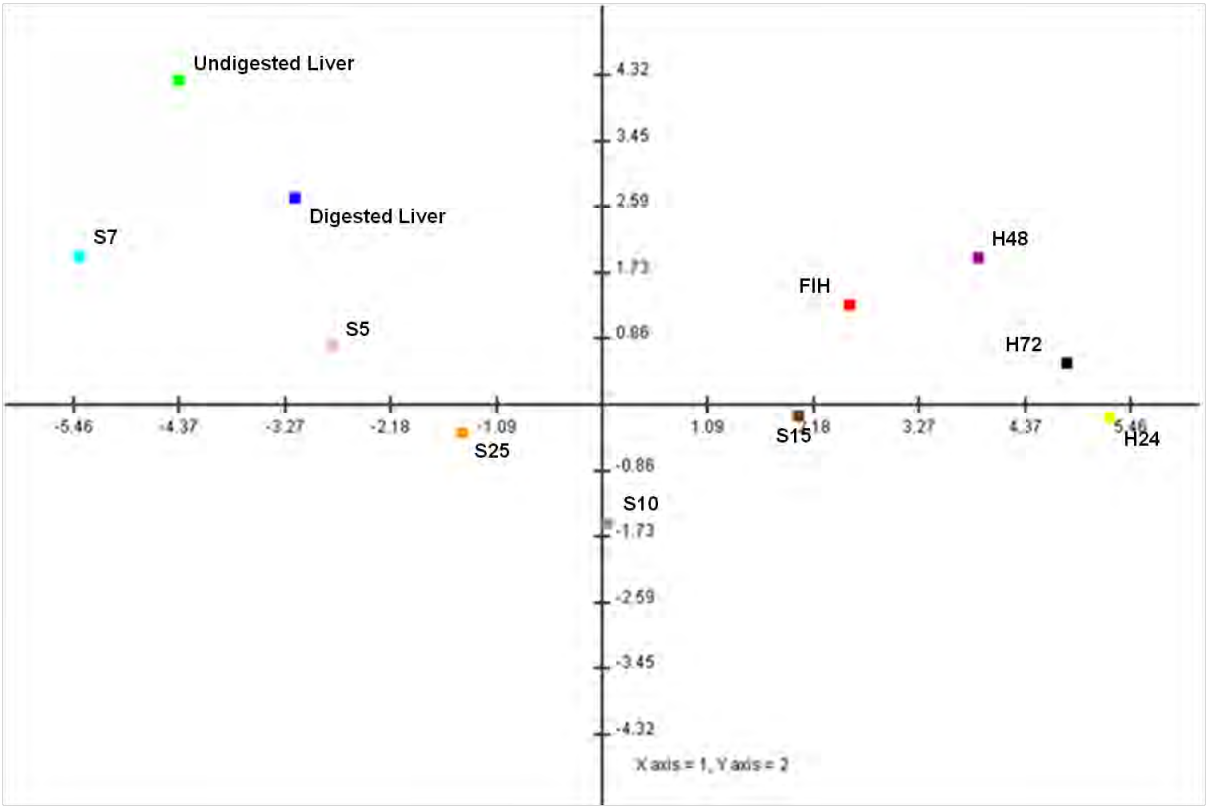


Figure 4.7: Principal components analysis (PCA) scores plot for the profile of expression of metabolic genes of interest in whole liver (undigested and digested) and all hepatocyte preparations. The plot shows separation of *in vivo* and *in* vitro preparations along the PC1 and PC2 axes. There is a clear separation of hepatocytes in monolayer culture at the greatest extreme from the *in* vivo samples. Variability in the profile of expression exists among spheroid hepatocytes according to stage of culture, however, spheroids at 5, 7 and 25 days post isolation show a better resemblance to the expression profile *in vivo* compared to freshly isolated hepatocytes and hepatocytes in monolayer culture. Undigested liver; Digested liver; FIH: Freshly isolated hepatocytes; H24: Monolayer culture 24 hpi; H48: Monolayer culture 48 hpi; H72: Monolayer culture 72 hpi; S5: Spheroid culture 5 dpi; S7: Spheroid culture 7 dpi; S10: Spheroid culture 10 dpi; S15: Spheroid culture 15 dpi; S25: Spheroid culture 25 dpi.

4.3 Discussion

The advantages of the use of intact hepatocytes over S9 fractions for metabolic assessment were seen in Chapter 3. In this study, the utility of 3D hepatocyte culture was investigated as an alternative approach to enhance the maintenance of differentiation in hepatocytes (and to overcome the loss of viability of cells maintained in suspension). The use of aggregate culture has received particular attention in mammalian cell culture (Abu-Absi et al., 2004, Brophy et al., 2009) and, as yet, spheroid hepatocytes from trout liver have not been investigated in the context of xenobiotic metabolism.

Spheroid cultures used in this study were maintained viable for 25 days and beyond. In cultures assessed at 10 days post isolation, spheroids showed EROD activity which was significantly greater than measured in monolayer cultures and which was not different to the rate observed in freshly isolated hepatocytes. Interestingly, the proportion of primary metabolites detected in a conjugated form in spheroids was also not different to that measured in freshly isolated cells. Despite this similarity, it is not possible to conclude that this indicated a similar rate of conjugation in the two preparations, as the proportion may be influenced by phase I product formation and potential deconjugation reactions.

The greater levels of metabolic activity measured in hepatocyte spheroids, compared to hepatocytes in monolayer culture, strongly support the use of spheroid cultures in predictive studies of persistence; particularly for the study of poorly metabolised substrates and chronic exposure assessment due to their extended longevity. This confers an advantage over several of the other intact cellular systems used in similar studies (see Section 1.10). Cryopreserved cells for example, have the advantage of ease of use with recovery of metabolic function (Mingoia et al., 2010) but, are only effective in short term incubations and

many other alternatives lack the flexibility and longevity of spheroids, making them less practical and less compliant with the 3Rs principle.

The hepatocyte isolation process for primary culture has been shown to affect the function of cells and reports in rats show that primary cell isolation causes alterations in CYP gene expression (Paine and Andreakos, 2004). This decline in gene expression, occurs in two stages and was proposed in Chapter 3 as a cause of the low levels of EROD activity seen in hepatocytes in monolayer culture 6 hours post isolation. Indeed, in this investigation, alterations in the gene expression of a range of metabolic enzymes were identified in sections of whole liver, following the 3 step collagenase digestion process. The expression levels of all of the genes of interest were lower in digested portions of liver in comparison to untreated segments of whole liver. Although these samples were taken from the same individual trout livers, it is difficult to make direct comparisons between undigested and digested liver fractions as their compositions differ. Undigested liver samples, taken prior to perfusion, contain blood which is removed from the organ during digestion, along with the hormones, differentiation factors and other components contained in it. The removal of blood has been implicated in hepatic gene expression changes in rats, through the loss of regulatory factors present (Padgham and Paine, 1993). Nonetheless, it is clear that the multi-step hepatocyte isolation procedure itself in trout contributes to alterations in hepatic gene function at the level of expression. Alternatives to isolation by collagenase digestion, EDTA isolation for example are available; however, comparable loss of CYP content has been reported with this and other perfusion methods due to reperfusion stress induced during the isolation process, disruption of the normal tissue architecture and adaptations of cells to the *in vitro* environment (Wright and Paine, 1992, Elaut et al., 2006).

Loss of CYP content and a dramatic decline in the expression of genes involved in xenobiotic metabolism has also been shown to continue when hepatocytes isolated from both humans and rats were subsequently cultured, reported to be as a result of the loss of cell-cell contacts and the absence of extracellular matrix deposits in conventional culture (Richert et al., 2006, Beigel et al., 2008).

Long term culture may provide an extended period during which alterations in morphology, expression and activities can be recovered as well as providing an environment where intercellular contacts between cells are maintained and there is enhanced secretion of extracellular matrix components, retaining cell-ECM interaction (Landry et al., 1985, Kunz-Schughart et al., 2004, Pampaloni et al., 2007). These features make spheroid hepatocytes more physiologically relevant.

The results from this investigation showed that, in general, levels of gene expression were greater in spheroid culture than measured in freshly isolated hepatocytes or cells in monolayer culture. With the exception of CYP1A expression, expression levels in spheroids did not fall below those measured in other hepatic preparations. The lowest levels of gene expression were measured in monolayer cultures 24 hours post isolation. Interestingly, this was consistent with the lower levels of EROD activity in this culture type (Chapter 3), however, the average expression of CYP1A (responsible, at least in part, for 7-ethoxyresorufin metabolism) in this preparation was greater than measured in freshly isolated hepatocytes and greater than or equal to the levels measured in spheroid culture. This, and the greater level of expression measured in monolayer culture 48 hours post isolation (which exhibited even lower EROD activity) suggests that other mechanisms may be responsible for the loss of CYP activity.

The gene expression profile of hepatocytes in spheroid culture was also most comparable to that recorded *in vivo*. It is difficult to make direct comparisons of expression levels between isolated hepatocyte preparations and whole liver due to the differences in cellular composition. Hepatocytes have been shown to be the quantitatively dominant cell type in trout liver, contributing around 80–85% liver volume (Hampton et al., 1989, Segner, 1998). Hepatocytes express the greatest level of CYPs in the liver, however, whole liver samples include at least 10 other, non-parenchymal cell types (Lester et al., 1993) which have been shown to play a role in the function, differentiation and gene regulation of hepatocytes, through inter cellular signalling (Altin and Bygrave, 1988). It has been reported that co-culture systems maintaining parenchymal and non-parenchymal cells concurrently, show improved morphological characteristics and increased drug metabolism capabilities (Van Berkel and Van Tol, 1979, Padgham and Paine, 1993, Cross and Bayliss, 2000) and this could be applied to trout hepatocyte spheroid culture in the future. Despite this, as expected, hepatocytes in spheroid culture retained a more representative profile of expression of genes related to xenobiotic metabolism.

Although gene expression in spheroids was greater than in other hepatocyte preparations, there appeared to be a transient decline in gene expression at 10 and 15 days post isolation, with a subsequent recovery at 25 days post isolation. This has also been reported for the expression of genes related to xenobiotic metabolism in hepatocyte aggregates isolated from other species. The greatest levels of variation in expression in rat hepatocyte spheroids was seen in genes related to phase I metabolism and despite this variation, metabolic activities in these cultures remained significantly higher than in rat hepatocyte monolayer cultures. The expression levels of genes related to phase II metabolism remained stable at various stages of spheroid culture (Brophy et al., 2009). This was also true

in the trout hepatocyte spheroids used in the present study, as UGT expression remained relatively stable at all stages of spheroid culture in comparison to CYPs.

The results of this investigation show the potential for the use of hepatocytes in spheroid culture in metabolic assessments and suggest that they may provide the ideal system for use in ERA. The *in vitro* system can now be used to estimate intrinsic clearance (CL_{int}) using V_{max} and K_m measurements from future metabolic studies (see equations 1–4). This is a measure of hepatic enzyme activity, independent of the effects of substrate binding to plasma proteins and hepatic delivery.

Based on the Michaelis-Menten equation:

$$v = \frac{Vmax\ [S]}{Km\ + [S]} \tag{1}$$

When [S] is substantially lower than K_m,

$$V \sim \frac{Vmax\ [S]}{Km} \tag{2}$$

Therefore:

$$V/_{[S]} \sim Vmax/_{Km} \tag{3}$$

$$\therefore CL_{int\ (in\ vitro)} = {Vmax}/{Km} \quad (4)$$

Under these conditions, it will be possible to predict hepatic clearance (CL_h), using estimated rainbow trout scaling factors including blood flow, liver size and plasma protein binding (Nichols et al., 2006) which would allow comparison to the CL_h of compounds measured *in vivo.* CL_h is a measure of compound clearance taking into account hepatic delivery, plasma protein binding, hepatic metabolism and biliary excretion. Once CL_h is derived from in vitro data, CL_h can then be fitted to physiologically based phamacokinetic models (PBPK) to optimise and improve the prediction of *in vivo* pharmacokinetics taking into account uptake parameters and other excretory routes (Rostami-Hodjegan, 2012, Gill et al., 2013).

In conclusion, on account of the greater levels of EROD activity and gene expression measured, the use of primary spheroid cultures of trout hepatocytes may be particularly suited to the *in vitro* assessment of metabolism and bioaccumulation potential of chemicals, suggesting that following assessment with a wider range of substrates and of a wider range of genes, they should be considered for routine screening, in place of currently used inferior microsomal and S9 systems. Indeed, spheroids are also superior to shortly lived freshly isolated cells in suspension (maintaining viability for only a matter of hours) and de-differentiated monolayer cultures (of lower functionality). As a result, hepatocytes in spheroid culture have the potential to be used in studies on the metabolism and potential bioaccumulation of compounds (particularly those of high persistence) that require long term incubations. This is especially important with the recent concerns raised over the environmental impact of contaminants exhibiting chronic toxicity. Another potential advantage of the use of hepatocytes in spheroid culture is the incorporation of measurements

of transporter function in these preparations. Efflux transporters may also be expressed to a greater extent in spheroids than in suspension and monolayer culture and as a result may prove to be a superior model for the assessment of compound elimination from hepatocytes. This potential will be assessed in the following chapter.

Chapter 5

Expression of drug transporters in hepatocyte spheroids: are additional advantages conferred to the system for use in ERA?

5.1 Introduction

In Chapter 4, the superiority of hepatocytes in spheroid culture for metabolic assessment was illustrated due to the extended longevity, enhanced levels of EROD activity and generally greater expression levels of genes involved in xenobiotic metabolism in spheroids, when compared to primary hepatocytes in monolayer culture. Together with these features which make hepatocyte spheroids a more reflective model of *in vivo* metabolism, measurements of compound uptake and efflux using spheroids might also be incorporated into such assessments, as a result of their potential enhanced drug transporter expression and function. However, there is no information on such systems in fish hepatocyte spheroids and indeed very little in spheroids of any species. Recently, a greater level of expression and functional activity of MDR1 in human hepatic HepG2 cells cultured as spheroids compared to those measured in monolayer culture, was reported (Oshikata et al., 2011).

Elimination is an important feature of the xenobiotic detoxification process and has a key influence on the pharmacological and toxicological potential of compounds. Impaired elimination of a xenobiotic can result in elevated plasma concentrations, affecting the pharmacodynamics of compounds and altering their potential for accumulation within an organism (Caminada et al., 2008). ABC efflux transporters facilitate the excretory function of organisms, transporting exogenous and endogenous compounds and/or their metabolites out of cells, effectively illustrated in hepatocytes, where they are heavily expressed. In fish, as in mammals, a common route of xenobiotic excretion is via bile, with proteins of the ABC superfamily playing a critical role in the transport of compounds and metabolites into bile (Guarino, 1987, Zaja et al., 2008b). Recent publications have shown that a variety of toxicologically relevant ABC efflux transporters are expressed in trout, with a wide tissue distribution pattern. MRP2 (ABCC2), MDR1 (ABCB1), BSEP (ABCB11) and BCRP

(ABCG2) have been identified as the efflux transporters with the highest levels of expression in trout liver, forming the multixenobiotic resistance mechanism (MXR) (Zaja et al., 2008b, Hildebrand et al., 2009, Loncar et al., 2010).

Despite the importance of the function of ABC transporters in the regulation of ADME properties of compounds, their role is often neglected in the assessment of xenobiotic fate (Kim, 2002, Bains and Kennedy, 2005). In recent years however, a greater recognition of the role of hepatic transporters on the disposition and elimination of compounds, and how these features combine with the metabolic aspects of hepatic clearance, has led to investigations into the development of effective methods to accurately assess substrate specificity and affinity for efflux transporters (Chandra and Brouwer, 2004, Szakacs et al., 2008). Conventional measurements of hepatic clearance in S9 fractions, as used in bioaccumulation assessment, assume the cellular uptake of compounds and totally efficient efflux activity of hepatic transporters. If a compound is metabolised, but the metabolites are not readily transported out of cells, they have the potential to accumulate. This information would be missed by study in subcellular fractions alone, as metabolic screening would suggest that the compound is metabolised and therefore unlikely to bioaccumulate.

Hepatocytes in spheroid culture may prove to be a superior model to subcellular fractions for such bioaccumulation studies, as assessment of compound elimination from hepatocytes may also be incorporated, providing a more accurate comparison to *in vivo* hepatic clearance. The profile of efflux transporter expression in hepatocytes in spheroid culture may also be more reflective of the *in vivo* situation when compared to freshly isolated hepatocytes and hepatocytes in monolayer culture, due to the extensive maintenance of the differentiation status in spheroids.

The aim of this investigation was to assess the expression and functionality of the main trout hepatic ABC efflux transporters (ABCB1, ABCB11, ABCC2, ABCG2) in hepatocytes in spheroid culture. Comparisons of the pattern and level of gene expression between hepatocytes in spheroid culture, monolayer culture and freshly isolated hepatocytes were made and the extent to which these profiles reflected *in vivo* expression was examined. Functional assessment of transporter activity was conducted using known specific fluorescent substrates and inhibitors of mammalian transporters as detailed in Tables 2.4 and 2.5, however, specificities of these compounds in fish species is not fully understood. Uptake and accumulation of fluorescent markers was measured by fluorimetry and intracellular uptake was visualised using confocal microscopy.

5.2 Results

5.2.1 Changes in efflux transporter gene expression in hepatocyte preparations

The expression of MDR1, BSEP, MRP2 and BCRP was assessed in conventional hepatocyte preparations (freshly isolated suspensions and monolayer cultures 24 and 48 hours post isolation) and in spheroid culture (5, 7, 10, 15 and 25 days post isolation). RNA was isolated from hepatic preparations and cDNA synthesised as described previously (Chapter 2).

A number of differences in transcript levels of the transporters of interest were seen in different hepatocyte cultures (Figure 5.1). In general, as seen with the expression of genes involved in xenobiotic metabolism (Chapter 4), expression levels measured in monolayer culture were lower than those measured in freshly isolated hepatocytes and, following a recovery in early spheroid culture, an increase in expression was evident in mature spheroids.

In conventional monolayer culture, there was a decline in the expression of both BSEP and BCRP at 24 and 48 hours post isolation, when compared to the level measured in freshly isolated hepatocytes. This was seen by an increase in Ct value and a corresponding fold change of 0.55 ± 0.18 ($p < 0.05$, Mann-Whitney *U* test) and 0.63 ± 0.13 ($p < 0.05$, Mann-Whitney *U* test) for BSEP and BCRP respectively in monolayer culture 24 hours post isolation and 0.51 ± 0.20 ($p < 0.05$, Mann-Whitney *U* test) and 0.79 ± 0.13 ($p > 0.05$, Mann-Whitney *U* test) respectively in monolayer culture 48 hours post isolation. In contrast to these declines, increases in the expression of MRP2 and MDR1, relative to levels measured in freshly isolated hepatocytes, were measured in monolayer cultures at the same time points. MRP2 expression in hepatocytes 48 hours post isolation exhibited a significant fold change of 2.07 ± 0.44 ($p < 0.05$, Mann-Whitney *U* test) in comparison to freshly isolated hepatocytes and there were also significant fold changes of 1.52 ± 0.25 and 2.09 ± 0.49 ($p < 0.05$, Mann-

Whitney *U* test) in the expression of MDR1 in monolayer cultures 24 hours and 48 hours post isolation respectively, when compared to freshly isolated hepatocytes.

In general, the expression levels of the efflux transporters measured in hepatocytes in spheroid culture were greater than, or equal to, levels measured in conventional primary hepatocyte cultures. During the development of spheroid structures (between 5–10 days post isolation), expression of genes for efflux transporters remained relatively stable (with the exception of BCRP expression in spheroids 10 days post isolation) although, gradual increases in the expression of MRP2 and MDR1 were seen. This increase in MRP2 expression in early spheroids, continued at subsequent time points. The highest levels of expression of all efflux transporters were measured in mature spheroids, with significant increases in the expression of MRP2 and MDR1 (7.88 ± 1.02 and 2.59 ± 0.28 fold respectively) recorded in spheroids 25 days post isolation.

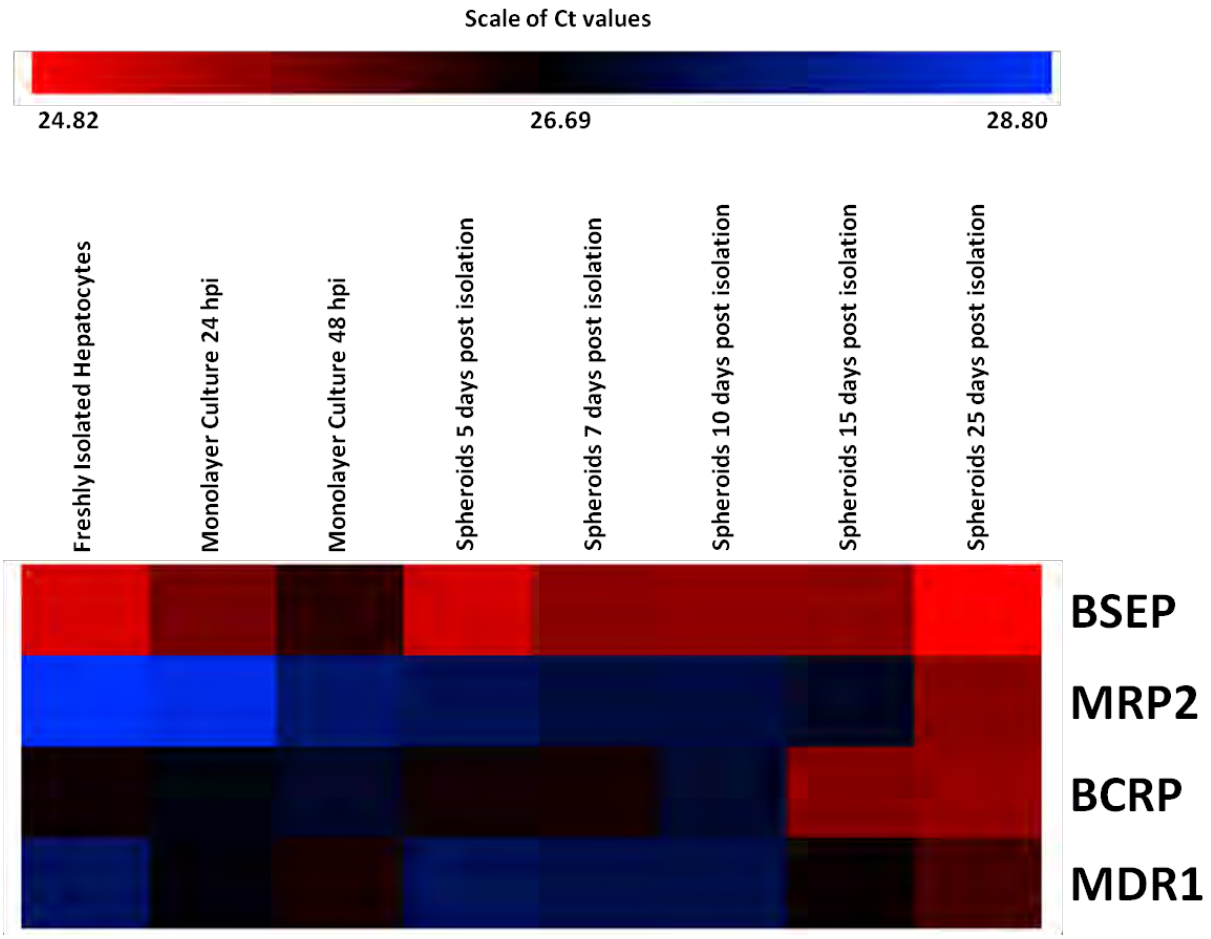


Figure 5.1: Expression levels of BSEP, MRP2, BCRP and MDR1 in freshly isolated hepatocytes, hepatocytes in monolayer culture (24 and 48 hours post isolation) and hepatocyte spheroids. Level of expression is indicated by colours representing deviation of Ct value from the median expression level of all genes measured at all conditions. The bar above the heat map represents the scale of Ct values; red representing the lowest extreme Ct value measured (therefore a greater level of expression), blue representing the highest extreme Ct value measured (therefore the lowest level of expression) and black representing the median level of expression. In general, a greater level of transporter gene expression was seen in spheroid culture, evident by the higher distribution of black and red to the right of the map. Expression levels of BSEP and BCRP appeared to decline in conventional monolayer culture, with concurrent increases in the expression of MRP2 and MDR1. Expression of transporters was relatively stable during spheroid development, with gradual increases in the expression of MRP2 and BCRP in early spheroid culture. Expression levels were greatest in the most mature spheroids, 25 days post isolation.

In all hepatocyte preparations and at all time points, BSEP was the efflux transporter exhibiting the greatest level of expression. This observation was in accordance with the expression of BSEP in whole liver samples taken from the same fish and in accord with published data (Loncar et al., 2010). In contrast, MRP2 exhibited the lowest level of expression in freshly isolated hepatocytes and hepatocytes in monolayer culture (comparable to the expression in whole liver) and the level of MDR1 expression was lowest in hepatocyte spheroids at all time points. Differences in the pattern of expression of the transporters of interest were seen between the various hepatocyte preparations. Freshly isolated hepatocytes and whole liver samples exhibited the same pattern of expression, with an altered pattern in hepatocytes in monolayer culture due to the increase in MDR1 expression. Hepatocytes in spheroid culture exhibited a different pattern of expression from isolated hepatocytes, but this was identical at all stages of spheroid culture.

Principal component analysis was conducted on samples separated by method of preparation, to compare the expression profile of the different hepatic preparations. The *in vitro* systems investigated were separated along the PC2 and PC3 axes, accounting for 79.8% and 5.0% of the observed variations respectively. Separation by these factors displayed a range of distinct differences in the profile of expression of efflux transporters between preparations (see Figure 5.2). In comparison to the profile of expression of efflux transporters *in vivo*, there was a clear grouping of hepatocytes cultured using traditional methods, which displayed the greatest difference. In contrast, hepatocyte spheroids were positioned closest in comparison to the *in vivo* profile of expression. Although there was variability in the positioning of spheroids relating to differences in the stages of maturation, in general, as seen for the expression of metabolic enzymes, the expression profile of hepatocytes in spheroid culture appeared to be closer to the *in vivo* profile than that of monolayer cultures and freshly

isolated hepatocytes. On the balance of evidence, the gene expression profiles of hepatocytes in monolayer culture and freshly isolated in suspension, offered the poorest representation of the *in vivo* profile, with hepatocytes in spheroid culture providing the best analogy to the expression profile *in vivo*.

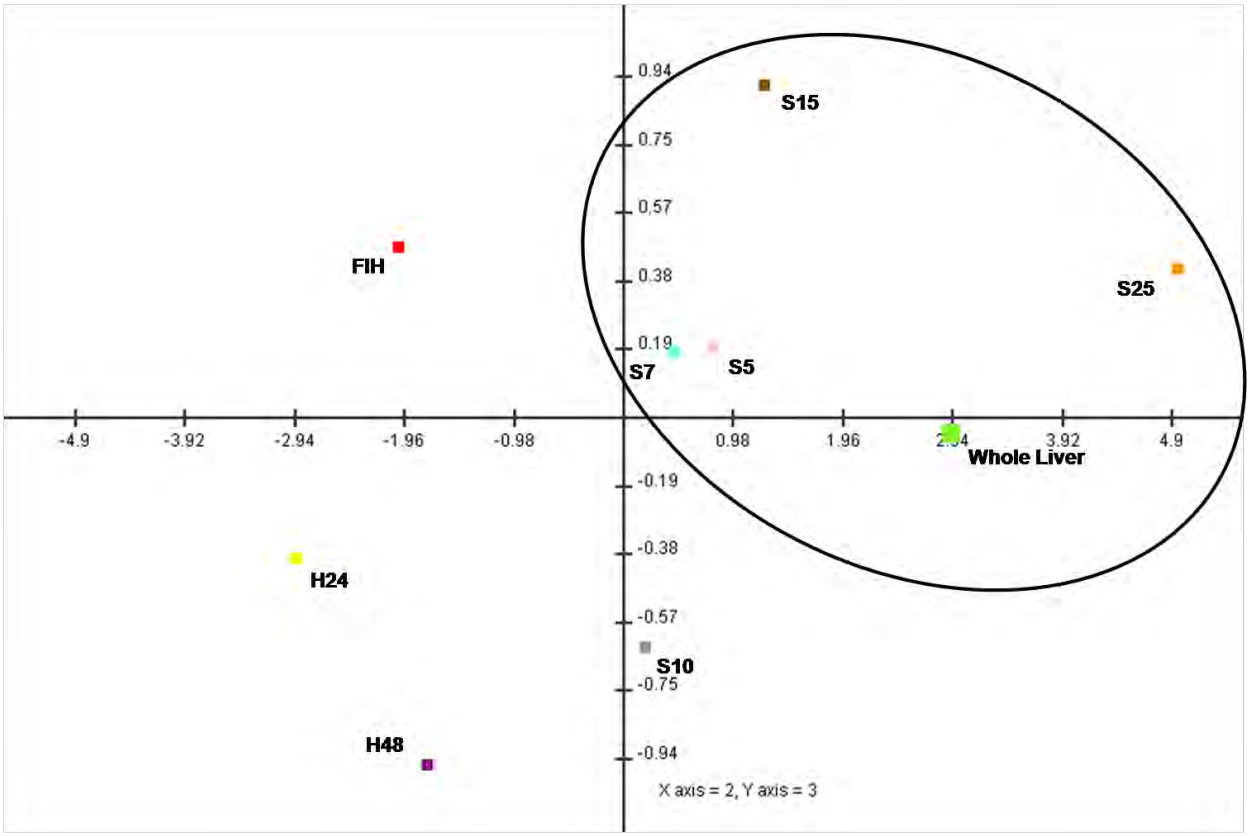


Figure 5.2: Principal components analysis (PCA) scores plot for the profile of expression of genes related to xenobiotic efflux in whole liver and all hepatocyte preparations. The plot shows separation of *in vivo* and *in vitro* preparations along the PC2 and PC3 axes. There was a clear separation of hepatocytes in monolayer culture at the greatest extreme (bottom left quadrant) from the whole liver sample. In comparison, the expression profile of freshly isolated hepatocytes was closer to that of the whole liver than to the other conventional cell culture types. Variability existed in the expression profile of spheroid hepatocytes according to stage of culture, however, spheroids at all time points displayed a better resemblance to the expression profile of the whole liver. Spheroid hepatocytes 5, 7 and 25 days post isolation showed the best similarity to the expression profile *in vivo*. FIH: Freshly isolated hepatocytes; H24: Monolayer culture 24 hpi; H48: Monolayer culture 48 hpi; H72: Monolayer culture 72 hpi; S5: Spheroid culture 5 dpi; S7: Spheroid culture 7 dpi; S10: Spheroid culture 10 dpi; S15: Spheroid culture 15 dpi; S25: Spheroid culture 25 dpi.

5.2.2 Functional activity of efflux transporters in spheroid hepatocytes

The function of the efflux transporters of interest by specific inhibition was assessed using the fluorescent substrate and inhibitor combinations outlined in Chapter 2. The intracellular presence and accumulation of these substrates in response to inhibition was observed and confirmed by confocal microscopy (Figures 5.3–5.6). Greater accumulation of the fluorescent substrates calcein-AM, rhodamine 123, H33342 and 2',7'-dichlorodihydrofluoroscein-diacetate (DCDHFDA) in spheroids at maximum inhibitor concentrations used was evident, when compared to uninhibited controls.

Increased intracellular accumulation of substrates as a result of specific inhibition was quantified using a fluorescence plate reader and compared to the fluorescence measurements recorded in control cells. All inhibitors investigated caused significant changes in substrate accumulation (Figures 5.7–5.10). The highest maximal fluorescence accumulation in spheroids was seen with sodium taurocholate, used to inhibit BSEP. A 3.70 fold increase in DCDHFDA accumulation was measured at 1.0 and 1.5 mM concentrations of sodium taurocholate. Inhibition of DCDHFDA efflux was not significant below 500 μM sodium taurocholate concentrations (Figure 5.7).

In comparison, fluorescence accumulation as a result of BCRP inhibition by Ko143 was considerably lower, with a maximum 1.38 fold increase in H33342 accumulation. BCRP inhibition by Ko143 caused significant increases in fluorescence at concentrations above 2 μM ($p < 0.05$, Mann-Whitney U test) but this inhibitor did not exhibit a distinct concentration-dependent increase of H33342 (Figure 5.8).

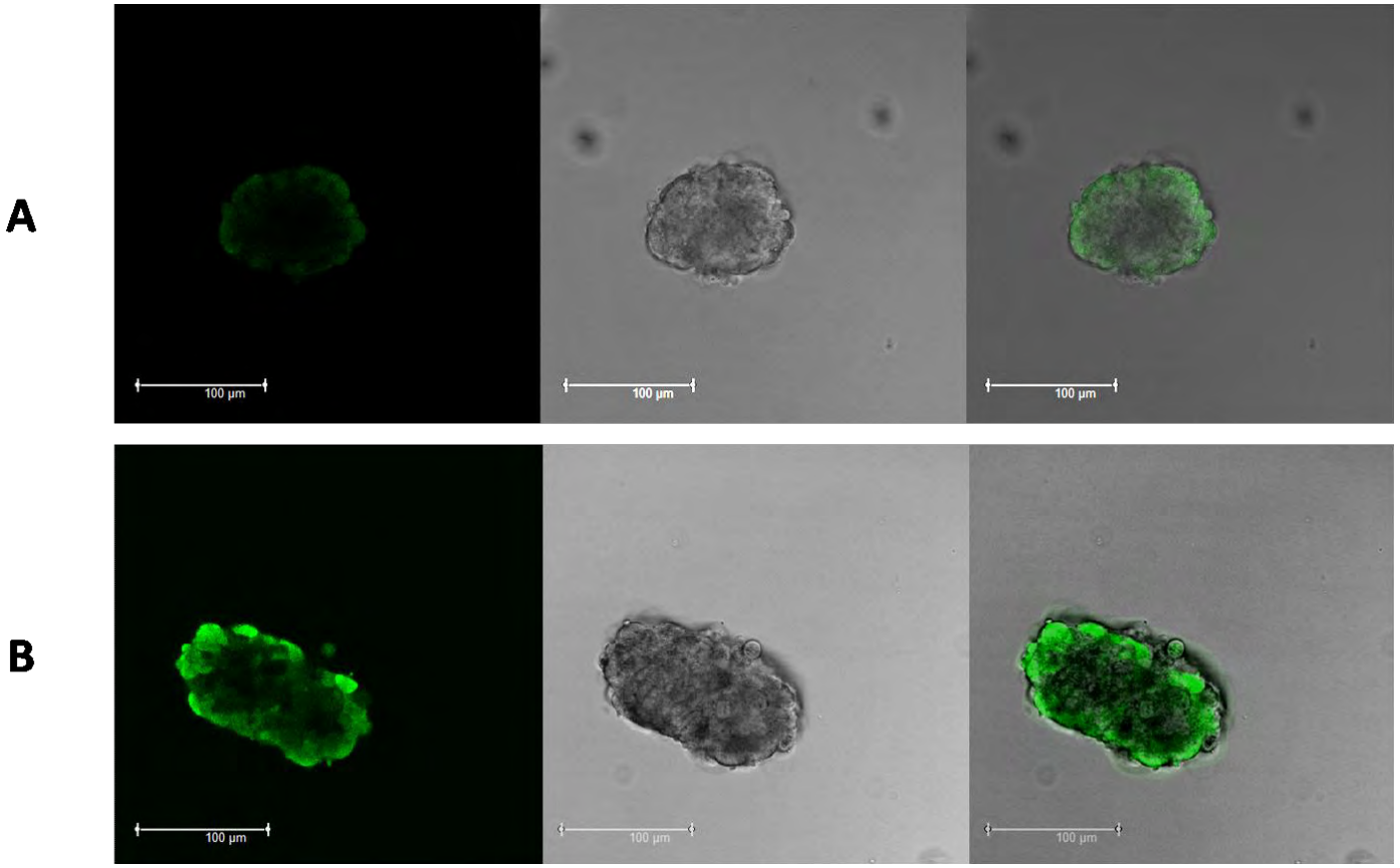


Figure 5.3: Visualisation of the accumulation of DCDHFDA in hepatocytes in spheroid culture following treatment with 1500 µM sodium taurocholate. The intracellular accumulation of DCDHFDA in spheroids was observed using confocal microscopy. A greater accumulation of fluorescence was observed in cells treated with sodium taurocholate (panel B) in comparison to untreated cells (panel A). Scale bars indicate 100 µm.

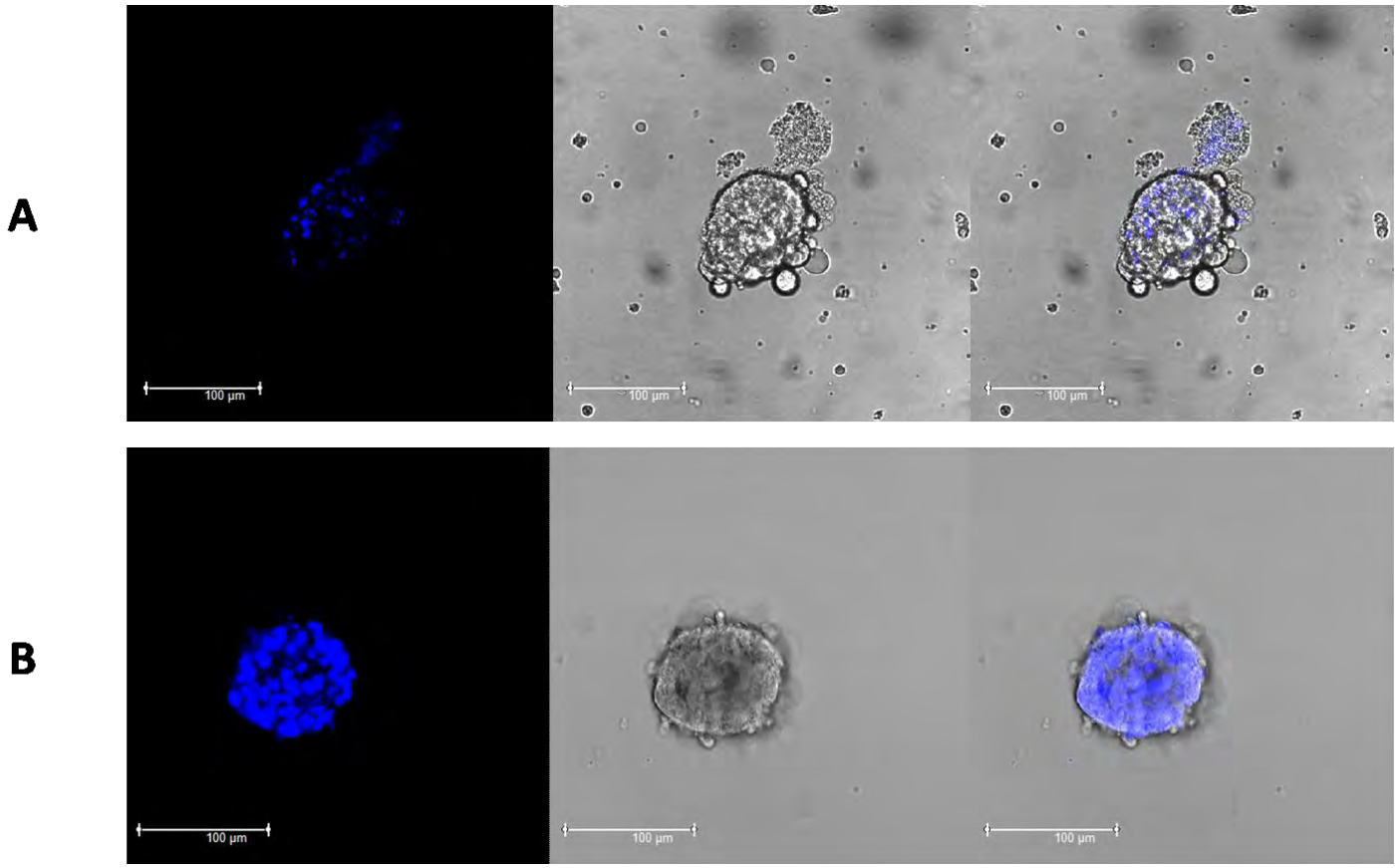


Figure 5.4: Visualisation of the accumulation of H33342 in hepatocytes in spheroid culture following treatment with the BCRP specific inhibitor Ko143 (20 µM). The intracellular accumulation of H33342 in spheroids was observed using confocal microscopy. A greater accumulation of fluorescence was evident in cells treated with Ko143 (panel B) in comparison to untreated cells (panel A). Scale bars indicate 100 µm.

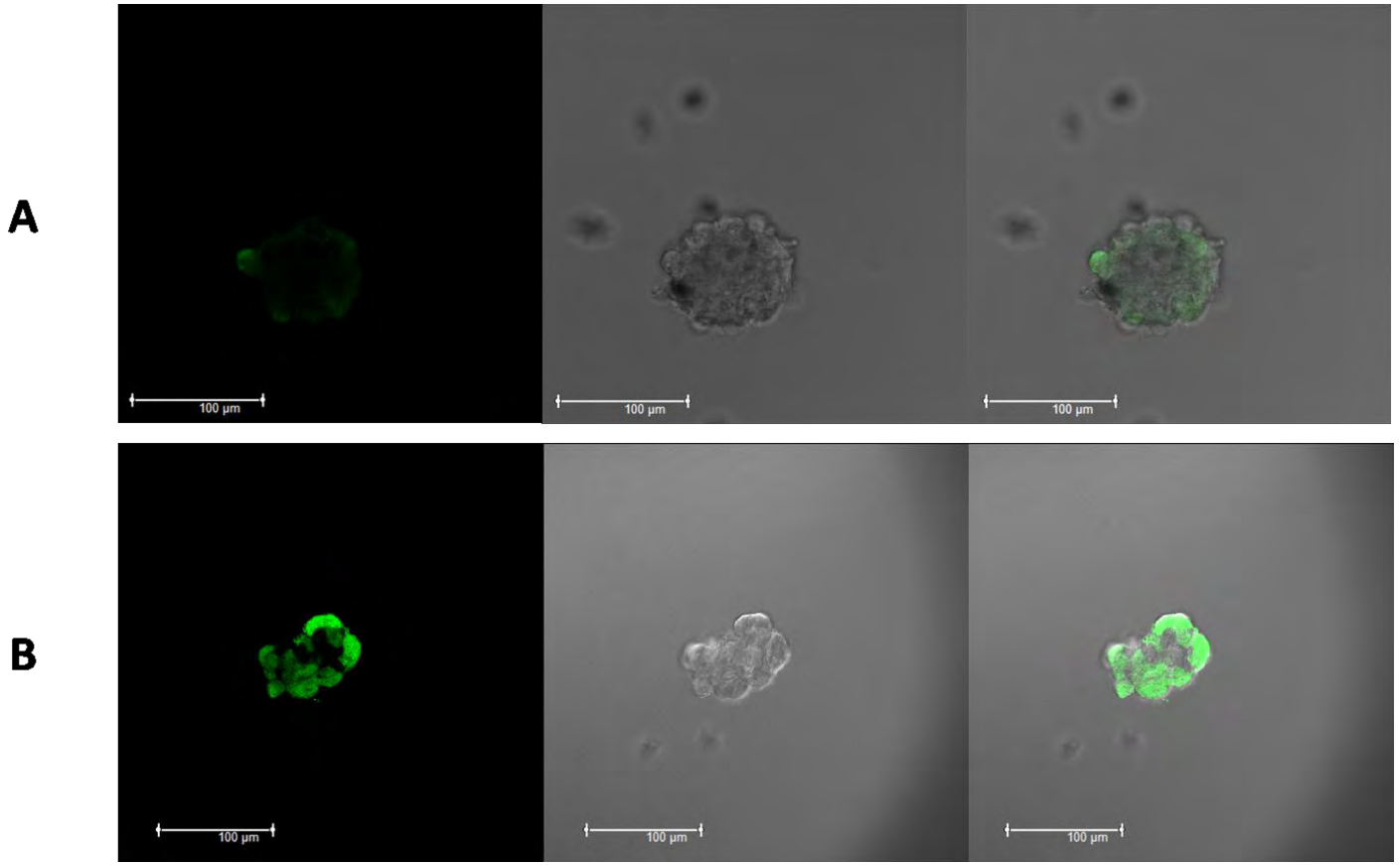


Figure 5.5: Visualisation of the accumulation of calcein-AM in hepatocytes in spheroid culture following treatment with the MRP2 inhibitor probenecid (1000 µM). The intracellular accumulation of calcein-AM in spheroids was observed using confocal microscopy. A greater accumulation of fluorescence was evident in cells treated with probenecid (panel B) in comparison to untreated cells (panel A). Scale bars indicate 100 µm.

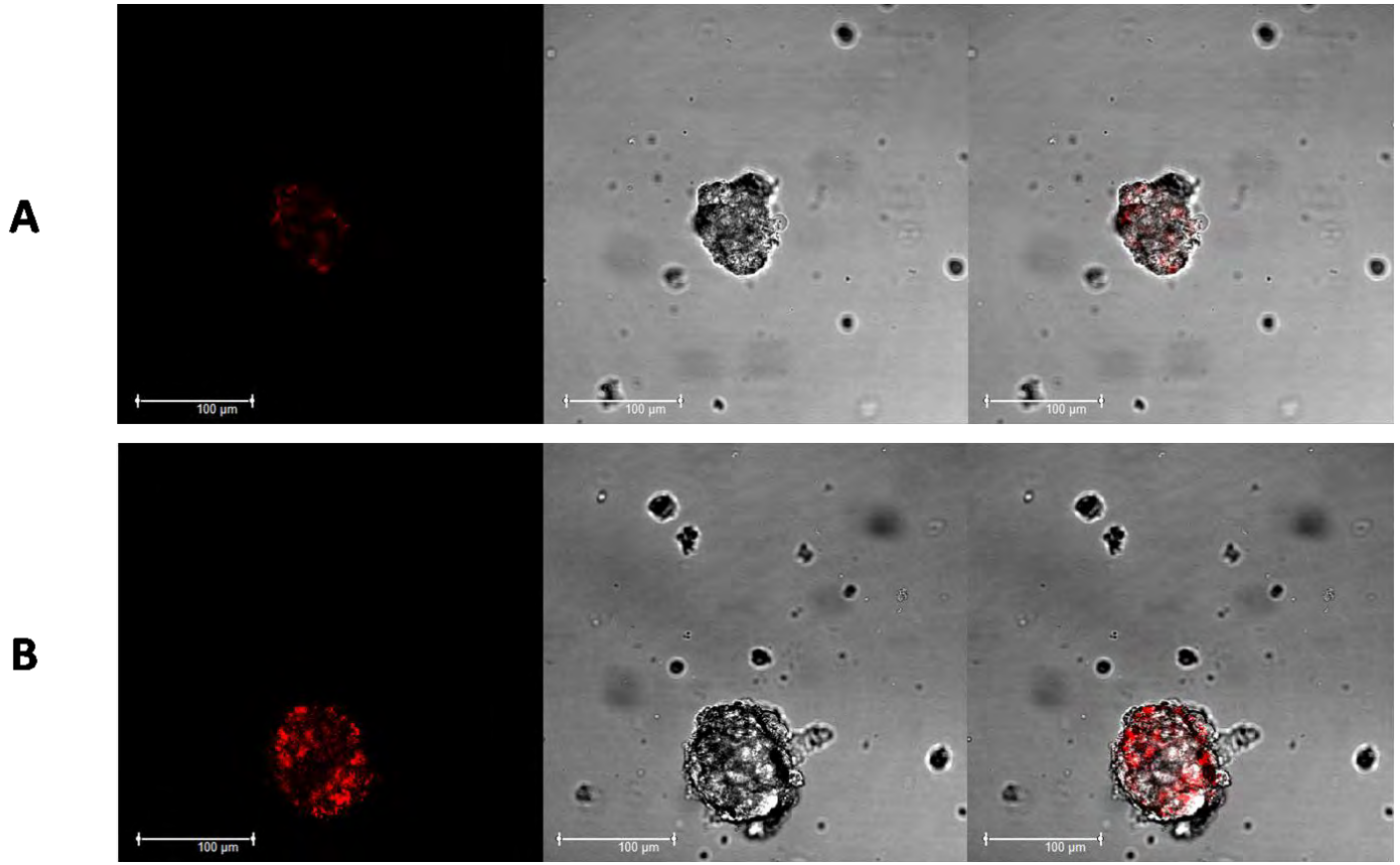


Figure 5.6: Visualisation of rhodamine 123 accumulation in hepatocytes in spheroid culture following treatment with the MDR1 inhibitor cyclosporin A (20 µM). The intracellular accumulation of rhodamine 123 in spheroids was observed using confocal microscopy. A greater accumulation of fluorescence was observed in cells treated with cyclosporin A (panel B) in comparison to untreated cells (panel A). Scale bars indicate 100 µm.

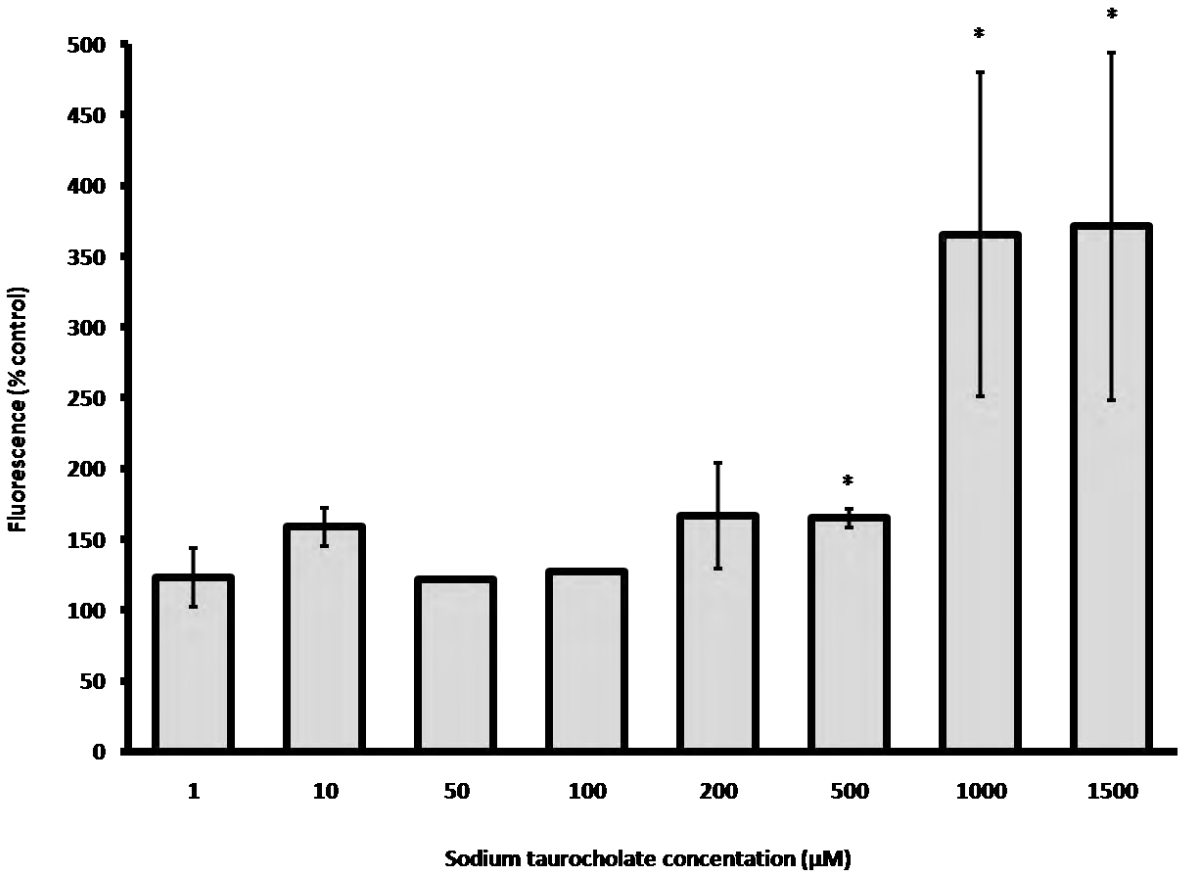


Figure 5.7: The effect of sodium taurocholate on the accumulation of DCDHFDA in hepatocytes in spheroid culture. The intracellular accumulation of DCDHFDA in spheroids was measured when exposed to different concentrations of the BSEP inhibitor sodium taurocholate. Data represent mean fluorescence accumulation relative to uninhibited controls and expressed as a percentage increase ± SEM. Control fluorescence accumulation = 100%; $^{*}p < 0.05$ (significantly greater than control), Mann-Whitney *U* test (n = 3 fish).

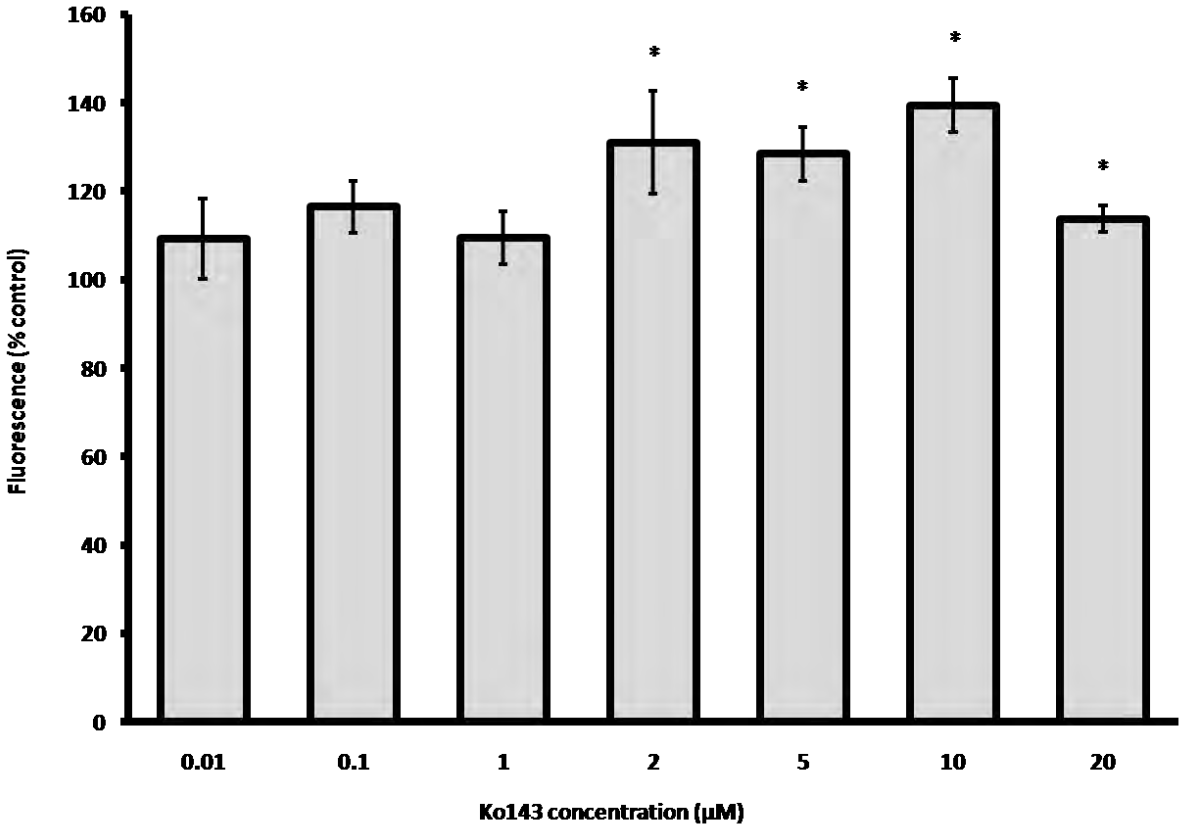


Figure 5.8: The effect of Ko143 on the accumulation of H33342 in hepatocytes in spheroid culture. Intracellular accumulation of H33342 in spheroids was measured following incubation with different concentrations of the BCRP specific inhibitor Ko143. Data represent mean fluorescence accumulation relative to uninhibited controls and expressed as a percentage increase ± SEM. Control fluorescence accumulation = 100%; $^{*}p < 0.05$ (significantly greater than control), Mann-Whitney *U* test (n = 3 fish).

Probenecid on the other hand, caused concentration-dependent increases of calcein-AM accumulation, through the inhibition of MRP2 in spheroids. At the lowest concentrations investigated (0.5–10 μM), up to a 1.8 fold accumulation of calcein-AM was measured, however this was not significantly greater in comparison to the fluorescence in control cells. Subsequently, at 30 and 50 μM concentrations, it appeared that there was reduced inhibition of MRP2, as fluorescence levels were similar to those measured in control cells. Significant increases in fluorescence accumulation ($p < 0.05$, individual samples *t*-test) were measured in cells exposed to probenecid at concentrations above 100 μM, with a maximum 2.95 fold accumulation measured at 1 mM (Figure 5.9).

Cyclosporin A appeared to stimulate MDR1 activity at concentrations ≤ 2 μM as there was a decline in rhodamine 123 fluorescence measured in treated samples when compared to uninhibited controls, most likely due to sample variance. Fluorescence was significantly lower than measured in control cells at the 0.01 μM cyclosporin A concentration ($p < 0.05$, Mann-Whitney *U* test). Inhibition of MDR1 was seen at cyclosporin A concentrations ≥ 5 μM with subsequent concentration-dependent increases in intracellular fluorescence accumulation. These increases were statistically significant at 10 μM and 20 μM concentrations, with 1.5 fold and 1.8 fold increases in intracellular rhodamine 123 accumulation respectively (Figure 5.10).

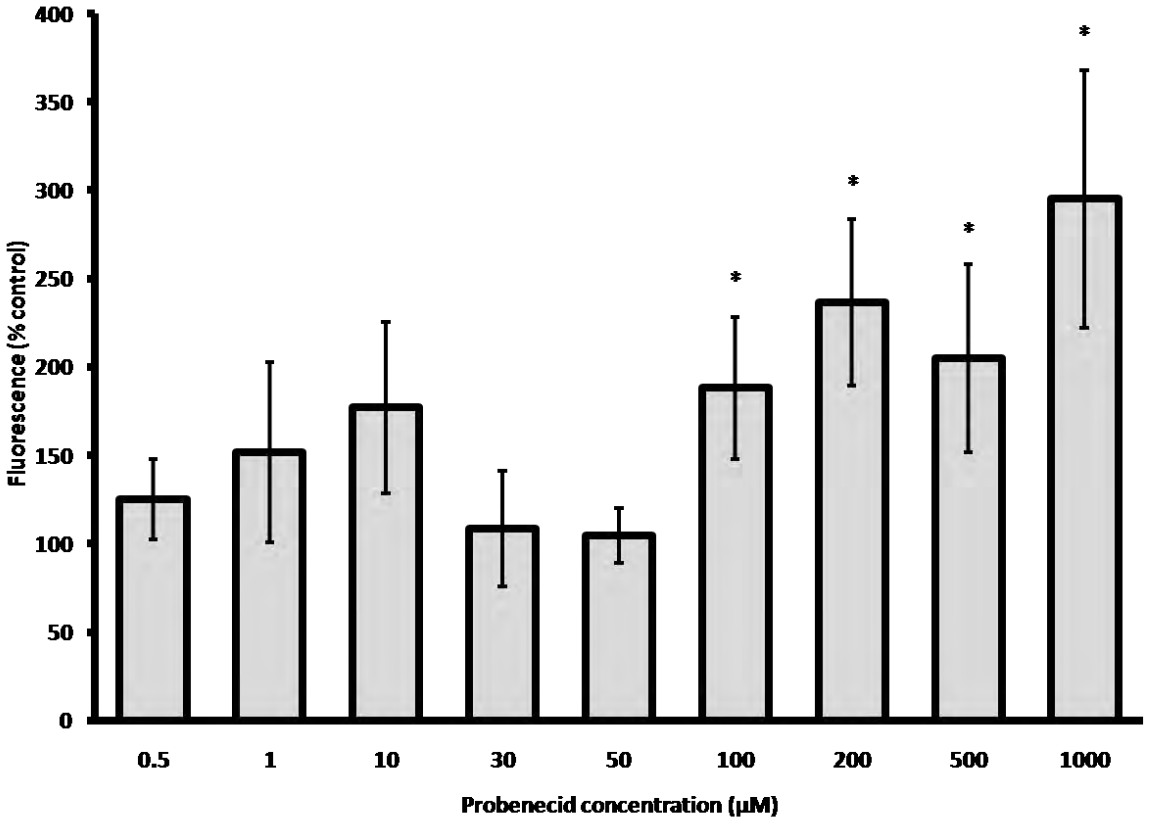


Figure 5.9: The effect of the MRP2 inhibitor probenecid on the accumulation of calcein-AM in hepatocytes in spheroid culture. The intracellular accumulation of calcein-AM in spheroids was measured when exposed to different concentrations of the MRP2 inhibitor probenecid. Data represent mean fluorescence accumulation relative to uninhibited controls and expressed as a percentage increase ± SEM. Control fluorescence accumulation = 100%; $^{*}p < 0.05$ (significantly greater than control), Mann-Whitney *U* test (n = 3 fish).

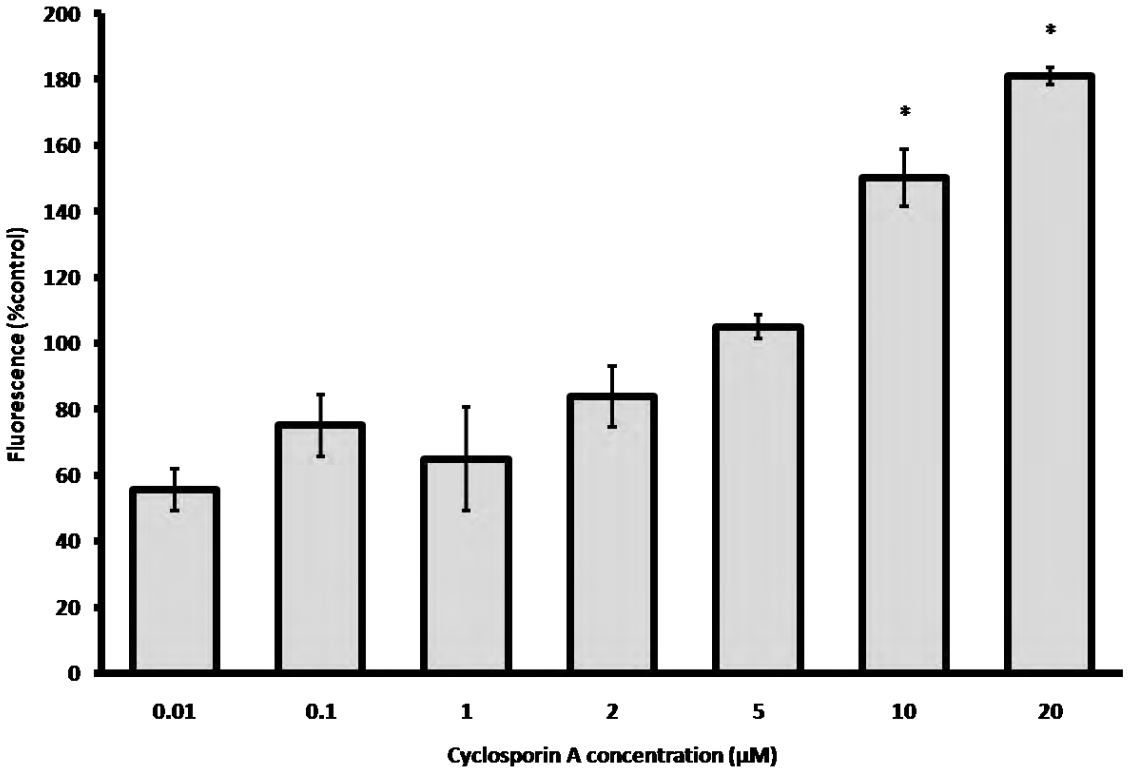


Figure 5.10: The effect of Cyclosporin A on the accumulation of rhodamine 123 in hepatocytes in spheroid culture. Intracellular accumulation of rhodamine 123 in spheroids was measured following incubation with different concentrations of the MDR1 inhibitor cyclosporin A. Data represent mean fluorescence accumulation relative to uninhibited controls and expressed as a percentage increase ± SEM. Control fluorescence accumulation = 100%; $^{*}p < 0.05$ (significantly greater than control), Mann-Whitney *U* test (n = 3 fish).

The fluorescent bile acid CLF was found to be taken up into spheroids and subsequently released with time. This release was inhibited by sodium taurocholate as an inhibitor of BSEP (Figure 5.11). CLF accumulation was significant at concentrations ≥ 50 μM. Figure 5.12 shows spheroids which were treated with fluorescent CLF and allowed to efflux the marker over a period of 30 minutes, revealing some evidence of the concentration of pools of fluorescence which was punctate and did not correspond to the structure of whole cells. It is possible that this punctate labelling is reflective of the presence of canalicular structures from which bile release from the spheroids is mediated.

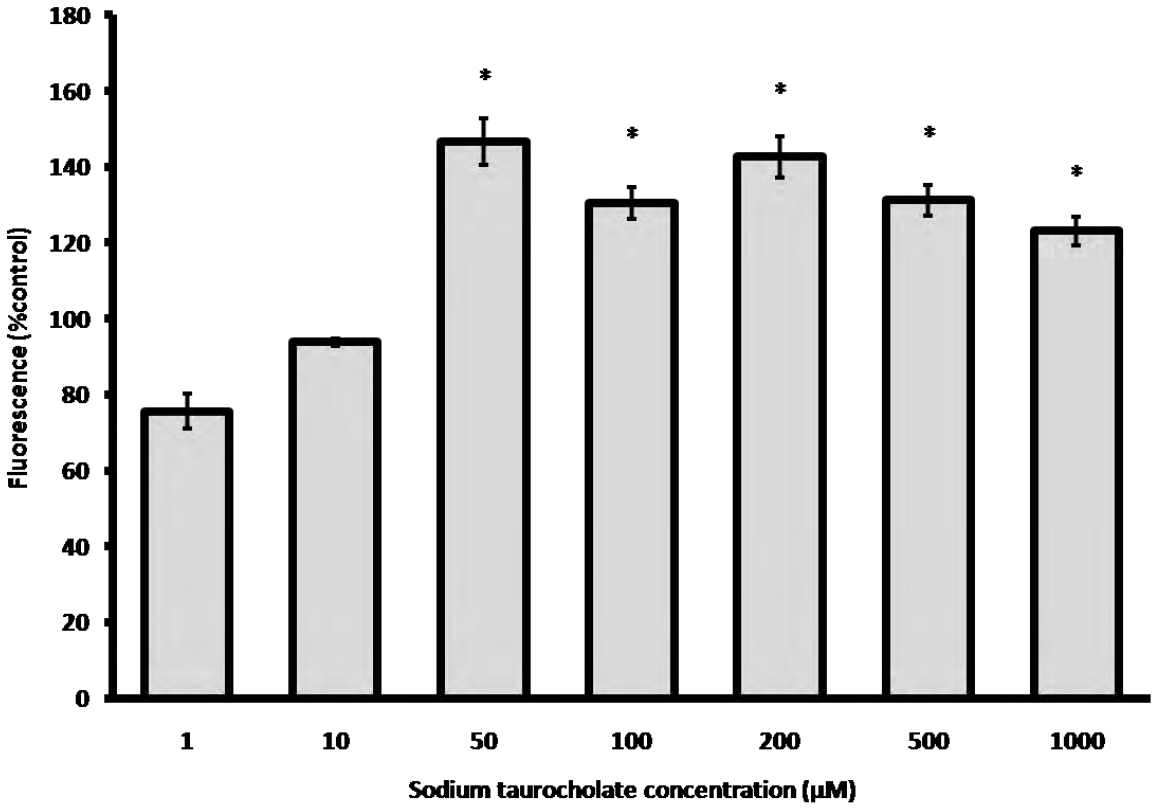


Figure 5.11: Cholyl-lysyl-fluorescein (CLF) accumulation following sodium taurocholate treatment in hepatocytes in spheroid culture. Intracellular accumulation of CLF in spheroids was measured following incubation with different concentrations of the BSEP inhibitor sodium taurocholate. Data represent mean fluorescence accumulation relative to uninhibited controls and expressed as a percentage increase ± SEM. Control fluorescence accumulation = 100%; $^{*}p < 0.05$ (significantly greater than control), Mann-Whitney *U* test (n = 3 fish).

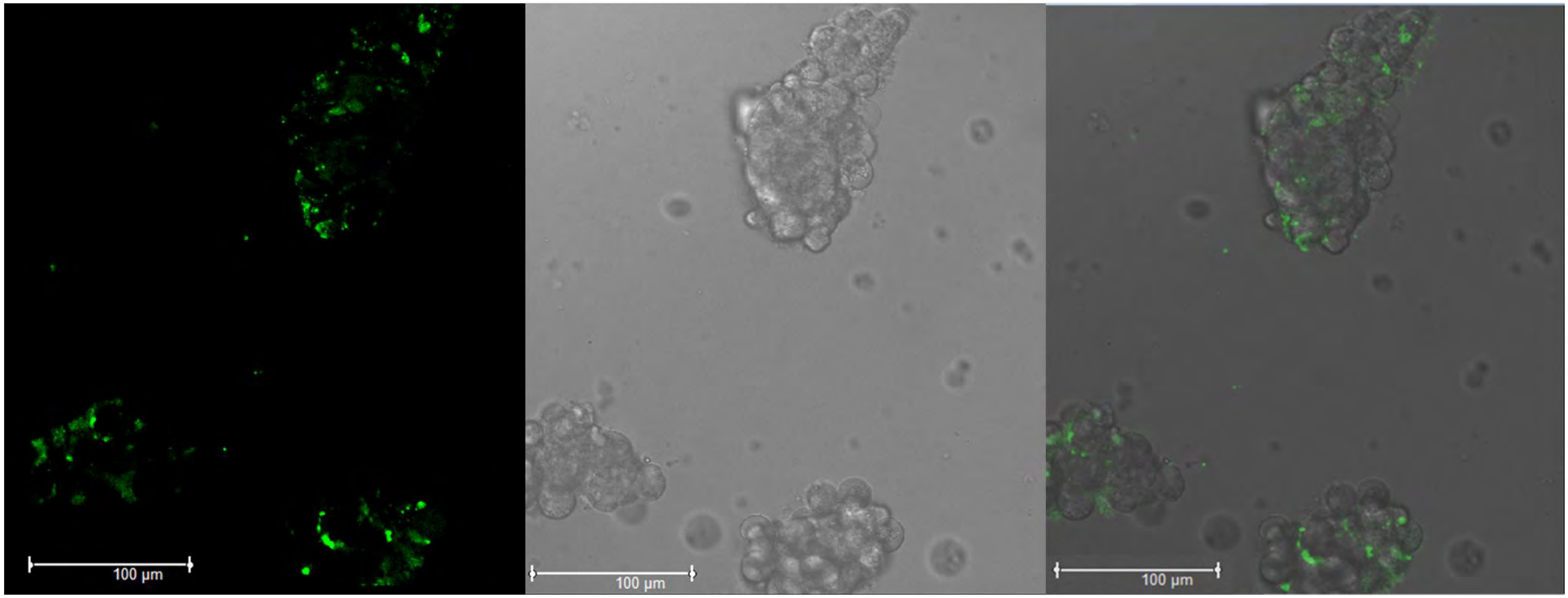


Figure 5.12: Confocal imaging of CLF distribution within spheroids. Spheroids were treated with CLF and allowed to efflux the compound over a period of 30 minutes and images show evidence of the concentration of the fluorescent compound in these structures. Staining was punctate and did not directly correspond to localisation within individual whole cells. This may reflect the presence of canalicular structures from which bile release from hepatocyte spheroids is mediated.

5.3 Discussion

The transport of compounds into and out of cells is an extremely important feature of xenobiotic detoxification, with the process having a key influence on the absorption, distribution, elimination, toxicity and efficacy of compounds. For the majority of compounds, transport and metabolism must be assessed together to allow accurate predictions of *in vivo* disposition, physiological effects and xenobiotic fate to be made (Kim, 2002, Brandon et al., 2003). Importantly, this is a feature lacking in conventional metabolic assessments implicated in ERA, conducted using subcellular fractions, where the cellular uptake and elimination of a xenobiotic is assumed. The aim of this investigation was to explore additional advantages of the use of hepatocytes in spheroid culture to those identified in Chapter 4. The potential for the incorporation of measurements of transporter function in metabolic assessment would make such assays more informative, with respect to the evaluation of bioaccumulation potential and therefore more relevant to the detoxification of compounds *in vivo*.

In this study, the expression of all of the MXR transporters investigated was identified in hepatocytes in spheroid culture at all stages of development. Although some variation in levels of expression was evident at different time points in spheroid culture, transcript levels were generally greater than those measured in conventional cell cultures. As seen with the expression of genes involved in xenobiotic metabolism (Chapter 4), there was a general down-regulation of expression in monolayer culture, most likely to be associated with cellular de-differentiation. Following a recovery period in early spheroid culture, a general increase in transporter expression was evident in mature spheroids, with the greatest expression levels measured in culture 25 days post isolation. The 3-dimensional structure of hepatocytes and enhanced cell-cell contacts in spheroid culture is thought to be responsible for the restoration and maintenance of cellular polarisation and differentiation status, lost during the isolation

process and in conventional culture, which is likely to be a contributing factor to such maintenance of liver specific functional activity (Landry et al., 1985, Ma et al., 2003). The importance of differentiation status on the expression of the uptake transporter organic anion transporter polypeptide (OATP) and the efflux transporter MRP1 has also been shown previously in trout cell lines (Boaru et al., 2006, Loncar et al., 2010), therefore, maintenance of this feature in spheroid hepatocytes confers significant advantages, making this model an attractive alternative for the *in vitro* study of drug transporters.

Although the expression of BSEP and BCRP remained relatively stable in early spheroid culture (5–10 days post isolation), a gradual increase in the expression of MRP2 and MDR1 was evident at these time points. This was in common with increases in the expression of the same transporters in monolayer culture, both of which continued with time in culture, when compared to expression in freshly isolated hepatocytes. MRP2 is involved in the transport of a variety of conjugated xenobiotics and endogenous compounds including bilirubin and estradiol (Kubitz et al., 1999, Smeets et al., 2004). A plethora of such physiological substrates are present in cell culture media and serum supplements and so may maintain the function of this transporter, as seen with the high expression of transporters of the MRP subfamily in rainbow trout cell lines (Fischer et al., 2011). Similarly, the increases in MDR1 expression may be as a result of the presence of antibiotic and antimycotic solutions included in cell cultures and therefore present for a longer period with spheroids. Antibiotics are known exogenous substrates of MDR1 along with a wide range of unmodified pharmaceuticals, including ibuprofen (Kim, 2002, Faassen et al., 2003, Fischer et al., 2011). Despite these increases, in common with the general pattern of expression in the liver, MRP2 and MDR1 expression levels measured in spheroids were the lowest of all transporters investigated.

As was true with the expression of genes related to xenobiotic metabolism, hepatocytes in spheroid culture were most representative of the profile of expression of efflux transporters *in vivo*. Despite the difficulties in making direct comparisons due to the differences in cellular composition mentioned previously (Section 4.3), it was again clear that the profile of expression of hepatocytes in monolayer culture and in freshly isolated cells exhibited a greater difference to whole liver samples, compared to spheroids. Temporal differences were evident in the profile of expression of efflux transporters in hepatocyte spheroids, with cells 10 days post isolation showing the greatest deviation with respect to the *in vivo* profile. Hepatocytes in spheroid culture 25 days post isolation demonstrated the greatest analogy to the expression profile of whole liver samples. This temporal and transient decline in gene expression was concurrent with that of the expression of genes responsible for xenobiotic metabolism at 10 and 15 days post isolation (Chapter 4). This correlates with the reported timing of the cessation of the fusion process in matured spheroids (Baron et al., 2012).

In common with liver expression, in all *in vitro* preparations and at all time points in culture, the expression of BSEP was the greatest of all transporters investigated. This was in accordance with a study in which BSEP expression in trout liver tissue samples was measured as 750 fold and 114 fold greater than MDR1 and MRP2 respectively (Zaja et al., 2008b). High hepatic BSEP expression is also a common feature measured in mammalian systems, however in contrast, expression of the transporter in a study using a variety of trout cell lines (including 3 of hepatic origin) was among the lowest of those investigated (Fischer et al., 2011). In addition to this, no functional activity of BSEP or BCRP was identified in trout hepatocyte-derived cell lines, despite them being the highest expressed transporters *in vivo*. BSEP is involved in the efflux of a wide range of both endogenous and exogenous

compounds, with a particular role in the secretion of bile acids into the bile canaliculus (Wang et al., 2003, Loncar et al., 2010). The lack of OATP which imports bile acids into hepatocytes and the absence of a canalicular structure has been suggested as a cause for the low expression and lack of function of BSEP in trout cell lines (Alrefai and Gill, 2007, Fischer et al., 2011). Rat hepatocyte couplets exhibit bile canalicular structures into which bile constituents are secreted (Orsler et al., 1999, Coleman and Roma, 2000) indicating the importance of cell-cell structural interactions. In the current investigation, it was observed that hepatocytes connected via spheroid structure retain BSEP expression and function and there is some evidence of canalicular structure. The uptake and release of the fluorescent marker CLF as a bile constituent occurred in spheroids, and release was inhibited by sodium taurocholate. These findings again suggest advantageous features of spheroids for use in studies of xenobiotic transport in comparison to the use of hepatocyte derived cell lines.

To assess whether the related protein products from the genes of interest were also functional in spheroid cultures, transporter activities were investigated, using known specific fluorescent substrates and inhibitors used in mammalian efflux transporter assays. Many of the substrate and inhibitor combinations used in this study have also been used in previous studies involving fish *in vitro* hepatic preparations (Zaja et al., 2007, Caminada et al., 2008, Fischer et al., 2011).

Significant differences in the accumulation of fluorescent substrates were seen in spheroids at the highest inhibitory concentrations of all compounds used, suggesting that all of the transporters of interest identified in spheroids were functional and susceptible to inhibition. In common with the high level of expression of BSEP in spheroids, the inhibition of this transporter resulted in the greatest substrate accumulation. A 3.7 fold increase in intracellular DCDHFDA accumulation in comparison to control was seen when exposed to

1.0 and 1.5 mM concentrations of sodium taurocholate. High levels of DCDHFDA accumulation in spheroids was in agreement with the inhibition of BSEP in trout hepatocytes in monolayer culture (Zaja et al., 2008b). This highlights the potential importance of biliary excretion pathways in rainbow trout.

MRP2 inhibition by probenecid resulted in a marked increase in the intracellular accumulation of calcein-AM, with a 2.95 fold increase in the accumulation of the substrate at the maximum inhibitor concentration used. Despite having a low level of expression relative to other efflux transporters, MRP2 inhibition resulted in the second greatest level of fluorescence accumulation. This feature may be related to the maintenance of physiological function of MRP2, through the presence of substrates provided in the cell culture environment. Although one study in rat isolated kidneys showed, in contrast, a stimulation of MRP2 and MRP4 mediated transport at probenecid concentrations in the region of 10 μM (Smeets et al., 2004), possibly due to calcein-AM being a substrate of MDR1 (Essodaigui et al., 1998). No evidence of such an effect was seen in the present investigation.

A lack of knowledge of the exact specificities of inhibitors and the broad and overlapping substrate specificities of efflux transporters proves problematic in the accuracy of functional transporter assays. This is especially true for the assessment of transporters in fish as the assumption of activities is based on the mammalian literature. Recent studies have highlighted the relatedness of MDR1, BSEP and MRP-group transporters, raising questions on the inhibitory specificity of cyclosporin A and the transporter specificity of rhodamine 123 and calcein-AM among an extensive list of others (Qadir et al., 2005, Caminada et al., 2008, Zaja et al., 2008b, Fischer et al., 2011). An important feature to explore further in hepatocyte spheroids is the use of transporter inhibitor co-exposure, to block multiple efflux pathways, in an effort to circumvent the lack of knowledge of substrate specificity for transporters.

Although BCRP was the efflux transporter with the second greatest level of expression, inhibition of BCRP resulted in the lowest accumulation of fluorescent substrate. Maximal H33342 accumulation in spheroids reached 140% of control when incubated with 10 μM Ko143. The high levels of BCRP expression in spheroids and indeed in whole liver suggest that the protein is a key mediator of xenobiotic efflux in rainbow trout hepatocytes (Zaja et al., 2008c). As a result, the lack of fluorescent accumulation may be due to a discrepancy of transcript and protein expression levels in spheroids or that higher concentrations of inhibitor are required. It is also possible that inhibition of this transporter, resulted in the up-regulation of another, closely related transporter (e.g. a member of the ABCC subfamily), with overlapping specificity, allowing for the continued efflux of the fluorescent substrate.

A number of environmentally relevant compounds have been shown to modify the expression and function of transporters in aquatic organisms, which could lead to the accumulation of compounds in organisms as a result of exposure to mixtures of environmental contaminants (Daughton and Ternes, 1999, Caminada et al., 2008). As a result, information on the efflux of compounds and their potential to inhibit transporter function is a feature which should be included in the assessment of environmental contaminants. The presence of these transporters in the intestinal lumen also show that they play a role in the limitation of compound uptake (Glavinas et al., 2004). In this study, emphasis has been placed on the efflux of compounds, however in the prediction of the potential of a compound to bioaccumulate, the kinetics of uptake, influenced by factors such as lipid solubility and intestinal transporter activity should also be considered.

This study is the first to assess trout ABC transporters in long term culture, showing the improved expression of the key efflux transporters, in comparison to other cell culture models. The system has the potential to be adapted for routine use in the assessment of

environmental contaminants as this would not only confer advantages of longevity and maintained metabolic activity as discussed previously, but also will enable the incorporation of measurements of compound efflux and inhibition, affirming such cultures as a much more informative model for the screening of bioaccumulation potential.

Chapter 6

General Discussion

6.1 General discussion

Bioaccumulation potential is a key parameter in the environmental risk assessment of pharmaceuticals and chemical contaminants. *In vivo*, this type of assessment often uses in excess of 300 fish per compound, can last for more than 4 weeks and can prove technically difficult. In an effort to reduce the number of organisms used, increase the throughput of screening and to provide more economical alternatives, robust *in vitro* testing strategies have been sought (Caminada et al., 2006, Halder et al., 2010). This has become especially important since the introduction of regulations for the testing of chemical contaminants, resulting in the potential requirement of the assessment of more than 140,000 substances in Europe, a process that has been projected to require the use of around 54 million vertebrate animals (Hartung and Rovida, 2009).

Studies using subcellular fractions, especially hepatic S9, have long been promoted and recently advocated as an effective *in vitro* alternative, due to the ease with which they can be prepared and used (Johanning et al., 2012). Whilst being compliant with the 3Rs principles of reduction, refinement and replacement, this study has identified a number of limitations to the use of subcellular fractions for this type of assessment and recognises the utility of trout hepatic cells as a superior alternative. However, the use of these cells in conventional culture is also not without limitation, which is also documented in the scientific literature (Walker and Woodrooffe, 2001, Castell and Gomez-Lechon, 2009). A number of cell culture techniques can be employed to circumvent these limitations and this project introduces the application of fish hepatic 3D aggregate spheroid cultures to the ecotoxicology arena. The use of this *in vitro* system confers a number of advantages over other types of cell culture, the key feature being the potential for use in the assessment of highly persistent chemicals that require long term incubation. Spheroids also offer a timely advantage for assessment of toxicity, with

the recent concerns raised over the environmental impact of contaminants exhibiting chronic effects (Hutchinson, 2008).

6.2 Limitations of S9 fractions in metabolic assessment

The work in Chapter 3 identified a number of major limitations, both quantitative and qualitative, in the ability of liver S9 fractions to reflect hepatic xenobiotic metabolism. Metabolic studies relating to bioaccumulation assessment are based on the principle that there is an inverse relationship between the degree of metabolism of a compound and its bioaccumulative potential. When compared to intact primary trout hepatocytes, the remarkably lower metabolic activity towards 7-ethoxyresorufin, short-lived activity in incubations and qualitative differences in production of ibuprofen metabolites, showed the potential of S9 fractions to significantly under-predict metabolic potential and therefore over-estimate bioaccumulation. Lack of cellular integrity is likely to correspond to a lack of *in vivo* representation. Indeed, it has been shown that human liver microsomes, were also ineffective at predicting *in vivo* hepatic clearance for similar reasons (Ito and Houston, 2005, Brown et al., 2007). These features highlight S9 fractions as an inferior model for use in ERA studies in comparison to studies using whole cells. The results from the ibuprofen investigation presented in Chapter 3 are in accord with a study assessing the ability of rat *in vitro* preparations to mimic *in vivo* drug metabolism, in which S9 fractions did not produce the same metabolites of a number of compounds as intact cells or *in vivo* systems and a discrepancy in phase II metabolism was also seen (De Graaf et al., 2002). The use of S9 fractions is further limited by the association with a number of inherent assumptions, including the cellular uptake and elimination of all compounds; processes known to play key

roles in xenobiotic detoxification and therefore have an impact on bioaccumulation (Kim, 2002).

Despite their ease and rapidity of preparation and the requirements of reduced maintenance in comparison to the use of whole cells, a number of practical disadvantages are associated with the use of S9 fractions. No standardised method for the preparation or use of these fractions is available, providing a number of parameters through which inter-laboratory variation may be introduced, at levels which may directly influence metabolic profiles. For example, often, such incubations do not include co-factors for conjugation reactions and frequently, incubations persist for extensive time periods, often longer than one hour, thus beyond initial reaction rates (Cowan-Ellsberry et al., 2008, Han et al., 2009, Gomez et al., 2011). Furthermore, although the term S9 refers to the use of 9000 g supernatants, this is not globally defined and, often, alternative speeds for centrifugation are used whilst still maintaining use of the term S9 (Johanning et al., 2012). As a result, this would alter the composition of the fractions used in metabolic assessment and may have a direct effect on the metabolic outcome. For this reason, a degree of harmonisation is required to confer uniformity and provide greater comparability for bioaccumulation predictions in regulatory assessment. Recently major concerns in industry have also been raised over both inter- and intra-laboratory variability of the outcomes of studies involving subcellular fractions (Owen, S.F., pers. comm.).

6.3 Assessment of the use of conventional cell cultures

In the current investigation, it has been demonstrated that the metabolic potential of whole hepatocytes is superior to subcellular fractions and therefore may prove a model more reflective of *in vivo* metabolism. Although previous studies have assessed the activity of trout liver subcellular fractions, no direct comparison has been made between cellular and subcellular preparations from the same liver in trout. The evidence from Chapter 3 confirmed that whole cells provide a more complete and integrated metabolic profile. Isolated hepatocytes maintain many of the functional and morphological properties of hepatocytes *in vivo* retaining a greater degree of complexity in comparison to subcellular fractions. A key example from this study is the presence of cofactors of phase II metabolic enzymes. Inclusion of these cofactors showed the potential effects of phase I product inhibition in EROD assays.

Although there is a significant improvement in terms of metabolic activity afforded by whole hepatocytes both in suspension and in monolayer culture, these *in vitro* systems were also shown to have their own limitations. A decline in metabolic activity with time in culture was shown; with sequentially lower EROD activities measured in hepatocytes in monolayer culture 24 hours and 48 hours post isolation when compared to freshly isolated hepatocytes. An even greater reduction measured in hepatocytes 6 hours post isolation also demonstrated the fact that the activities of freshly isolated hepatocytes are short lived and the restoration of inter-cellular connections provided in monolayer culture may play a key role in the maintenance of CYP activities. Loss of CYP activity in 2 dimensional culture is likely to arise from the widely reported and rapid cellular de-differentiation of hepatocytes in conventional cultures (LeCluyse, 2001, Binda et al., 2003, LeCluyse et al., 2012).

6.4 The utility of hepatocytes in spheroid culture

Numerous examples of other cell culture techniques can be employed to aid the maintenance of the differentiation status of hepatocytes in culture, in an effort to overcome the above limitations. One of these, the use of hepatocytes maintained in 3D aggregate culture is championed in this project. As supported by the results in Chapter 4, trout hepatocytes in spheroid culture 10 days post isolation, maintained a similar level of metabolic activity to freshly isolated hepatocytes but excel in utility for bioaccumulation studies due to their extensive longevity. In this project, hepatocytes in spheroid culture have been maintained viable and active for up to 40 days, with enhanced expression of genes related to xenobiotic metabolism compared to conventional cultures. These advantages will enable the assessment of persistent compounds with slow metabolism, requiring long term incubation (e.g. polybrominated di-phenyl ethers, which are currently widely used (Chen et al., 2012, Shen et al., 2012, Waaijers et al., 2013)), conferring an advantage over several other intact cellular systems. Indeed, preliminary studies conducted as an aside to the work in this project have shown similar responses in metabolism and uptake measurements taken following subsequent exposure to compounds after a three day wash out period and that spheroids continually exposed to 10 μM ibuprofen retained their structural integrity and activity for up to 12 days. This shows the potential to use the system in studies of repeat and long term exposure, which may illustrate adaptive responses induced in cells, and the *in vitro* study of compounds exhibiting chronic toxicity.

The considerable advantage of extended longevity is lacking in many alternative culture systems. For example, although longevity can be achieved through the use of cryopreserved cells, conferred through storage and ease of use, with recovery of metabolic function (Mingoia et al., 2010), like freshly isolated hepatocytes, thawed cells are only

effective in short term incubations, as viability in subsequent monolayer culture has been shown to be as low as 40% in rats (Jackson et al., 1985). The continued deterioration of cellular function following thawing has also been shown (Cross and Bayliss, 2000, Cervenkova et al., 2001). The perfused liver model has also recently been proposed for use in metabolic studies, exhibiting hepatic clearance rates in agreement with estimates obtained by extrapolating published *in vitro* data. Perfused liver also has the advantage of allowing for the study of biliary excretion (Nichols et al., 2009) and use of the whole organ is directly representative of *in vivo* structure. An additional benefit of the system is the possibility of making accurate, direct assessments of CL_h, which is not possible in cell based assays due to the requirement of corrections using scaling factors to take into account plasma protein binding and hepatic delivery. However, the functional integrity and reproducibility of the perfused liver model has been reported to be limited (up to 3 hours (Brandon et al., 2003)). Importantly, this system can only use one animal per compound, is limited in time of incubation and a failure rate of 20% in trout has recently been reported (Nichols et al., 2009). As a result, the perfused liver technique lacks adaptability and is not as compliant with the 3Rs principle as would be the case with the use of hepatocytes in spheroid culture. Direct tissue assessment can also be conducted using liver slices, which also have the advantage of retaining high *in vivo* structural organisation and do not require digestion by proteolytic enzymes (Olinga et al., 1997, Cervenkova et al., 2001). However, it is difficult to accurately measure viability in slices and again, this *in vitro* system is relatively short lived, partly due to impaired oxygen diffusion. It has been shown that in rat liver slices, CYP activity declined by more than half of initial levels within 24 hours (Hashemi et al., 2000, Brandon et al., 2003).

Studies in Chapter 5 showed the enhanced expression and maintained functionality of efflux transporters in hepatocyte spheroids up to 25 days post isolation, highlighting their

potential use in studies to assess the effects of contaminants that modify the expression and function of aquatic organisms, which could lead to the accumulation of compounds in organisms as a result of exposure to mixtures in the environment (Daughton and Ternes, 1999, Caminada et al., 2008). Traditionally, transporter studies are conducted separately to those related to metabolism. The use of spheroids would allow these studies to be combined, enabling accurate predictions of *in vivo* disposition and xenobiotic fate to be made and may afford an even greater presentation of the polarity cues of hepatocytes that is achieved through the use of sandwich cultures (Kim, 2002, Brandon et al., 2003, Ng et al., 2006). The inclusion of studies on compound transport would make metabolic studies used in ERA much more informative than those focusing solely on biotransformation, as uptake and efflux have a key role in xenobiotic clearance and this would further enhance compliance with the 3Rs principle. This may also prove timely as there is now an increased focus on compounds which interfere with drug transporter activities (Giacomini et al., 2010). In this respect, spheroids also outperform the use of immortalised cell lines in efflux transporter studies, with the functionality of all transporters investigated evident in the former but a lack of functionality of BSEP and BCRP reported in trout hepatocyte derived cell lines (Fischer et al., 2011). It is also reported that the immortalisation and de-differentiation processes that take place during the stabilisation of permanent cell lines can affect mRNA expression of transporters (Zaja et al., 2008a, Fischer et al., 2011).

Prolonged viability and extensive maintenance of liver specific functionality are thought to be related to the enhanced cell shape, maintained polarity through 3D structure and retained inter-cellular contacts afforded to hepatocytes in spheroid culture. In 2-dimensional culture, *in vivo* hepatocyte shape is lost as cells flatten, altering structural integrity and diameter (LeCluyse et al., 2012). Maintenance of *in vivo* characteristics is thought likely to

provoke the enhanced levels of gene expression identified in Chapters 4 and 5 (Abu-Absi et al., 2004, Brophy et al., 2009, Sakai et al., 2010a, Sakai et al., 2010b). These results show that in spheroid culture, the gene expression profiles (both of genes related to xenobiotic metabolism and of efflux transporters) were most representative of those measured *in vivo* when compared to hepatocytes in conventional culture. It is also possible to achieve enhanced cell shape in monolayer cultures by supplementation with extracellular matrix components or culturing on various types of substrata (Ichihara, 1991, LeCluyse et al., 2012). However, whilst some reports suggest that the addition of such components increases the mRNA levels of some liver-specific genes, it has been shown that many liver-specific functions continue to be lost progressively (LeCluyse et al., 2012).

In the form of 3D aggregates, hepatocytes in spheroid culture retain the optimal hepatic cyto-architecture, which is considerably more representative of the environment in which cells exist *in vivo*, surrounded by other interacting cells. A number of physiological characteristics are reformed and maintained in spheroid culture including cell-cell contacts, tight junctions and bile canaliculi which reform as an enclosed vacuole into which bile may be secreted, maintaining the polarities of the cell following the relocation of membrane material (Coleman and Roma, 2000, Walker and Woodrooffe, 2001, Fukuda et al., 2006, Brophy et al., 2009). *In vivo*, hepatocytes in fish have a tubular arrangement with apices directed to the centre of the tubule. The plasma membranes of adjacent cells form the bile canaliculi and separation from the sinusoidal membrane is responsible for the maintenance of polarisation (Di-Giulio and Hinton, 2008) (see Figure 6.1). Maintenance of these features, the retention of native cell shape and the enhanced secretion of extracellular matrix compounds by hepatocytes in aggregate culture are thought to confer the long term advantages, enhanced liver specific functional activity, maintained differentiation status and gene expression of

hepatocytes in spheroid culture (Landry et al., 1985, Flouriot et al., 1993, Ma et al., 2003, Brophy et al., 2009).

Investigations in Chapter 5 showed some evidence of the retention of canalicular structure in hepatocytes maintained as spheroids. The uptake and concentration of the fluorescent marker CLF as a bile constituent occurred and punctate staining, which did not correspond to a pan-cellular localisation within individual cells, was seen. The release of CLF, known to be exported by BSEP, was also inhibited by the BSEP specific inhibitor sodium taurocholate. Despite this, the fate of bile within these structures, irrespective of species, remains unclear and further investigation of the mechanism and kinetics of efflux is required.

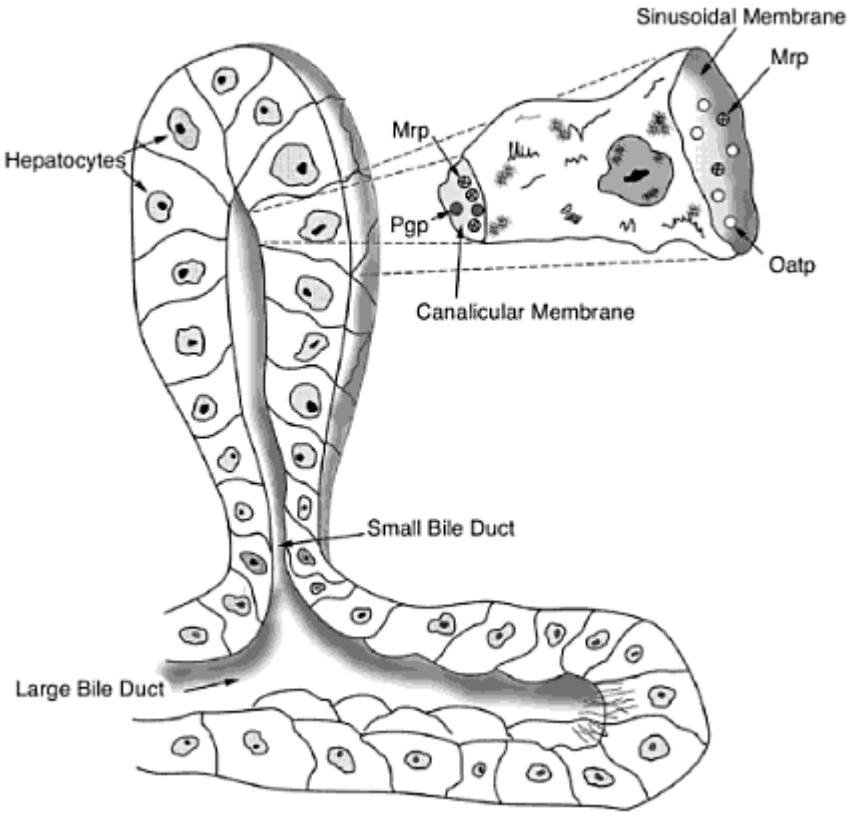


Figure 6.1: The tubular arrangement of hepatocytes in the liver of fish. Hepatocytes are polarised cells; with a sinusoidal membrane (interacting with the blood), representing the basal pole and the canalicular membrane representing the apical pole, where bile is transferred to the bile canaliculus. The loop in-between opposite hepatocytes forms the canalicular space with the apical poles of the hepatocytes forming the walls of the biliary passageway. Blood flows along the sinusoid surrounding the tubule. Hepatocytes in spheroid culture present a structural conformation significantly more representative of that seen *in vivo*. When cultured as spheroids, hepatocytes are in a 3D conformation, surrounded by other interacting cells, maintaining native cell shape and polarity; features necessary for many of the specialised functions of hepatocytes and thought to be responsible for the conservation of differentiation status, gene expression and longevity. However, in monolayer culture, hepatocytes flatten, losing their natural 3D shape and cellular polarity (From (Di-Giulio and Hinton, 2008).

One feature of *in vivo* hepatic structure not maintained in spheroid cultures in this study, is the inclusion of non-hepatocytes. Trout livers include at least 10 other, non-parenchymal cell types which constitute up to 20% of liver volume (Hampton et al., 1989, Lester et al., 1993). Non-parenchymal cells have been shown to play a role in the function, differentiation and gene regulation of hepatocytes, through inter cellular signalling (Altin and Bygrave, 1988, Abu-Absi et al., 2004, Elliott and Yuan, 2011). It has been reported that co-culture systems maintaining parenchymal and non-parenchymal cells concurrently, show improved morphological characteristics and increased drug metabolism capabilities (Van Berkel and Van Tol, 1979, Padgham and Paine, 1993, Cross and Bayliss, 2000). This evidence suggests that the levels of gene expression in the cultures used in this project could be improved by further enhancing *in vivo* reproducibility. Studies into the production of trout heterotypic liver spheroids should be conducted to investigate the potential to further enhance the qualities of the system, however there has been evidence to date suggesting that the inclusion of non-hepatocytes in aggregate suspensions hinders spheroid production (Baron et al., 2012). Development of such cultures would open a number of avenues of further investigation, including the possibility to culture cells from other organs involved in the detoxification of xenobiotics in fish (e.g. gill and intestines), as spheroids. Furthermore, it may later be possible to produce scaffold co-cultures of cells in spheroid culture from multiple organs in a layered arrangement, creating an even more representative *in vivo* model. The combination of cultures from multiple tissues would recreate multicompartmental metabolism, enabling complete uptake, distribution, metabolism, excretion, bioaccumulation and toxicity studies to be conducted, taking into account the involvement of other important organs.

Hypoxia as a result of limited oxygen diffusion to cells at the core of rat spheroid structures with a diameter > 150 μm has also been widely reported (Abu-Absi et al., 2004, Brophy et al., 2009, Stevens, 2009). The regulation of spheroid size and shape during development is difficult and these features have also been shown to affect cell specific function. Methods to exert greater control over size are emerging and the use of polymeric scaffolds with a set pore size has been shown to successfully restrict spheroid diameter to 100 μm (Glicklis et al., 2004).

6.5 Additional advantages of the application of spheroids in ecotoxicology

The application of novel *in vitro* testing strategies has received considerable interest in recent years, especially due to the concept of the 'intelligent testing strategy', which has become increasingly important in environmental toxicology, particularly from a pharmaceutical industry perspective (De Graaf et al., 2002, Lange and Dietrich, 2002). The introduction of chemical testing regulations invoked a strong focus on the refinement of current testing approaches, especially with the use of short-term toxicity testing (Owen et al., 2009). A number of alternative strategies to the conventional testing battery are being implemented to aid *in vivo* screening prioritisation, including the use of quantitative structural activity relationship predictions and the use of preclinical and clinical pharmacology data. The aim is to make use of the extensive datasets generated in mammalian species during drug development, to enable a transfer of knowledge for application in environmentally relevant species and is applicable due to similarities in physiology and conservation of drug targets (Gunnarsson et al., 2008, Owen et al., 2009). Data obtained during drug development provide valuable information on the mechanism of action, physical properties and safety of compounds and may prove useful in predicting potential effects in non-target organisms. This

knowledge transfer, or ‘read-across’ to environmental organisms, is analogous to the use of non-human mammalian species to predict the safety and efficacy of compounds in humans (Winter et al., 2010). The adaptation of this principle to direct risk assessment, aims to make the process more efficient by making greater use of existing data; and ethical by reducing the number of animals used in testing through the elimination of inappropriate assays (Hutchinson, 2008). The potential to read-across in the opposite direction has also been proposed, to potentially reduce the number of *in vivo* studies conducted in non-human mammalian species, by identifying the attrition of compounds at earlier stages, in lower vertebrates. *In vitro* studies using trout hepatocyte spheroids could be used for compounds which exhibit a similar metabolic profile as seen in mammalian species. For this to be possible, a comprehensive understanding of the metabolic profiles and pathways, in a number of aquatic species is required for well characterised model substances already established in mammalian systems.

6.6 Future work

1. Further characterisation of the spheroid system is required, especially in the identification of the ideal stages of use. It remains to be seen whether functional activity is enhanced, with the recovery of expression at 25 days post isolation. If so, even greater levels of metabolic activity than measured in this study may be achieved. Once this knowledge is acquired, it will be possible to assess the possibility of use of spheroids in 28 day chronic exposure studies.

2. Assessment of the metabolism of a wider range of substrates is also required to identify the range of metabolic capabilities of the system. Crucially, direct *in vivo* comparisons must be made to elucidate true relatedness to the metabolic profile and kinetics of the whole organism.

3. Although it is clear that trout hepatocyte spheroids possess the molecular machinery to detoxify and eliminate compounds, the potential for these systems to be modulated has not been investigated. A number of environmental contaminants alter the expression and function of metabolic systems and these should be assessed to determine whether responses correspond to those induced *in vivo*. The involvement of nuclear receptors would be central to this and such studies would enable the effects of mixtures of environmental contaminants to be evaluated for interactive effects.

4. A number of difficulties surround the use of specific inhibitors of transporters in trout studies. In this investigation, functional assessment was conducted using known fluorescent substrates and inhibitors of mammalian transporters, however their specificity

in mammals, let alone fish is equivocal. This lack of knowledge of specificity could be circumvented in further studies through the down regulation of expression of individual transporters. This may be possible using RNAi, however problems with transfection efficiency in primary fish cells has been reported. Development of such a technique could be beneficial in the study of cholestasis in fish species as no such cholestatic model currently exists.

6.7 Concluding remarks

The ultimate goal of alternative testing strategies is to use systems which generate reliable data that relate to *in vivo* metabolic activity as accurately as possible. In this project, the appropriateness of the use of liver S9 fractions has been challenged and the incorporation of studies using whole hepatocytes advocated. Further to this, the application of hepatocytes in spheroid culture is proposed as an even more superior alternative, due to the extended longevity, maintenance of functional activity and enhanced expression of a number of genes. This investigation has proved hepatocyte spheroids to be more representative of the activities of the *in vivo* liver compared to conventional culture and the system excels through the potential for use in studies on the metabolism and potential accumulation of highly persistent compounds that require long term incubations. It is proposed that following assessment with a wider range of substrates, hepatocyte spheroids should be considered for the routine screening of environmental contaminants.

Chapter 7

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Chapter 8

Appendix

8.1 Publications

Uchea, C., Sarda, S., Schulz-Utermoehl, T., Owen, S., Chipman, J.K. (2013) In vitro models of xenobiotic metabolism for use in environmental bioaccumulation studies. *Xenobiotica*, 43, 421-431.

8.2 Oral presentations

- Biosciences graduate research school (BGRS) symposium. University of Birmingham, UK. April 2012
- AstraZeneca Safety Health and Environment Research Forum. Brixham, UK. March 2012
- 16th International symposium on pollutant responses in marine organisms (PRIMO). California, USA. May 2011
- Clinical Pharmacology, drug metabolism and pharmacokinetics symposium, AstraZeneca, Alderley Park, UK. November 2010

8.3 Poster presentations

- 21st Society of Environmental Toxicology and Chemistry (SETAC) Europe annual meeting. Milan, Italy. May 2011
- British Science Festival, Birmingham. September 2010
- Clinical Pharmacology, drug metabolism and pharmacokinetics symposium, AstraZeneca, Alderley Park, UK. November 2009

8.4 Awards

- AstraZeneca Global 3Rs Award- Second place. December 2012
- MedImmune UK 3Rs Award Winner. October 2012
- Award for best presentation at University of Birmingham School of Biosciences graduate symposium. April 2012
- BBSRC policy placement award. December 2011