Development and characterisation of a three-dimensional *in vitro* hepatic fish model to investigate xenobiotic metabolism

by

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AUTHOR'S DECLARATION

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List of Abbreviations

2-D	Two-dimensional
3-D	Three-dimensional
3Rs	Reduction, Refinement and Replacement
7-ER	7-ethoxyresorufin
ACN	Acetonitrile
ADME	Adsorption, Distribution, Metabolism and Elimination
ATP	Adenosine triphosphate
BER	Base excision repair
BCF	Bio-concentration factor
BDDCS	Bio-pharmaceutics Drug Disposition Classification System
BF-2	Bluegill sunfish
BNF	β-napthoflavone
BSA	Bovine serum albumin
C-SEM	Cryo-scanning electron microscopy
CV	Co-efficient of variation
CYP450	Cytochrome P450
DAPI	4'6-diamidino-2-phenylindole
DMEM:F12	Dulbecco's modified eagle medium:Ham's F12 mixture
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ECM	Extra-cellular matrix
ECVAM	European Centre for the validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
EROD	7-ethoxyresorufin-O-deethylase
FBS	Foetal bovine serum
FHM	Fathead minnow
GC-MS	Gas chromatography mass spectrometry
GST	Glutathione S-transferase
HBSS	Hank's balanced salt solution
HepG2	Human liver carcinoma
HPLC	High performance liquid chromatography
KCL	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
K_{met}	Metabolic loss of parent rate
L-15	Leibovitz's media
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDH	Lactate dehydrogenase
Log K_{ow}	Octanol-water partition co-efficient
LOP	Loss of parent metabolism assay
MSCs	Mesenchymal stem cells
MTC	Maximum tolerated concentration
NADH	Nicotinamide adenine dinucleotide
N_aHCO_3	Sodium bicarbonate

N_a2HPO_4	Sodium phosphate monobasic monohydrate
NSAIDs	Non-steroidal anti-inflammatory drugs
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbon
PBDEs	Polybrominated diphenyl ethers
PBT	Persistent, bio-accumulative and toxic
PBTK	Physiologically based toxicokinetic
PCB	Polychlorinated biphenyl
PIE	Pharmaceuticals in the environment
p-HEMA	poly(2-hydroxyethyl methacrylate)
PLHC-1	Clearfin livebearer hepatocellular carcinoma
POPs	Persistent organic pollutants
QSARs	Quantitative Structure Activity Relationships
REACh	European Registration, Evaluation and Authorisation of Chemicals
RPM	Revolutions per minute
RTG-2	Rainbow trout gonad
RTL-W1	Rainbow trout liver
S9	Sub-cellular fraction
SEM	Scanning electron microscopy
SOP	Standard operating procedure
SR	Serum-replacement mixture
SSRI	Selective serotonin re-uptake inhibitor
TCDD	Tetrachlorodibenzo-p-dioxin
TEM	Transmission electron microscopy
TIE	Toxicity identification evaluation
Tris-HCL	Trizma® hydrochloride
UDPG-t	UDP-glucuronosyl transferase
USWT	Ultrasound standing wave trap
Vg	Vitellogenin
WST-1	Tetrazolium salt reduction assay
GGT	ɣ-glutamyltransferase activity

ABSTRACT

Development and characterisation of a three-dimensional *in vitro* hepatic fish model to investigate xenobiotic metabolism

Matthew G. J. Baron

Regulatory and academic studies use large numbers of fish annually. The use of fish primary cells offers an *in vitro* alternative for the assessment of chemical toxicity and the evaluation of environmental samples in ecotoxicology. Their uses however are not without limitations which includes short culture periods (i.e. longevity and loss of organ-specific functionality over time. Three-dimensional (3-D spheroid) technology is now established for *in vitro* mammalian toxicity studies and offers significant advantages for environmental applications in a model fish species. This thesis reports development of a reproducible six-well plate, gyratory-mediated method for rainbow trout (*Oncorhynchus mykiss*) hepatocyte spheroid culture and compares morphological, functional and biochemical status with two-dimensional (2-D) monolayer hepatocytes. The work further assesses the bio-transformation potential of developed 3-D spheroids to a range of environmentally relevant pharmaceuticals. The study suggests that mature spheroids retain some organotypic responses over-time in culture including morphological (*viz.,* smooth outer surface, tight cell–cell contacts); functional (*viz.,* histo-architecture; cell adhesion molecule expression) and biochemical properties (*viz.,* protein, glucose, albumin- and enzyme levels) that is superior to conventional 2-D monolayer cells. These 3-D spheroids also demonstrate a capacity for the metabolism of environmentally-relevant pharmaceuticals that could be utilised to better understand their bio-accumulation potential in fish. This is an important step forward for developing alternative *in vitro* tools in future fish ecotoxicological studies as well as for fundamental understanding of the interaction of chemicals with biomolecules which could potentially lead to detrimental responses at different levels of biological organisation.

CHAPTER 1

General introduction

1.1 Alternative *in vitro* methods in eco-toxicological studies

The development and validation of reliable *in vitro* methods that offer an alternative to conventional *in vivo* studies in experimental animals is rapidly becoming an important requirement, particularly in toxicological studies to reduce the use of animals in line with the Replacement, Refinement and Reduction ($3R_s$) principles as well as to elucidate the modes or mechanisms of action of chemicals (Mazzoleni et al. 2009; Scholtz et al. 2013). In common with mammalian toxicological studies, *in vitro* assays with the use of fish cells are being promoted in ecotoxicology for testing chemicals (Sanchez-Fortun et al. 2008), effluents (Dayeh et al. 2002) and pharmaceuticals (Bartram et al. 2012; Uchea et al. 2013) and in toxicity identification evaluation (TIE) studies (Castano and Gomez-Lechon 2005). The use of fish primary cells and cell lines has been therefore of growing importance in these assessments with research suggesting their suitability in replacing or supplementing *in vivo* animal tests (Babich and Borenfreund 1987a; Castano et al. 1994). The use of alternative experimental test methods, however, has been limited to date, perhaps not due to a lack of available methods, but instead to a lack of consensus on their applicability domains, international validation and wider regulatory acceptance including appropriate recommendations and test guidelines by authorities such as the Organisation for Economic Co-operation and Development (OECD) (Scholz et al. 2013).

Conventional *in vitro* culture methods such as two-dimensional (2-D) primary tissue culture and immortalised cell lines have been used extensively in a wide-range of toxicological fields, in particular to identify *in vitro* endpoints that may address the

issue of specific toxic effects and mechanistic action of chemicals in target organs or different cell types (Raisuddin and Jha 2002; Zucco et al. 2004; Xiao et al. 2007; Bornschein et al. 2008; Reeves et al. 2008; Vevers and Jha 2008; Papis et al. 2011). Despite their distinct economic and ethical advantages over large-scale animal studies, these conventional *in vitro* models in particular can lack many important characteristics of intact tissue and consequently, the *in vivo* environment. Many of the cell lines used in toxicological studies, for instance, are unable to perform *in vitro* all the stages of development, differentiation and ageing that are characteristic of *in vivo* tissue (Tiffany-Castiglioni et al. 1999).

Fish primary cells can overcome some of these issues and can express many of the differentiated cellular structures and functions of their source tissues (Dowling and Mothersill 2001; Segner and Cravedi 2001). However, short culture periods and loss of organ functionality limit their use in long-term, chronic toxicity and bio-accumulation studies. Fish cell lines, although considered to be less differentiated than primary cultures, do provide an easier and more standardised system. The nature of their culture conditions (a monolayer grown in a 2-D structure on plastic or glassware), however, does not fully represent the growth conditions or the three-dimensional (3-D) architecture of *in vivo* tissues, nor accurately detect organ-specific toxicity.

Despite the application of *in vitro* cell culture methods for screening environmental pollutants, aquatic animals (fish in particular) are still the most commonly used experimental system for ecotoxicity testing. For instance, the number of experimental animals in the European Union reported for 2008 that were used for regulatory

toxicological and safety evaluations was ~12 million and ~1.9 million of these were fish, amphibians and birds, which comprise those vertebrates generally used in environmental hazard and risk assessment (EU 2010c). Also, for the implementation of the European Registration, Evaluation and Authorisation of Chemicals (REACh) strategy due to the higher number of animals needed per compound, chronic fish toxicity testing has been estimated to contribute to the majority of vertebrates needed for environmental hazard assessment in Europe (van der Jagt et al. 2004). In addition, 3 - 6 million fish per year are currently being used for whole effluent testing in the United States and ~100,000 fish for regulatory testing of products for the protection of humans, animals, or the environment in Canada (CCAC 2012), highlighting the need for alternative methods on a global scale (Schultz et al. 2013).

Toxicity testing using whole fish can be expensive, time-consuming, ethically controversial and not always an accurate predictor of the human response. Surveys of drug screening tests in mammalian *in vivo* studies have shown that 57% of the toxicological data derived from experiments with rodents do not correlate with the results of human trials (Olson et al. 2000). Similarly, pre-clinical *in vivo* toxicity tests with multiple animal species have been demonstrated to be poorly predictive (DeBoo and Hendriksen 2005; Knight 2007a; Knight 2007b), highlighting the importance of investigate alternative *in vitro* testing strategies.

Pharmaceuticals in the environment (PIE) have gained increasing attention due to their potential for short and long-term biological effects in aquatic organisms with particular concern for their potential to bio-accumulate in tissue. Current alternative, bio-accumulation assessments for fish rely mainly on (a) Quantitative Structure

Activity Relationships ($QSAR_s$) that relate molecular properties of a compound to a measure of a particular activity (i.e. acute toxicity) and (b) octanol-water partition coefficients (log K_{ow}) that measure the hydrophilic or hydrophobic nature of a test chemical (Arnot and Gobas 2006). However, these are not validated for many chemical classes, including pharmaceuticals, and do not account for the impact of metabolism in the organism (Johanning et al. 2012). Existing *in vivo* fish bio-concentration factor guidelines (OECD 305) account for metabolism, yet require large numbers of animals (fish) per test. Coupled with the increasing numbers of compounds that will require testing under the REACh strategy (van Hemmen 2009), alternative testing strategies are urgently required to reduce the gap between *in silico* and *in vivo* approaches (Cowan-Ellsberry et al. 2008; Johanning et al. 2012; Scholtz et al. 2013). One such strategy could be to integrate more physiologically relevant *in vitro* methods, such as 3-D spheroids, into QSAR models to measure the metabolism of chemicals and use the information to create a physiologically based toxicokinetic (PBTK) model for fish. *In vivo* metabolism rates could then be extrapolated from intrinsic *in vitro* clearance rates (Scholtz et al. 2013) that could allow for the calculation of more accurate and predictive bio-centration factor (BCF) values.

1.2 Methods for generating 3-D cell cultures

Various methodologies have been developed for generating 3-D cell cultures, some requiring external matrices to simulate the *in vivo* extra-cellular matrix (ECM), with more recent simplified techniques relying on gyratory-mediated methods to aggregate cells into multi-cellular aggregates or spheroids, without the need for

additional, often animal-derived substrates. The most commonly used 3-D culture methods are discussed here, highlighting the advantages and disadvantages of each (Table 1.1).

In the *in vivo* situation, tissues have a 3-D geometry, gel-like (soft) stiffness, and a specific biochemistry determined by the proteins of the ECM, examples of which include collagen, laminin, and fibronectin (Pampaloni and Stelzer 2009). Growing cultures in a 3-D protein structure such as ECM hydrogels has proved a popular culturing method (Chevallay and Herbage 2000a), with previous research suggesting that collagen scaffolds provide signalling as well as a physical contribution to produce a more complex organisation and differentiation (Jacquot et al. 1994). More recent research has demonstrated how collagen-coated micro-patterned substrates can improve the differentiation efficiency of multipotent mesenchymal stem cells (MSC_s), improving their application in clinical therapy (Wang et al. 2009). Despite its advantages, collagen is an animal-based component (connective tissue of tendons), limiting its appropriateness for investigations using animal replacement methods. Studies have however, used alternative methods, where alginate hydrogel structures are combined with ultrasound standing wave trap (USWT) technology, to produce rapid, hydrogel cell encapsulated spheroid models for functional hepatocyte studies (Bazou et al. 2008).

Others types of ECM or media have been used in 3-D culture to force cells to adhere to each other as opposed to the substratum often referred to as liquid overlay techniques.

Table 1.1 Summary table of some common 3-D culture methodologies.

3-D culture method	Advantages	Disadvantages	References
Hydrogel structures	* Provides a physical 3-D ECM * Easy to set-up	* Animal derived products * Expensive	Chevallay and Herbage 2000a; Bazou et al. 2008; Wang et al. 2009
Liquid overlay	* Maintain cellular composition and differentiation of intact tissue * Cheap and easy set-up * Rapid and easy screening	* May alter cell-adhesion behaviour * Small amount of spheroids produced * Higher adherence rate of fish cells	Folkman and Hochberg 1973; Yuhas et al 1977; Bradley and Pitts 1994
Micro-carrier beads	* Supports aggregation of attachment dependent cells * Large-scale generation of spheroids * Cheap and easy set-up * Allows co-culture of different cell types	* High shear stress forces (when combined with spinner flasks)	Clark and Hirtenstein 1981; Cheng et al. 2009; Tsuda et al. 2009; Tsoy et al. 2010
Porous substrates / scaffolds	* Large-scale generation of spheroids * Bio-degradeable	* Expensive	Bell 1995; Chevallay and Herbage 2000b; Sun et al. 2005; Cantòn et al. 2010
Gyratory-rotation cultures	* Large-scale generation of spheroids * Reduced variability in experimental set-up * Gentle rotation (orbital) reduces level of adherence * Cheap and easy set-up * No additional ECM required	* High shear forces (spinner flasks)	Sutherland et al. 1970; Sutherland 1988; Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996; Walker et al. 2000; Morgan et al. 2004: Xu et al. 2003a; Xu et al. 2003b; Xu et al. 2003c; Sales et al. 2004; Baron et al. 2012; Uchea et al. 2013

Cells are grown on a thin layer of agar attached to the base of a growth vessel (Folkman and Hochberg 1973; Bradley and Pitts 1994), but other substrates such as agarose and reconstituted basement membrane have also been used (Yuhas et al. 1977). 3-D cultures grown in this manner maintain the cellular composition and differentiation of intact tissue; however the cell adhesion behaviour may be altered due to contact of the cells with the substratum (Kim 2005). There is also the consideration that some cell types, in particular those from fish, can have a greater affinity for substrate adherence, despite the presence of an overlay material, making cell removal difficult and hence potentially damaging the 3-D architecture. Until recently, the liquid overlay technique has been the most widely used technique in 3-D cell culture, because of its economic and ethical advantages over animal derived hydrogels.

The use of micro-scale material beads has been a popular culture method for generating 3-D cultures, with the primary advantage of supporting the aggregation of attachment-dependent primary cells and cell lines that do not spontaneously aggregate (Kim 2005). Micro-carrier beads have been used to investigate the role of micro-environmental mechanical stress in tumour spheroid growth dynamics (Cheng et al. 2009); their applicability for cell based drug testing assays (Tsoy et al. 2010; Tsuda et al. 2009); and as a potential new culture method in disease therapy (Shimony et al. 2006). Micro-carrier beads have many advantages which make them suitable for large scale generation of 3-D cultures; however, it is worth noting that cell type, choice of micro-carrier material and culture vessel are important factors that will affect both cell adhesion and attachment. Similarly, culturing 3-D aggregates in this way requires cultivation in gyratory or spinner flasks, which may add additional

problems associated with these culture methods (i.e. shear forces) (Lin and Chang 2008).

The use of commercially available porous substrates such as filter-well inserts and fibre meshes have been demonstrated as suitable substrates with which to generate 3-D cultures. This technology has contributed to advancements in replacing controversial invasive dermal irritation tests (Draize et al. 1944), with 3-D *in vitro* epidermal models (Canton et al. 2010). In 2007, a reconstructed human skin model (EPISKIN® produced by SkinEthic and L'Oreal – Nice, France) consisting of normal human keratinocytes cultured on a collagen matrix, was approved as an alternative to the rabbit Draize skin irritation test (de Brugerolle 2007) after evaluation by the European Centre for the Validation of Alternative Methods (ECVAM).

Similarly, 3-D cultures can be generated by growing cells on prefabricated scaffolds; the large surface area allows cells to attach and divide and form 3-D aggregates within the gaps of the scaffold structure (Bell 1995). For *in vitro* dermatotoxicity studies, scaffolds are developed using non-degradable polystyrene or cellulose (Sun et al. 2005). This has been further adapted to develop a real-time method using transfected human fibroblasts containing pNF-kB/d2EGFP for directly detecting inflammatory signals within scaffolds (Canton et al. 2007). Natural polymer scaffolds such as collagen tend to be used for clinical studies, where scaffolds need to be biodegradable (Chevallay and Herbage 2000b).

Perhaps the most popular method for culturing spheroids in the last decade has involved rotational methods, where large numbers of 3-D cultures, or spheroids, can

be prepared in suspension without the need for additional ECMs or substrates and where cells are forced to aggregate by simple stirring. Spinner flask culture, developed in the 1970^{s} (Sutherland et al. 1970) has been a popular method for culturing large numbers of multicellular tumour spheroids (Sutherland 1988), but expensive set-up costs and exposure of cells to strong shear forces which may affect cell physiology, have limited its application (Lin and Chang 2008). Simple gyratory-shaking culture methods have addressed many of the problems associated with other 3-D culture methods. Spheroids can be prepared in a Petri dish or micro-plate format at low rotation speeds and in controlled temperature incubators, reducing variability in experimental set-ups (Walker et al. 2000; Ma et al. 2003; Xu et al. 2003a; Xu et al. 2003b; Xu et al 2003c; Morgan et al. 2004; Sales et al. 2004; Baron et al. 2012).

These rotational culture methods have been the culture method of choice for comparison studies between primary hepatocyte 2-D monolayer and 3-D aggregates in fish (Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996; Baron et al. 2012; Uchea et al. 2013). There is no need for additional ECM materials as the aggregates themselves form an external structure, corresponding to the secretion of an extracellular matrix (Landry et al. 1985; Cravedi et al. 1996). There is a reduced risk of adherence to the base of the growth vessel as cells are kept in suspension and low shaking speeds reduce the risk of high shear forces. Most importantly, this format can provide enough samples to allow a range of assays to be performed for toxicity and functional studies (Xu et al. 2003b) (Fig. 1.1).

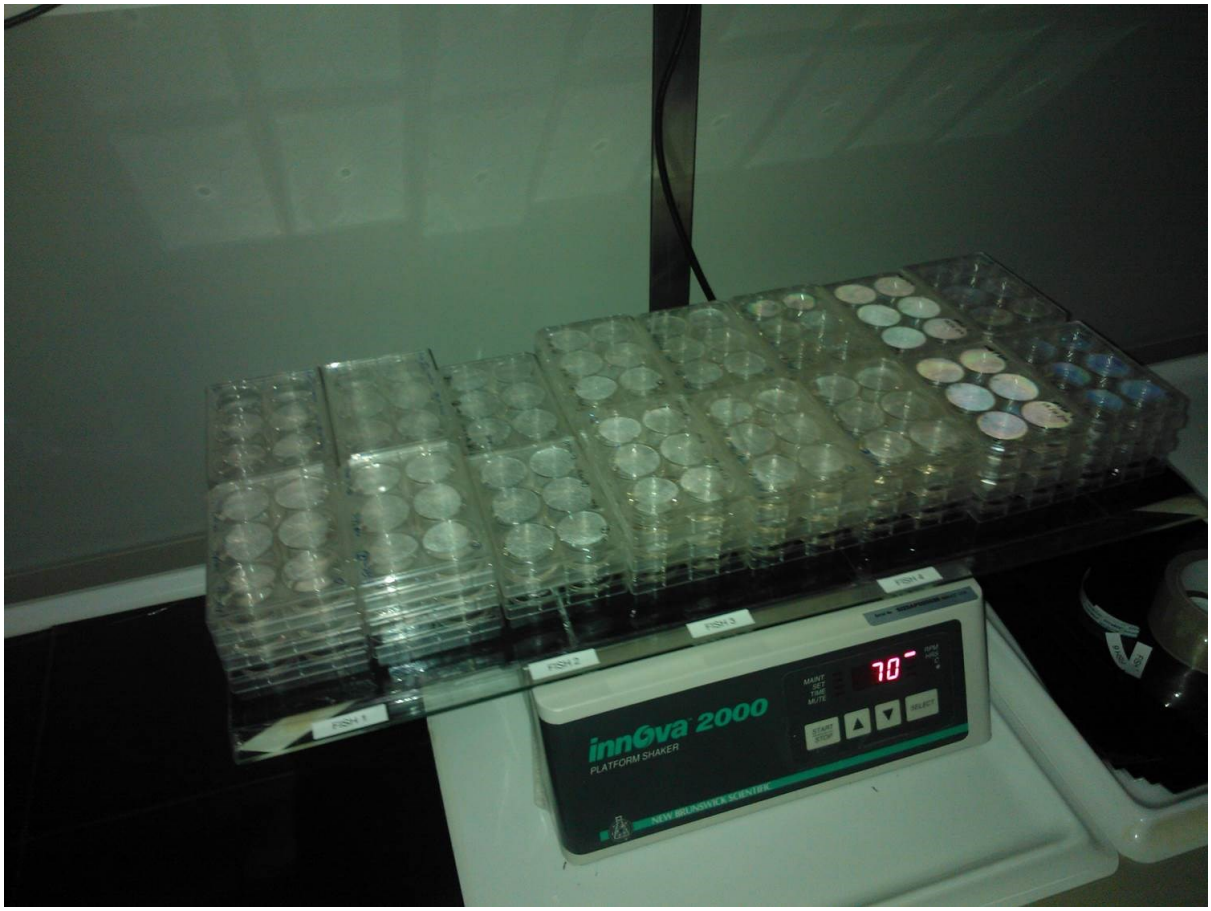


Figure 1.1 Image of rotary-mediated set-up for spheroid formation taken from our laboratory. Cultures from six individual fish are rotated simultaneously producing a large number of spheroids for a range of assays.

1.3 Physiological endpoints for biochemical and cytotoxic characterisation

The importance of the 3-D architecture of tissues and organs being preserved in *in vitro* models is not a new concept (Elsdale and Bard 1972; Hay and Dodson 1973; Bissell 1981; Ingber and Folkman 1989), where limited technology and expensive set-up costs have perhaps favoured conventional, less-expensive 2-D culture techniques. Over two decades of research have demonstrated, however, that with respect to traditional 2-D cell culture systems, 3-D cell models have the potential to

advance the quantitative modelling of biological systems from cells to living organisms (Pampaloni et al. 2007; Mazzoleni et al. 2009) and improve the physiological relevance of cell-based assays, an important criteria particularly for toxicological-based studies.

Cells grown in 3-D systems develop into tissue-like structures, more similar to those formed in living organisms. Their shape and environment are important in determining their growth behaviour and gene expression (Kim 2005), which can be quite dissimilar from conventional 2-D monolayer models (Bissell et al. 1982; Chang and Hughes-Fulford 2008). Specific biochemical organ functions such as glucose, albumin, urea and enzyme release (Ma et al. 2003; Xu et al. 2003a; Xu et al. 2003b) and important enzyme bio-transformation systems (Flouriot et al. 1995; Cravedi et al. 1996; Liu et al. 2007) are maintained over-time in culture, highlighting their stability in functionality and biochemical performance. In addition, 3-D cultures can be composed of cells with different phenotypes such as proliferating, non-proliferating and necrotic cells (Kim 2005), the latter making them a particularly useful comparative model to intact tumours. Also, unlike monolayer cultures, where growth is typically unrestricted if unlimited space and nutrients are supplied, growth in three dimensions tends to be self-regulating (Folkman and Hochberg 1973), meaning cells will tend not to grow beyond a critical diameter and cell number, important characteristics of regulated tissue growth.

Characterisation of biochemical endpoints using rat liver and human liver carcinoma (HepG2) spheroids have demonstrated that combining functional endpoints together with two or more indicators of enzyme release, can provide a reliable evaluation of

cytotoxicity (Xu et al. 2003b). Exposure of model hepatoxicants inhibits glucose secretion in rat liver and HepG2 spheroids (a functional indicator of cytotoxicity), suggesting an effect on mitochondrial function, in particular adenosine triphosphate (ATP) depletion (Xu et al. 2003b). Lactate dehydrogenase (LDH) release has been commonly used to test the viability and/or cytotoxicity of a test agent in *in vitro* studies (Walker et al. 2000; Sales et al. 2004) and ɣ-glutamyltransferase activity (GGT) has shown a six-fold increase in HepG2 spheroids exposed to hepatoxicants, suggesting a greater sensitivity of hepatic spheroids as a diagnostic marker for liver disease and xenobiotic transformation (Xu et al. 2003b).

Enzyme (e.g. LDH) and metabolic (e.g. ATP) endpoints are less frequently used in fish toxicology studies in comparison to cellular viability assays. Studies have reported increased LDH leakage in a time and concentration-dependent manner in rainbow trout gonad (RTG-2) cells after exposure to polybrominated diphenyl ethers (PBDEs) (Jin et al. 2010) and exposure to zinc metals induces enzyme leakage in fish cell lines, but only at high exposure concentrations (Ni Shuilleabhain et al. 2004). Good correlations between *in vivo* LC50s and *in vitro* EC50s for intracellular ATP content have been found using an RTG-2 test, indicating the applicability of the test as an alternative protocol to estimate the acute toxicity of chemicals on fish without using live animals (Castano et al. 1996).

Previous work has also demonstrated the use of aggregate culture systems for characterising levels of estrogen receptor and vitellogenin (Vg) mRNAs in rainbow trout and has been described as a promising *in vitro* model to investigate the bio-transformation pathways in fish and their regulation by endogenous and exogenous

compounds (Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996), yet in the last two decades, to our knowledge and for work from our group and collaborators (Baron et al. 2012; Uchea et al. 2013), no new research into the suitability of 3-D cell culture technology in ecotoxicity testing has emerged. Needless to say, understanding the physiological, biochemical and metabolic characteristics of 3-D models are important criteria for promoting their application in the field of ecotoxicology.

1.4 Hepatic metabolic assessment using fish *in vitro models*

Liver cells are convenient and useful candidates for investigating xenobiotic metabolism in fish, as the liver plays an integral role in the detoxification of xenobiotics. Sub-cellular fractions derived from liver homogenates (S9) are commonly used for assessing the metabolic potential of compounds in fish (Gomez et al. 2010; Johanning et al. 2012; Connors et al. 2013). Although assays utilising these can be fast and relatively cost effective, the rate of metabolism can be vastly influenced by the addition of different co-factors (Uchea et al. 2013), plus the inherent assumption that there is uptake of the chemical into the system in the first place. They also lack the cellular complexity and organelle composition of intact cell systems, potentially affecting the prediction of metabolic rates (Han et al. 2009).

Freshly isolated hepatocytes have the capacity for all phase I and phase II biotransformation reactions (Cravedi et al. 1996) and primary monolayer cultures can be used to study the regulation of xenobiotic metabolising enzymes by exogenous (Vaillant et al. 1989; Pesonen and Anderson 1991) and endogenous compounds

(Devaux et al. 1992). However, their short culture periods limit their use in mechanistic studies concerning regulation systems of enzyme activities (Cravedi et al. 1996). Studies have shown that levels of fish liver specific functions such as cytochrome P450 (CYP450)-dependent (Vaillant et al. 1989) and conjugating enzyme activities (Klaunig 1984) in primary monolayer cultures remain close to those of freshly isolated '*in vivo*-like' cells for short periods (5 d), but rapidly lose their functionality beyond this period.

Several permanent cell lines of liver origin such as PLHC-1 (clearfin livebearer hepatocellular carcinoma) and RTL-W1 (rainbow trout liver) and some fibroblastic-like cells such as RTG-2, BF-2 (bluegill sunfish) and FHM (fathead minnow epithelial) retain at least a basic CYP450-dependent monooxygenase activity for investigating xenobiotic metabolism (Fent 2001). CYP1A is induced on exposure to selected polychlorinated biphenyl (PCB) congeners in PLHC-1 (Bruschweiler et al. 1996; Hahn et al. 1996) and both RTG-2 and RTL-W1 (Bols et al. 1999; Babin & Tarazona 2005) cell lines have exhibited some degree of CYP1A induction after exposure to selected pesticides and polycyclic aromatic hydrocarbons (PAHs), with RTL-W1 cells in particular exhibiting a higher sensitivity in their 7-ethoxyresorufin O-deethylase (EROD) response when compared to primary hepatocytes (Behrens et al. 2001). It has also been reported that representative enzymes from other CYP sub-families (CYP2M, CYP2K and CYP3A) show much lower (≤ 17 fold) activity in PLHC-1 and RTL-W1 cells when compared with reported activity levels in primary cultures (Thibaut et al. 2009).

In order to consider 3-D spheroids as a suitable alternative to *in vivo* fish toxicity testing, it must be established whether the spheroids possess a degree of metabolic function, and whether this can be maintained, over time, in culture. Freshly isolated fish hepatocytes grown as aggregated spheroids demonstrate stable liver enzyme activities for up to 30 d in culture (Flouriot et al. 1995), such as CYP450 content, EROD and UDP-glucuronosyl transferase (UDPG-t) activity, and demonstrate increased levels of glutathione S-transferase activity (GST). CYP450 content and EROD activity levels are similar to those seen in adult rat hepatocyte aggregates, with UDPG-t activity levels being markedly higher (Tong et al. 1992). After assessment of a number of *in vitro* systems (S9, fresh hepatocytes, 2-D monolayer and 3-D spheroids), the use of primary 3-D spheroid cultures has been shown to be particularly suited to the *in vitro* assessment of metabolism and bio-accumulation potential of chemicals, due to maintenance of functionality and longevity in culture but most importantly, the expression of liver-specific CYP450 and glucuronosyl-transferase genes involved in bio-transformation are maintained over time in these culture systems (Flouriot et al. 1993; Liu et al. 2007; Sakai et al. 2010a; Sakai et al. 2010b; Baron et al. 2012; Uchea et al. 2013) (Fig. 1.2).

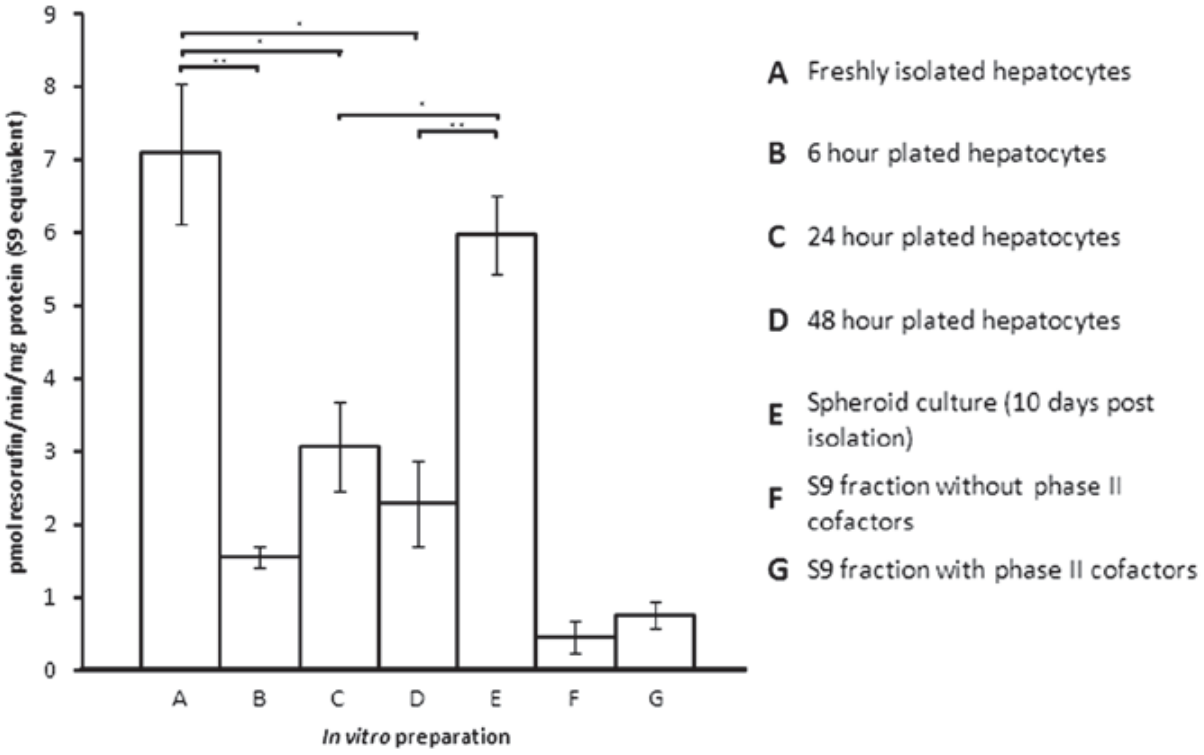


Figure 1.2 Comparative rates of total resorufin production by different hepatocyte preparations. Spheroids demonstrate a significantly higher baseline EROD rate to conventional 2-D monolayer and microsomal S9 fractions. Taken from Uchea et al. 2013.

1.5 CYP450s as potential biomarkers

These enzymes are perhaps the most important family of metabolising enzymes (Table 1.2), used as biomarkers, to indicate the level of pollution of the aquatic environment. They belong to the super family of proteins called haemoproteins that catalyse the oxidation of organic substances via a mono-oxygenase reaction (insertion of one atom of oxygen into an organic substrate (RH) while the other oxygen atom is reduced to water). They are present at high levels in the liver, accounting for 1 to 2% of the mass of hepatocytes in fish (Lester et al. 1993) and the

cytochrome isoforms whose primary functions are to metabolise xenobiotics in fish arise from the CYP1, CYP2 and CYP3 families.

To detect pollution in aquatic environments, the CYP1 family members have been shown to be the most sensitive with polycyclic aromatic hydrocarbons (PAHs) (Jung et al. 2001); polychlorinated byphenyls (PCBs) (White et al. 1997); tetrachlorodibenzo-p-dioxin (TCDD) dioxins (Machala et al. 2000); pesticides (Fent et al. 1998) and pharmaceuticals (Bartram et al. 2012) known to be effective inducers. CYP2B fails to be induced in fish (Machala 1997; Petrivalsky et al. 1997), whereas CYP3A is induced in fish by some organochloride compounds or PCBs (Machala 1997). However, care must be taken when choosing reference compounds, as the presence of some PAHs or PCBs may not induce elevated CYP1A levels due to either high concentrations of pollutants (Stegeman et al 1995; Schlezinger and Stegeman 2001) or the presence of metals that may inhibit CYP450s.

Despite the majority of research focussing on the induction of CYP1A in fish, more attention is now being focussed towards the CYP3A family, the largest family of CYP enzymes in both mammals and fish. Several CYP450 3A enzymes have been identified via cloning and gene sequening in several teleost species, including CYP3A27 and CYP3A45 in rainbow trout (Lee et al. 1993; Lee and Buhler 2003); CYP3A38 and CYP3A40 in medaka (Kullman et al. 2000; Kullman and Hinton 2001) and CYP3A30 and CYP3A56 in killifish (McArthur et al. 2003). The role of CYP3A enzymes has been investigated in pharmaceutical metabolism such as that of the bactericidal antibiotic drug, rifampicin and of the glucocorticoid steroid, dexamethasone, which have both been shown to induce the enzyme CYP3A65 in

larval zebra fish (*Danio rerio*) (Tseng et al. 2005). Recent research has demonstrated the development of a fluorescent-based CYP3A high-throughput assay for fish cell lines that uses human CYP3A substrates, suggesting that CYP3A expression is modulated by the same receptors in mammals and fish (Christen et al. 2009).

Table 1.2 Nomenclature of cytochrome P450s and the classes of substrates they metabolise (Siroka and Drastichova 2004).

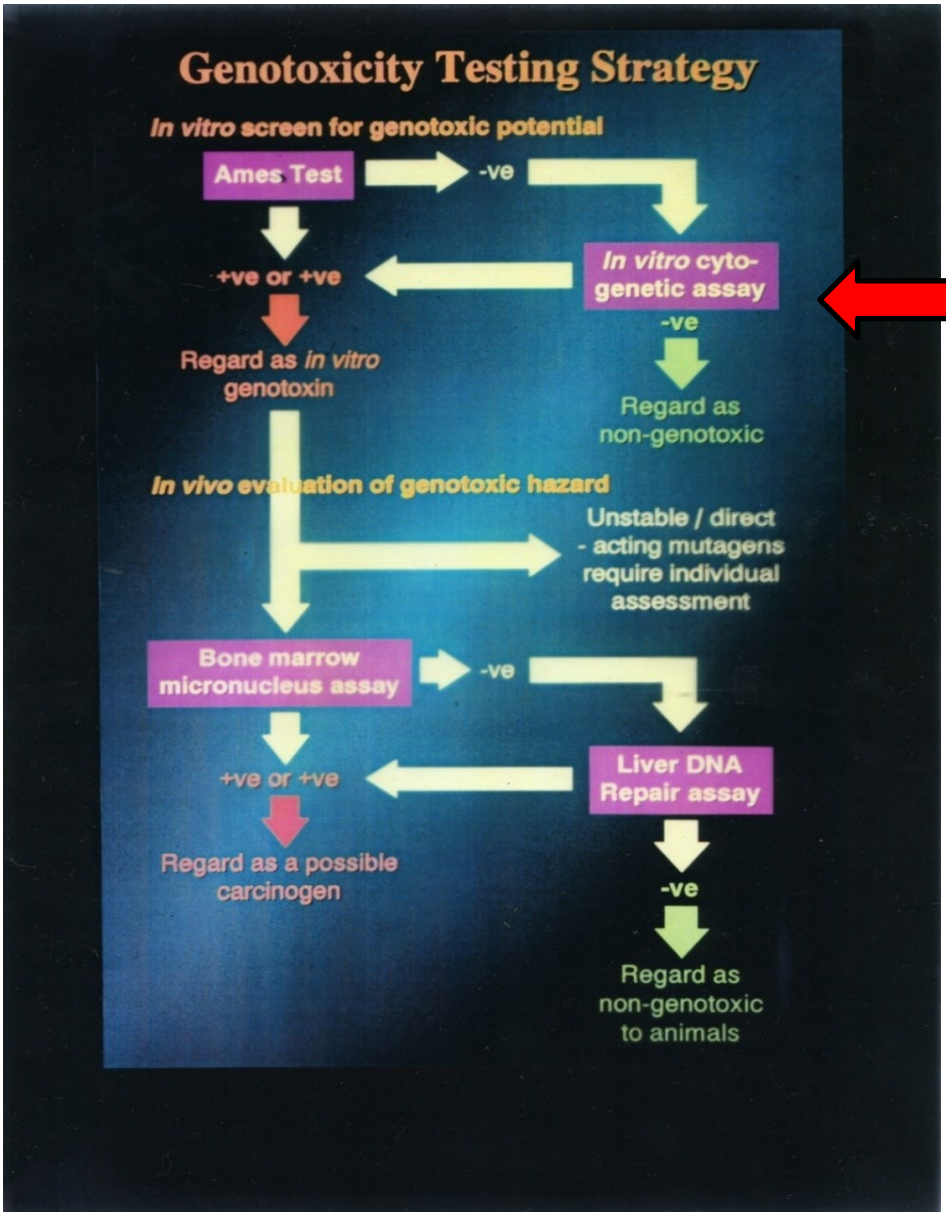
Cytochrome P450 family	Characteristics
CYP 1	Metabolism of foreign compounds
CYP 2	Metabolism of foreign compounds
CYP 3	Metabolism of foreign compounds
CYP 4	Metabolism of long-chain fatty acids
CYP 5	Thromboxane biosynthesis
CYP 6	Insect isoforms
CYP 7	Metabolism of steroids
CYP 8	Prostacyclin biosynthesis
CYP 9	Insect isoforms
CYP 10	Mollusc isoforms
CYP 11	Biosynthesis of steroids
CYP 17	Biosynthesis of steroids
CYP 19	Biosynthesis of steroids
CYP 21	Biosynthesis of steroids
CYP 24	Metabolism of vitamin D
CYP 26	Metabolism of retinoids
CYP 27	Biosynthesis of bile acids
CYP 51-70	Fungal isoforms
CYP 71-100	Plant isoforms
CYP 101-140	Bacterial isoforms

1.6 3-D cultures in genotoxicity and carcinogenic studies

Toxicity of chemicals could be manifested in a variety of ways and it is well established that the genotoxic, carcinogenic and teratogenic potentials of chemicals are strongly linked (Jha 2008). With particular reference to the aquatic environment, there has been growing concern with respect to the presence of those contaminants which could be carcinogenic, mutagenic and reproductive toxicants (Jha 2008). In the human health arena, genotoxicity tests contribute to the assessment of whether a chemical has the potential to cause somatic or germ-cell effects in animals (i.e. the potential to induce cancer or heritable mutations) (Elliot 1994). Genotoxicity testing is usually undertaken as a two-stage process, firstly an *in vitro* assessment to determine intrinsic genotoxic activity followed by an *in vivo* evaluation to determine whether genotoxic activity is carried over into the whole animal. This is where the criticism of conventional *in vitro* culture methods lies, with poor crossover comparison between 2-D structures and whole animal models. The use of spheroids could act as a next-stage in a tiered testing strategy, if good correlation between 3-D *in vitro* and whole animal *in vivo* data is confirmed then spheroids could offer a more predictive *in vitro* model, without the need for large-scale animal *in vivo* tests (Fig. 1.3).

To refine, reduce and ultimately replace aspects of *in vivo* genotoxicity testing development of new complementary biological models and methods that will improve the ability to predict genotoxic risk with *in vitro* data are required. Recent studies have suggested the feasibility and relevance of the 3-D *in vitro* reconstructed human skin model, as a biologically active barrier for *in vitro* clastogenicity assays on

cultured cells (Flamand et al. 2006) and as a potential model for monitoring DNA damage response in a human skin tissue microenvironment (Su et al. 2010) that can be determined using an established genotoxic test, the single cell gel electrophoresis assay (comet assay). This test has been used extensively in fish studies to investigate the effects of genotoxin exposure on levels of DNA damage in established cell lines (Sebastian and Helumt 2001; Raisuddin and Jha 2004; Nehls and Segner 2005; Vevers and Jha 2008); *in vivo* fish tissue (Belpaeme et al 1998) and fish embryos (Kosmehl et al. 2008). It plays an important role in genetic ecotoxicology to determine induced genetic damage, which has significant consequences for short- and long-term survival of the natural or wild species (Jha 2008).



OECD Guideline
Approved: *In-vivo* Comet assay
Pending: *In-vitro* Comet assay

Figure 1.3 Genotoxicity battery testing strategy. Spheroids could provide a further stage of *in vitro* testing which could reduce the need for a large-scale *in vivo* animal test. Adapted from Elliot (1994).

Solid tumours are often found to have an altered extracellular condition called the tumour micro-environment (Seo and Kinsella 2009) which has been shown to impact on local disease progression, metastatic potential, and response to radiation and chemotherapy (Overgaard 2007). Recent research has demonstrated the importance of spheroids in elucidating the role of the base excision repair (BER) pathway on the survival of tumours, suggesting that BER inhibition may confer tumour microenvironment targeted cytotoxicity in human cancers (Seo and Kinsella 2009). Further studies have indicated that spheroids are a useful model in the detection of hypoxic cells in solid tumours, following exposure to bioreductive drugs such as tirapazamine (Olive 1995), which also induces significant DNA damage, detected at similar levels in both Chinese hamster V79 spheroids and solid tumours (Olive et al 1996). Such spheroids also exhibit a much lower sensitivity to cell killing than their monolayer counterparts, due to a decrease in growth fraction of large spheroids; protection afforded by nutrient deprivation causing cellular ATP reduction (Olive et al. 1993) and by the presence of resistant cell sub-populations to DNA-damaging agents (Olive et al. 1997). Multicellular spheroids have also been suggested to be an appropriate *in vitro* system for simulating 3-D tumour micro-milieu that can be used for predicting tumour response to therapeutic agents, including metabolic inhibitors (Khaitan et al. 2006).

1.7 Aims and objectives

Against the backdrop of above mentioned information, the three main aims and objectives of this study were as follows:

(a) To develop (3-D) and optimise (2-D and 3-D) *in vitro* hepatic cultures from rainbow trout through optimisation of the hepatocyte isolation process, cellular yield, viability and culture parameters to determine optimum conditions with which to provide prolonged culture periods in both model systems, in particular, to provide a detailed protocol for the routine formation of 3-D rainbow trout primary hepatocyte spheroids;

(b) To characterise baseline morphological and biochemical organ-functionality of 3-D fish hepatocyte spheroids and compare with 2-D monolayer hepatocyte culture models; and

(c) To determine metabolic substrate depletion of environmentally relevant pharmaceuticals in hepatic trout spheroids.

CHAPTER 2

Materials and methods

Materials and methods specific to each individual study in addition to those mentioned here are outlined in their respective chapters.

2.1 Chemicals and media

Propranolol hydrochloride, atenolol and metoprolol succinate were obtained from AstraZeneca (UK). All other chemicals and reagents were purchased from Sigma-Aldrich (UK) unless stated otherwise and of the highest purity available. All culture media and associated reagents were purchased from Life Technologies (UK) (Appendix I).

2.2 Fish husbandry

Female diploid rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were kept in 180 L holding tanks (de-chlorinated and aerated water; non re-circulating system) at a stocking density of ~40 fish per tank (Fig. 2.1). Water parameters (temperature: 16.0 ± 0.7 °C; pH: 6.7 ± 0.1; dissolved oxygen (DO): 94.0 ± 2.7%) were measured daily and were found to be in the acceptable range for rainbow trout maintenance. A photoperiod of 12 h light and 12 h dark was applied and fish were fed a daily ration of pellets (BioMar, UK) at ~2% body weight to maintain their weight and health. Fish were starved for 24 h prior to sampling.

2.3 Fish euthanasia

Donor animals were sacrificed by a blow to the head followed by immediate destruction of the brain via pithing, as per the Schedule I protocol of the Animals (Scientific Procedures) Act 1986 (UK). Fish were weighed, surfaced sterilised with 70% ethanol and transferred to a downdraft dissection table ready for tissue harvesting.

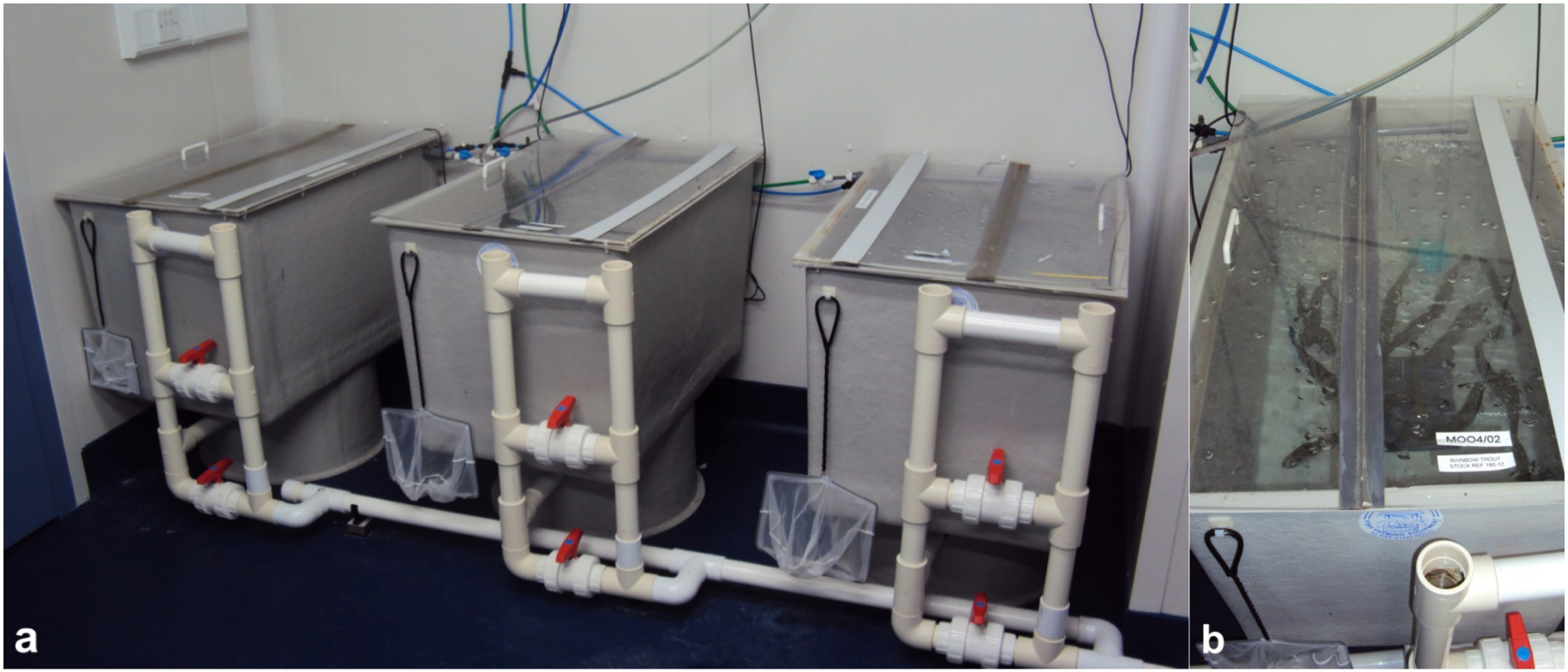


Figure 2.1 Husbandry set-ups for stock rainbow trout: (a) 180 L holding tanks on a non-recirculating system maintained at 16 ± 0.7 ºC; (b) stocking density was kept high at the start of experiments to reduce aggression between individuals. Fish were removed routinely (daily) as a control against the formation of a dominance hierarchy between individuals.

2.4 Tissue preparation

2.4.1 Fish dissection

Fish were placed on their right lateral flank and a small incision was made with a scalpel blade at the anus; then a ventral cut was continued with surgical scissors through the body tissue to the isthmus. This was followed by an incision from the anus along the lateral line to the operculum and then ventrally along the opercula opening, removing the pectoral fin in the same incision. The dissected fillet was then carefully removed, exposing the body cavity. The liver was carefully checked for any scarring or damage and the pyloric caecae gently displaced to reveal the hepatic portal vein. The tissue surrounding the pericardial cavity was carefully cut using micro-surgical scissors in order to expose the heart (Fig. 2.2).

2.4.2 Liver perfusion set-up

The liver was dissociated by a collagenase perfusion method (Nabb et al. 2006) with modifications in our laboratory for optimisation of hepatocyte yield and viability. Liver perfusion parameters were thoroughly optimised and validated and are described in more detail in Chapter 3. The method outlined here was implemented in all subsequent experimental procedures (Chapters 4 and 5). All solutions used during the perfusion process were first pre-filtered through a sterile 0.22 µm filter (Whatman® Puradisc) and then acclimated to 15 ºC in a Galaxy 170R cooled incubator (Eppendorf, UK), so as to avoid thermal shock to the liver. The entire perfusion was carried out *in situ.* A cannula comprising of a 30 x 0.6 mm (23G) hypodermic needle joined to a section of polythene Portex® tubing (0.76 mm internal diameter; 1.22 mm external diameter) (Smiths Medical, UK) connected to a calibrated peristaltic pump (Watson Marlow, UK) was used to perfuse the liver (Fig.

2.3) (Appendix II). A flow rate of 1 ml min^{-1} was chosen as the optimum flow rate that would not exert unnatural pressure on the organ, but would maintain a constant and efficient perfusion process. The cannula was pre-sterilised with 2 ml ethanol followed by 2 ml calcium and magnesium-free Hanks's balanced salt solution (HBSS).

Figure 2.2 Image of a rainbow trout. (a) Un-dissected; (b) dissected fish. Left lateral fillet has been removed to expose the body cavity. The liver (i) which is located in the cranial region of the cavity is healthy and undamaged and the heart (ii) has been exposed by carefully removing the surrounding tissue of the pericardial cavity. The pyloric cacae has been displaced to expose the hepatic portal vein (iii). (Bar = 5 cm).

2.4.3 Blood clearance

The liver was cleared of residual blood using calcium and magnesium-free HBSS (5.3 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 137.9 mM NaCl, 0.34 mM Na_2HPO_4 and 5.6 mM D-glucose, pH 7.4) supplemented with EDTA (2.3 mM) to interrupt calcium dependent cell-cell interactions and $NaHCO_3$ (4.2 mM) to increase the pH buffering capacity of the solution. The cannula was inserted into the hepatic portal vein, approximately 1 cm from the junction with the liver (Fig. 2.4a) and then pushed approximately 1 cm along the vein, whereupon the tissue surrounding the liver junction gradually turned from deep red to a straw yellow colour (Fig. 2.4b). The cannula was left for 1 min to ensure the liver perfused correctly before being clamped into place with a haemostat. The ventral aorta of the heart was then cut and kept open with surgical scissors to allow the perfusate to drain and the perfusion continued for 10 min (1 ml min^{-1}; 10 ml total). The colour change of the liver was observed throughout the 10 min period, with a fully straw coloured liver detectable by the end of the perfusion (Fig. 2.4c). The exposed liver was regularly washed with HBSS to eliminate dehydration and shrinkage.

2.4.4 Enzymatic digestion

Immediately following blood clearance, the liver was digested using calcium and magnesium-supplemented HBSS (1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$-$6H_2O$ and 0.4 mM $MgSO_4$-$7H_2O$, pH 7.4) supplemented with 0.1% collagenase D (Roche Scientific, UK) and $NaHCO_3$ (4.2 mM). While the cannula remained in the vein the pump was momentarily switched off, the other end of the tubing clamped and placed into a separate, sterile 50 ml centrifuge tube containing the collagenase solution, to ensure no air bubbles entered the line.

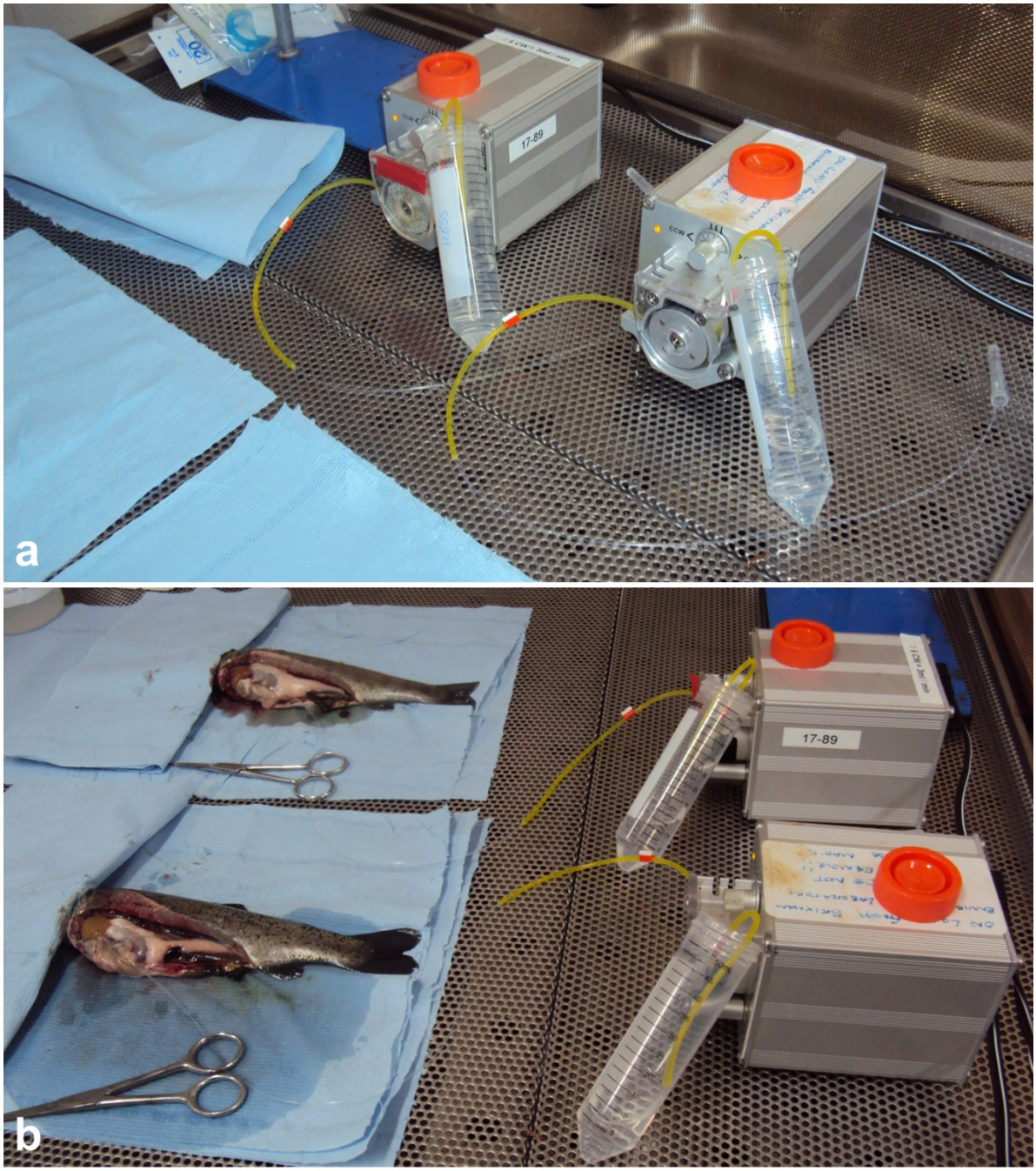


Figure 2.3 *In situ* liver perfusion set-ups. (a) Cannulae were attached to peristaltic pumps and calibrated to deliver a flow rate of 1 ml min^{-1}. (b) Fish perfusions were carried out in parallel. Haemostats have been removed in (b) to show attachment of cannula to hepatic portal vein.

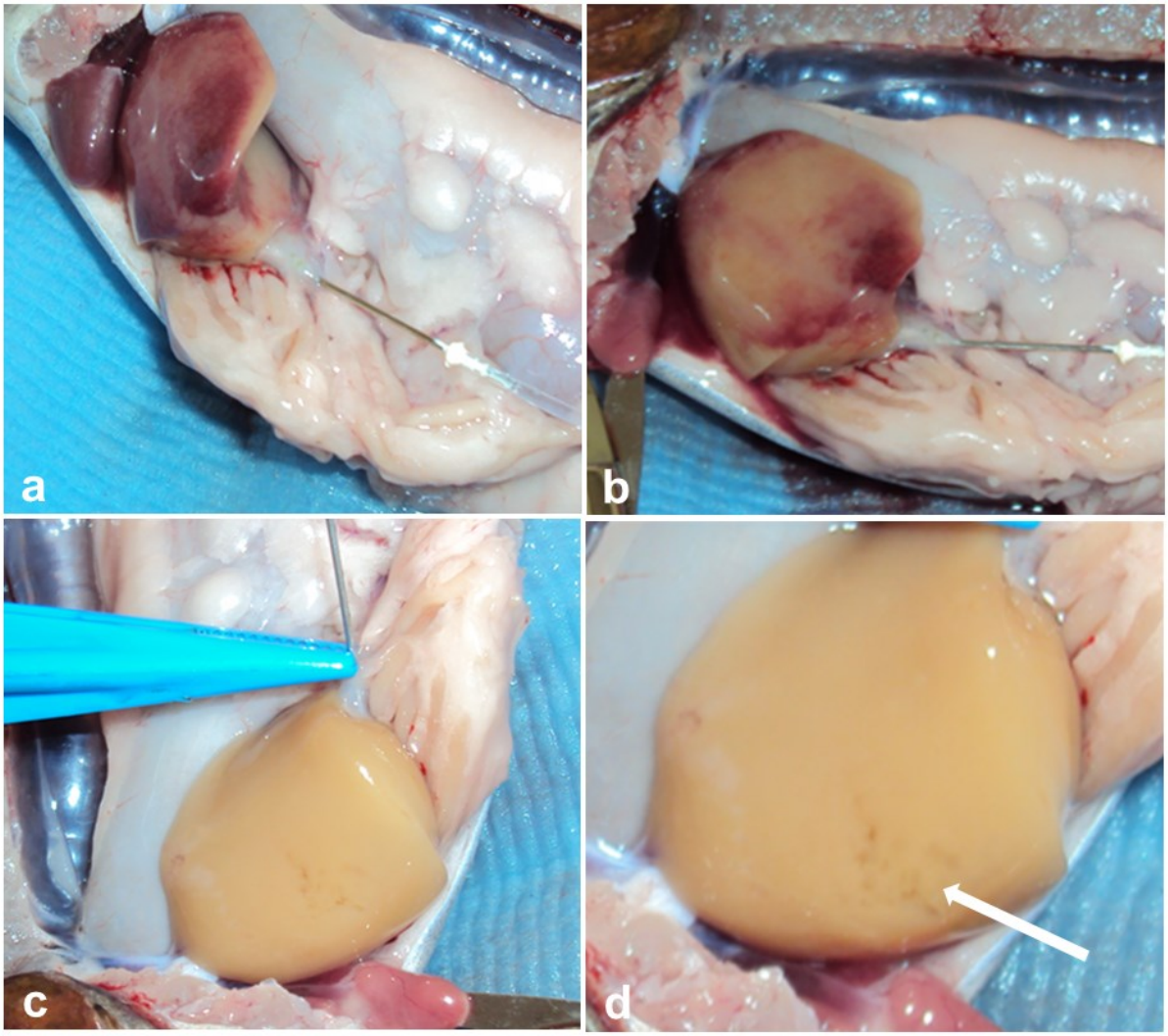


Figure 2.4 Stages of *in situ* liver perfusion. (a) The cannula is inserted into the hepatic portal vein approximately 1 cm from the liver junction and the perfusate (calcium and magnesium-free HBSS) pumped through and monitored for 1 min. (b) A colour change from deep red to straw yellow throughout the lobes indicate a flushing of residual blood from the liver at which point the ventral aorta of the heart is cut. (c) At the end of the first stage of perfusion (~10 min), the liver is completely cleared of blood. (d) During the enzymatic digestion stage with collagenase D solution, patches of disaggregated cells can be seen appearing below the surface of the liver (white arrow).

The pump was then switched back on and the perfusion continued for 20 min (1 ml min^{-1}, 20 ml total). During this time, the liver's appearance was observed, where patches of disaggregated cells appearing below the surface were an indicator of efficient digestion (Fig. 2.4d).

2.4.5 Enzyme clearance

The perfusion was continued as per the blood clearance and enzymatic digestion steps with Leibovitz's (L-15) media (with L-glutamine and without phenol red, pH 7.4) for 3 min (3 ml min^{-1}, 3 ml total) to clear residual collagenase solution from the liver. As blood is cleared from the liver within 1-2 min, a 3 min enzyme clearance period was considered to be sufficient. The pump was then stopped, the haemostat unclamped and the cannula removed from the vein.

2.4.6 Liver dissociation and hepatocyte isolation

The liver was removed from the body cavity by carefully cutting the hepatic portal vein, hepatic arteries, hepatic veins and adjoined connective tissue. The gall bladder was pulled away from the liver and cut, at the bile duct, while being sealed with tweezers to prevent bile from leaking onto the liver. The liver was briefly washed with calcium and magnesium-supplemented HBSS, pat dried with paper towel and weighed on a sterile; 60 mm untreated Petri dish, and then washed with 15 ml of L-15 media (without serum). The outer surface of the liver was cut open and the disaggregated cells gently raked out into the surrounding medium.

The cell suspension was first passed through 100 µm sterile filter gauze via a 20 ml sterile syringe and collected in a 50 ml sterile centrifuge tube. This was repeated with

40 µm sterile filter gauze to remove undigested sections of liver. The gauze was washed with medium and the cell suspension made up to a final volume of 20 ml. The cell pellet was collected by centrifugation (50 *g*; 5 min; 15 °C), the supernatant removed and the cell pellet re-suspended in 20 ml fresh medium. This was repeated twice more to remove dead cells. Following the final centrifugation and supernatant removal, the cell pellet was re-suspended in 20 ml L-15 medium supplemented with 10 % fetal bovine serum (FBS: pH 7.4). Cell suspensions were immediately transferred to micro-plates after isolation to maximise viability.

2.4.7 Cell viability and density counts

A 100 µl aliquot of cell suspension was added to 100 µl of trypan blue solution (0.2% final concentration) and incubated for 10 min at 15 °C. The solution was then briefly mixed and 100 µl placed on a haemocytometer slide. Live cells (viable) with intact cell membranes do not allow trypan blue to be absorbed. In dead cells (non-viable) the stain is absorbed across the membrane, appearing blue under a microscope. Viable, non-viable and total cell number were counted and cell viability was calculated (No. viable cells / Total cell count x 100 = % viable cells). Cell suspensions with a viability of ≥ 85% were used for subsequent experiments.

2.5 Primary cell culture

2.5.1 3-D (spheroid) culture

Isolated cell suspensions were diluted (1 x 10^6 cells ml^{-1}) in L-15 medium supplemented with 10% FBS (pH 7.4) and transferred to non-tissue culture treated six-well micro-plates (Falcon, VWR); 3 x 10^6 cells $well^{-1}$; 3 ml total volume) that had been pre-coated with 500 µl of 2.5% poly(2-hydroxyethyl methacrylate) (p-HEMA)

solution (plates dried for 48 h in a sterile, Class II culture cabinet) and placed at 15 ºC on an orbital shaking platform (Innova 2000; New Brunswick, UK) set at a constant rotation speed of 70 RPM. After 24 h, when aggregates had started to form, 1.4 ml of old medium was replaced with 1.5 ml of fresh medium to compensate for well evaporation as per the protocol followed for generating mammalian spheroids (Ma et al. 2003). Culture media was then changed every 2 days thereafter (1.3 ml replaced with 1.5 ml of fresh medium).

2.5.2 2-D (monolayer) culture

Diluted cell suspensions (1 x 10^6 cells ml^{-1}) in L-15 medium supplemented with 10% FBS (pH: 7.4), were plated (3 ml $well^{-1}$) in treated-tissue culture six-well micro-plates (Iwaki, Sterilin) and incubated statically at 15 ºC. After 24 h, 1.4 ml of old medium was replaced with 1.5 ml of fresh medium and thereafter every 2 days. Both spheroid and monolayer cultures were prepared from the same donor organ.

2.6 Fish serum preparation

Female rainbow trout (1900.0 ± 265.5 g; *n* = 9) were bled using 20 ml syringes and Na-heparinised 0.8 mm needles (5000 U ml^{-1} heparin sodium salt). Blood samples were pooled (~40 ml per fish), distributed between 50 ml centrifuge tubes and held on ice for 5 h. Tubes were then centrifuged at 3000 *g* for 20 min at 4 ºC. Serum layer was removed and stored at -80 ºC until use.

2.7 Microscopy

2.7.1 Spheroid size measurements

Diameters of individual spheroids were calculated from digital images acquired using a microscope mounted digital camera (OptixCam Summit, USA) attached to an inverted light microscope (Olympus® CK40-SLP) at a total 100 (10 X 10) magnification, at days 4, 6 and 8. Radius (*r*) measurements of spheroids and individual hepatocytes were also calculated to determine spheroid / hepatocyte volume $\left(V = \frac{4}{3}\pi r^3\right)$, which in turn was used to calculate the number of cells per spheroid $(\frac{spheroid\ volume}{hepatocyte\ volume})$. Digital images were analysed using Image J analysis software (Maryland, USA; http://image j.nih.gov/ij/).

2.7.2 Phase-contrast light microscopy

Morphological changes of developing spheroid and monolayer cultures were observed under light microscopy for ≤ 80 d.

2.7.3 Scanning electron microscopy (SEM)

Spheroids were removed from culture after 1, 2, 4 and 8 d, fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h then dehydrated with 30, 50, 70, 90 and twice in 100% ethanol, 5 min for each concentration. Liquid was removed from the samples by critical point drying for 1 h and samples were sputter-coated with gold. Surface structures of developing and mature spheroids were observed under a scanning electron microscope (JEM-5600LV, JEOL Ltd, UK).

2.7.4 Cryo-scanning electron microscopy (C-SEM)

Spheroids were removed from culture after 3 and 10 d, blotted onto filter paper and secured on metal stubs, whereupon any excess culture medium was removed. Samples were then plunge frozen in liquid nitrogen (-196 ºC) and inserted into a cryo-preparation chamber (Quorum Technologies PP3000T). Samples were then sublimed for 5 min at -35 ºC and sputter-coated with gold for 90 s. Samples were then observed under a scanning electron microscope (JSM- 6610LV, JEOL Ltd, UK).

2.7.5 Transmission electron microscopy (TEM)

Eight day old spheroids were fixed in two stages: (1) 2.5% glutaraldehyde dissolved in 0.1 M cacodylate buffer (pH 7.2) for 1 h, rinsed with distilled water, then (2) 1% osmium tetroxide (0.1 M cacodylate buffer; pH 7.2) for 1h. Samples were then rinsed with distilled water and dehydrated as per the protocol for SEM preparation (Section *2.9.2*). Samples were then immersed in acetone for two 15 min periods, followed by a sequence of propylene oxide : pure resin monomer concentrations: (a) 2 : 1, (b) 1 : 1, (c) 1 : 2 mix followed by (d) 100% resin for 1 h, except 100% resin which was left overnight. Samples were then transferred to a resin mould with fresh resin and left for 24 h at 60 ºC for polymerisation to occur. Resin blocks were sectioned (0.45 µm) using an ultra-microtome, mounted on a copper grid and stained in uranyl acetate and lead citrate for 1 h. Samples were observed under a transmission electron microscope (JEM-1200 EXII, JEOL Ltd, UK).

2.7.6 Confocal microscopy

Fresh hepatocytes (1 h post isolation), 1, 2 and 8 d old spheroids were removed from culture and placed into 1.5 ml Eppendorf tubes. Fresh hepatocytes and weak

aggregates were centrifuged briefly to pellet, or in the case of spheroids were allowed to sediment without centrifugation. Old medium was removed, suspensions washed twice with 500 µl Dulbecco's phosphate buffered saline (DPBS) and fixed with 2.5% glutaraldehyde dissolved in 0.1 M cacodylate buffer (pH 7.2) for 1 h. After fixation, suspensions were washed twice with 500 µl DPBS and extracted with 500 µl of 0.1% Triton X-100 (in DPBS) for 5 min. Washing with DPBS was repeated and suspensions were blocked with 500 µl 1% bovine serum albumin (BSA) (in DPBS) for 30 min. The washing step was repeated, tubes wrapped in foil and 200 µl DPBS added to each tube followed by 10 µl of Alexa flour 488 phalloidin (10 U ml^{-1} working solution) and left for 20 min. The washing step was repeated to remove excess stain, suspensions re-suspended in 2 ml DPBS followed by 0.4 µl 4´,6-diamidino-2-phenylindole (DAPI) (1 µg l^{-1} working solution; in DPBS). The washing step was repeated and suspensions re-suspended in 1 ml DPBS. All washing and incubation steps were carried out at room temperature.

DPBS was carefully removed and 100 µl Vectorshield® mounting medium (Vector Laboratories, UK) added to each tube. Suspensions were briefly mixed with a 200 µl pipettor and an 80 µl drop transferred to a cavity-glass microscope slide (Marienfeld-Superior, Germany). Slides were air dried for 1 min before sealing with a glass coverslip. Slides were covered in foil and the gel allowed to harden overnight at room temperature. Images were acquired using a confocal microscope (FV1200MPE, Olympus, UK).

2.8 Biochemical assays

2.8.1 Sample collection and preparation

Samples were prepared as described previously (Ma et al. 2003) with some modifications for fish tissue. Old culture medium from each well was completely replaced with 3 ml serum-free L-15 medium 24 h before sampling at each respective time point. Spheroids were collected 24 h after changing the medium in 2 ml aliquots and transferred to micro-centrifuge tubes. Monolayer cultures were carefully scraped into the surrounding media using a cell scraper. The tubes were centrifuged for 3 min at 900 *g* and the media decanted into separate 500 µl aliquots. The pellet was washed twice in 500 µl DPBS then homogenised for 60 s in 100 µl of homogenisation buffer (2 mM NaH_2PO_4, 2 mM Na_2HPO_4, 0.5 mM EDTA and 145 mM NaCl, pH 7.4) (Xu et al. 2003a) using a micro-tube pestle, followed by 30 s sonication in an ultra-sonic water bath. Homogenates were made-up to a final volume of 1 ml homogenisation buffer and split into 250 µl aliquots. Sample preparation was carried out on ice and media and homogenate samples were stored at -20 ºC prior to assay.

2.8.2 Protein

Total protein was determined using a Pierce BCA protein assay kit (Thermo Scientific, UK) following the manufacturer's instructions. Briefly, a 10 µl aliquot of neat homogenate sample was added to each well of a 96-well micro-plate (Iwaki, Sterilin) in duplicate, followed by 200 µl of BCA reagent. The plate was allowed to stand for 3 min at room temperature (21 ºC) then incubated for 30 min at 37 ºC. Absorbance was read on a micro-plate reader (SpectraMax M5, Molecular Devices, USA) at 595 nm. For protein per hepatocyte analysis, contents of individual wells

were disaggregated using 0.05% trypsin (1:5 dilutions with versene) for 10 min at room temperature and 50 µl aliquots used for cell counts. Total protein was determined as described.

2.8.3 Albumin

Albumin concentrations were determined using an albumin fluorescence assay kit (Fluka, UK) as described previously (Kessler and Wolfbeis 1992), modified in our laboratory to be suitable for a micro-plate reader in a 96-well assay format. A 50 µl aliquot of neat medium or homogenate sample was added to each well of a black 96-well micro-plate (Falcon, VWR) in duplicate, followed by 250 µl of assay reagent. The plate was allowed to stand for 2 min at room temperature and the fluorescence read on a micro-plate reader (SpectraMax M5, Molecular Devices, USA) with an emission wavelength of 600 nm and an excitation wavelength of 630 nm.

2.8.4 Glucose

Glucose concentrations were determined using an enzymatic glucose kit based on the oxidation of D-glucose to gluconic acid catalysed by the enzyme glucose oxidase. This method was modified in our laboratory to be suitable for a micro-plate reader in a 96-well assay format as outlined by (Xu et al. 2003a). A 50 µl aliquot of neat medium or homogenate sample was added to each well of a 96-well micro-plate in duplicate, followed by 250 µl of assay reagent. The plate was incubated for 30 min at 37 ºC and the absorbance read on a micro-plate reader (Spectra Max M5, Molecular Devices, USA) at 540 nm.

2.8.5 Lactate dehydrogenase (LDH)

LDH concentrations were determined based on the method described by (Scholtz & Segner 1999). A 50 µl aliquot of diluted medium (1:5) or homogenate (1:10) sample was added to each well of a 96-well micro-plate in duplicate, on ice, followed by 250 µl of reaction buffer (50 mM Tris-chloride, 0.14 mM NADH, pH 7.5). The plate was allowed to stand at room temperature for 5 min and the reaction started with the addition of 25 µl of 12.1 mM sodium pyruvate dissolved in 50 mM Tris-chloride reaction buffer (pH 7.5). The contents of the plates were briefly mixed and the enzyme activities recorded for 20 min at 25 ºC, in a micro-plate reader (SpectraMax M5, Molecular Devices, USA) at 340 nm.

2.8.6 Ethoxyresorufin-O-deethylase (EROD) activity

Live-cell EROD assays were carried out as previously described (Behrens et al. 1998) with modifications for spheroid cultures. Monolayer and spheroid cultures were prepared from the same individual fish and were seeded into black pHEMA-coated 96-well micro-plates (Falcon, VWR, UK in 50 µl serum-free L-15 media (pH 7.4). A positive control (0.36 µM β-napthoflavone (BNF); final well concentration) and a solvent control (0.1% DMSO; final well concentration) both dissolved in serum-free media were then added to wells in 50 µl volumes and plates were incubated at 15 ºC for 48 h. After the exposure period, 50 µl of reaction buffer (serum-free L-15 media, 16 µM 7-ethoxyresorufin (7-ER), 18 µM dicoumarol; final well concentrations; pH 7.4) were added to each well and the production of resorufin measured in a fluorescence plate reader (SpectraMax M5, Molecular Devices, USA; excitation, 544 nm, emission, 590 nm) immediately after addition of the medium and every minute thereafter for 60 min. Standard curves of resorufin (0 – 256 pmol $well^{-1}$) were produced for each

assay (150 µl well volumes). Stock solutions of 7-ER and resorufin were prepared in DMSO and dicoumarol in 1 M NaOH. Total protein concentration was determined using a Pierce BCA protein assay kit as described previously (Section *2.8.2*).

To calculate EROD activity of the live cell cultures, reaction linearity was plotted for each sample over time. For each reaction, resorufin production was determined my calculating the amount of resorufin (pmol) present in the well at two different time-points. Using the resorufin standard curve, fluorescence values were then converted to resorufin amount (pmol). The difference in resorufin amount was divided by the number of minutes over which the measurement was obtained (pmol min^{-1}). This value was then divided by the protein value (mg) obtained from the protein assay. EROD activity was then expressed as: pmol min mg^{-1} protein.

2.9 Viability assays

2.9.1 Tetrazolium salt reduction (WST-1) assay

Spheroid viability was determined in separate plates of mature spheroids via a tetrazolium salt reduction method (WST-1 reagent, Roche Scientific, UK). Spheroids produced via the 6-well plate, gyratory method were pooled, washed, counted and transferred to pHEMA-coated 96-well micro-plates (100 spheroids $well^{-1}$; 75 µl L-15 medium) using a multi-channel pipettor. Spheroids were checked for morphological integrity before addition of 75 µl L-15 medium containing the test concentrations (*n* = 6 wells). The final well test concentration range was 0 – 320 µg l^{-1}; 0.1% DMSO. A positive control (1% Triton X-100) was also included (*n* = 6 wells). Plates were incubated at 15 ºC for 48 h. Survival and viability was assessed first by visual observation (spheroids dissociate as viability decreases). After 24 h of exposure, 15

µl of WST-1 reagent was added directly to each well (1:10 dilution) and plates incubated for a further 24 h. Absorbance was read in a micro-plate reader (SpectraMax M5, Molecular Devices, USA) at 450 nm.

2.10 Metabolism assays

2.10.1 Substrate depletion assay

Spheroids were pooled (for each individual fish) from 6-well micro plates, into a pHEMA-coated 50 ml centrifuge tube and washed three times with 10 ml L-15 medium (no serum). As spheroids were too large to be counted on a haemocytometer, 4 x 5 µl drops of spheroid suspension were transferred to a glass microscope slide and spheroids were counted at X4 magnification. Spheroid suspensions were transferred to a sterile reagent reservoir, agitated with a multi-channel pipettor to maintain a homogenous solution and transferred to 96-well pHEMA-coated micro plates (Iwaki, Sterilin, UK) at a seeding density of 100 spheroids $well^{-1}$ (in 75 µl L-15 medium; pH 7.4). At this time, morphological integrity of spheroids was assessed under an inverted light microscope to determine their suitability for exposure.

Spheroids were then exposed to test chemicals (75 µl volume) for 24 - 72 h, depending on the test chemical. Solvent controls (spheroids + medium & solvent) and blanks (test chemical - spheroids) were also added to separate wells. Micro plates remained at 15 ºC for the entire exposure period. Metabolism was quenched with the addition of 150 µl acetonitrile (ACN) at set time points after which the plates were sealed tightly with plate sealers and analysed for substrate depletion by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Total protein

concentration was determined using a Pierce BCA protein assay kit as described previously.

2.10.2 LC-MS/MS

For LC-MS analysis, standard solutions were prepared for each compound to cover the range 1 to 1000 nM. These were made up to match the sample solvent composition i.e. 80:20 water:ACN containing 10 nM internal standard. Analyses were performed using a TSQ Quantum Access mass spectrometer (Thermo Scientific San Jose CA USA). Chromatographic separation was achieved by gradient elution on a Hypersil Gold 2.1 x 50 mm 3 µm C_{18} column (Thermo Scientific San Jose CA USA). The mobile phase was a mixture of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol programmed as follows: 80% A to 100% B over 1.5 min and held for 1.5 min, then reset to initial conditions. The flow rate was 500 µl min^{-1} with an injection volume of 20 µl. The mass spectrometer was operated in electrospray ionization mode using selected ion monitoring with a capillary temperature of 270 °C, vaporiser temperature 350 °C, spray voltage 3750 V, sheath gas nitrogen @ 50 (arbitrary units) and auxiliary gas nitrogen at 30 (arbitrary units). Each compound was automatically optimized for ion polarity, precursor ion, and product ion and collision energy using QuickQuan software (Thermo Scientific San Jose CA USA). Compound determination was by comparison to standard solutions of known concentration using an internal standard method. LC-MS/MS analysis was carried out by Mr. Rob Cumming at Brixham Environmental Laboratory.

2.11 Statistical analysis

*S*tatistical analysis was performed using Minitab version 15 software. Normal distribution and homogeneity of variance of data sets were assessed by Shapiro-Wilk and Levene's tests, respectively. Datasets were analysed using parametric tests; one-way ANOVA with post-hoc Tukey HSD test for comparisons between culture treatments (biochemical; WST-1 viability) or a paired-sample t-test (EROD activity; LOP seeding density). Depletion rate constants (*k*: h^{-1}) were calculated using non-linear regression analysis (Sigma Plot v12.5) and used to calculate the half-life ($t_{1/2}$) of the parent chemicals exponential decay. Coefficients of variance (*CV*) were also calculated to assess the degree of variability between fish.

CHAPTER 3

Development and optimisation of 2-D and 3-D liver cell cultures from rainbow trout (*Oncorhynchus mykiss*)

3.1 Introduction

The development, optimisation and validation of reliable and robust *in vitro* methods that offer an alternative to whole animal *in vivo* studies that can support regulatory ecotoxicology, is rapidly becoming an important requirement on a global scale (EMA 2006; Schrattenholz and Klemm 2006; Mazzoleni et al. 2009; PPPR 2009; REACh 2009; Scholtz et al 2013) in a response to the growing concern on the number of animals used for the environmental hazard assessment of chemicals (van der Jagt 2004). The use of conventional *in vitro* culture systems such as S9 microsomal preparations, freshly isolated primary cells, 2-D monolayer and immortalised cell lines have been used extensively in the ecotoxicological assessment of chemicals to identify end-points that may address specific toxic effects and/or the mechanistic action of chemicals in target organs or different cell types (Zucco et al. 2004; Xiao et al. 2007; Vevers and Jha 2008; Papis et al. 2011). However, concerns over their ability to maintain periods of organotypic functionality question there suitability for use in chronic toxicity and bio-accumulation studies (Baron et al. 2012).

Three-dimensional cell culture systems can address some of the problems associated with these conventional culture techniques, in particular the 3-D geometry and cyto-architecture can be more representative of *in vivo* tissues and they can maintain important biochemical organ functions, over time, in culture (Ma et al. 2003; Xu et al. 2003; Baron et al. 2012). However, investigations into the use of 3-D cell culture systems for application as a potential new tool for aquatic toxicology testing is limited, despite the extensive use of these 3-D systems for mammalian toxicological applications (Pampaloni et al. 2007).

Many methodologies have been proposed to generate 3-D micro-tissues (Table 1.1). Some require external matrices to simulate ECMs such as hydrogel structures, porous substrates / scaffolds (Chevallay and Herbage 2000a; Wang et al. 2009) and others that require only simple rotational forces to re-aggregate cells into 'ball-like structures (Walker et al. 2000; Ma et al. 2003; Xu et al. 2003). Due to the relatively simplistic nature of generating these spheroidal cultures via rotational techniques and previous investigations that have incorporated this technique for the generation of fish hepatic aggregates (Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996), it was decided to utilise this technique for the generation and optimisation of 3-D fish liver spheroids for use in further experiments.

3.2 Materials and Methods

3.2.1 Fish size / perfusion optimisation

Flow rates and perfusion times were optimised using different sized fish (Group A: 800-1800 g; B: 180-300 g and C: 80-150 g).

3.2.2 Primary cell culture

3.2.2.1 Seeding densities

Cell suspensions were adjusted to give a final concentration of 4×10^6 cells ml^{-1}. To optimise culture periods of hepatic models, cell suspensions were diluted and seeded at different densities (4×10^6; 2×10^6; 1×10^6; 0.5×10^6; 0.2×10^6 and 0.1×10^6 cells ml^{-1}; Fig. 3.1a).

Figure 3.1 (a) *In vitro* hepatic cultures under 'spheroidal' rotational conditions at different seeding densities. (b) pHEMA-coated 6-well micro-plate that has undergone routine culture maintenance. Clumps of hepatic tissue have been removed and medium has been exchanged to maintain a well volume of ~3 ml. Cultures were cleaned (by removing cellular debris) as thoroughly as possible at each respective culture maintenance time point so as to maximise the longevity of *in vitro* cultures.

3.2.2.2 Culture vessels

Cell suspensions for monolayer culture were seeded in tissue culture-treated 6-well micro-plates (3 ml well^{-1}). For spheroid culture, suspensions were seeded in pHEMA-coated 6 (3 ml well^{-1}); 24 (1.5 ml well $^{-1}$) and 96 (250 µL well^{-1}) well micro-plates (treated and non-treated) and 10 ml Pyrex® beakers (2 ml beaker^{-1}). Suspensions in 96-well micro-plates were seeded between 0.1 – 1 x 10^6 cells ml^{-1} only. Pyrex® beakers were autoclaved then covered with a Petri dish lid prior to *in vitro* culture.

3.2.2.3 Culture media

Monolayer and spheroid cultures were maintained in either (1) Dulbecco's modified eagle medium:Ham's F12 mixture (DMEM:F12) or (2) L-15 media with the following serum supplementations: (a) no serum; (b) 2% FBS; (c) 5% FBS; (d) 10% FBS; (e) 2% serum-replacement mixture (SR); (f) 5% SR; (g) 2% trout serum and (h) 5% trout serum. All culture media solutions were pre-filtered through a 0.22 µm sterile filter and adjusted to pH 7.4.

3.2.2.4 Temperature

Cultures were incubated at 15 and 18 °C (in an incubator) and 21 °C (on open bench).

3.2.2.5 Rotation speeds

Spheroid cultures were incubated (1) statically and (2) under different incubator / orbital shaker conditions: (a) IKA KS 260 rotating platform (10 mm orbit), Scientific Laboratory supplies, UK; (b) Innova 2000 rotating platform (19 mm orbit), both of which were housed in a Galaxy 170 R refrigerated incubator (New Brunswick, UK)

and (c) MaxQ 4000 refrigerated incubator with integrated shaker platform (19 mm orbit), Thermo Scientific, UK. Orbital rotation speeds were set at 50, 60, 70 and 80 RPM from initial seeding and aggregation was observed after 1 h then every 12 h thereafter. Monolayer cultures were incubated statically in their respective culture vessels for 24 h in the IKA KS 260 and Galaxy 170 R incubators to allow cells to attach and reach confluency.

3.2.2.6 Media and culture maintenance

After 24 h in culture, 1.4 ml of old medium was replaced with 1.5 ml of fresh medium to compensate for evaporation as per the protocol followed for generating mammalian spheroids (Ma et al. 2003). During medium changes, clumps of non-viable hepatic tissue were removed using a 200 µl pipette (Fig. 3.1b). Old medium from monolayer cultures (1.4 ml) was replaced with 1.5 ml fresh medium using a Pasteur pipette after 24 h, leaving 1.5 ml of medium in the well so as not to disturb the monolayer. Medium was then replaced every 48 h thereafter (1.3 ml replaced with 1.5 ml fresh medium) in all cultures.

3.3 Results

3.3.1 Fish and perfusion optimisation

During this preliminary period of culture method development, liver size, perfusion periods / flow rates, enzyme concentrations and digestion times were optimised to maximise cellular yield and viability (Table 3.1). Perfusions with larger fish (Group A - 1300 ± 368.8 g; *n* = 10) were carried out to determine whether greater hepatic yields could be produced from larger livers (13.0 ± 1.8 g; *n* = 10). These fish proved difficult

to perfuse, with extended perfusion and enzymatic digestion periods (> 1.5 h); increased flow rates (3-4 ml min^{-1}) and higher enzyme concentrations (0.5%) required to only partly perfuse the liver. Cellular yield was lower and more variable ($8.9 \times 10^6 \pm 16.0$ cells g^{-1} liver; *n* =10) than those acquired from smaller fish, as was cell viability (72 ± 5%; *n* = 10).

Perfusion of livers (2.5 ± 0.4 g; *n* = 8) from medium-sized fish (Group B – 233.9 ± 32.5 g; *n* = 8) resulted in greater cellular yields ($119.6 \times 10^6 \pm 57.9$ cells g^{-1} liver), ~17-fold higher than those acquired from larger livers and a higher cell viability (85 ± 5%; *n* =8), although on occasions the viability acceptance criteria (≥ 85%) were not met. Perfusion / digestion periods, flow rates and enzyme concentrations were all reduced accordingly (≥ 45 min; 1 ml min^1 and 0.1%, respectively). Patches of digested cells could be seen during enzymatic digestion using a collagenase concentration of 0.1%. Higher (0.5%) and lower (0.02%) collagenase concentrations resulted in lower cell viability and poor tissue digestion, respectively. Livers (1.3 ± 0.3 g) perfused from small-sized fish (Group C – 106.4 ± 19.3 g; *n* = 10) produced a less variable hepatic yield (67.3 ± 19.6 cells g^{-1} liver; *n* = 10). Average viability was 92 ± 5% (*n* = 10).

Table 3.1 Liver biometry and cellular viability (measured by trypan blue exclusion) of perfused livers from different-sized rainbow trout. Values are expressed as mean ± SD.

Fish group	Fish Size (g)	*n*	Body weight (g)	Liver weight (g)	Total hepatocyte yield (x 10^6)	Cells g^{-1} liver (x 10^6)	Viability (%)
A	800-1800	10	1300.0 ± 368.8	13.0 ± 1.8	115.7 ± 208.0	8.9 ± 16.0	72 ± 5
B	180-300	8	233.9 ± 32.5	2.5 ± 0.4	295.9 ± 130.5	119.6 ± 57.9	85 ± 5
C	80-150	10	106.4 ± 19.3	1.3 ± 0.3	85.8 ± 33.1	67.3 ± 19.6	92 ± 5

Cell suspensions were purified by low-speed centrifugation prior to seeding as whole liver cell suspensions (i.e. suspensions that contained viable and non-viable hepatic cell types) did not re-aggregate into spheroids under gyratory-mediated conditions. Cell suspensions may have contained a mixture of hepatic cell types (viz. sinusoidal endothelial cells, stellate cells, Kuppfer cells and hepatocytes) but were hard to differentiate under light microscopy after cells had rounded-up and further SEM analysis of single cell suspensions could only confidently identify hepatocytes and residual blood cells. Based on previous observations, hepatocytes contribute ~80% of the cell suspension (Segner 1998). Following low-speed centrifugation steps, hepatocytes represented ~95% of the total cell population. Despite this still potentially remaining a mixed cell type suspension, it was decided to refer to cell / hepatic suspensions as 'hepatocytes' for all further experimental chapters.

3.3.2 Primary cell culture

3.3.2.1 3-D (spheroid) culture

Hepatocytes cultured statically on pHEMA-coated culture vessels did not aggregate, regardless of vessel type or seeding density. Cultures seeded in 24 and 96-well micro-plates and 10 ml beakers and placed under orbital-rotation conditions, formed poorly-defined aggregates with a high degree of clumping between individual aggregates, independent of seeding density. Single-cell cultures (Fig. 3.2a) seeded at 1-2 x 10^6 cells ml^{-1} with a constant rotation speed of 70 RPM and with additional 10% FBS added to the culture media, formed weak aggregates after just 24 h (Fig. 3.2b) with spheroids developing after 3 d in culture (Fig. 3.2c). After 4 d the aggregation process was near completion with only a few single cells remaining in the culture medium (Fig. 3.2d).

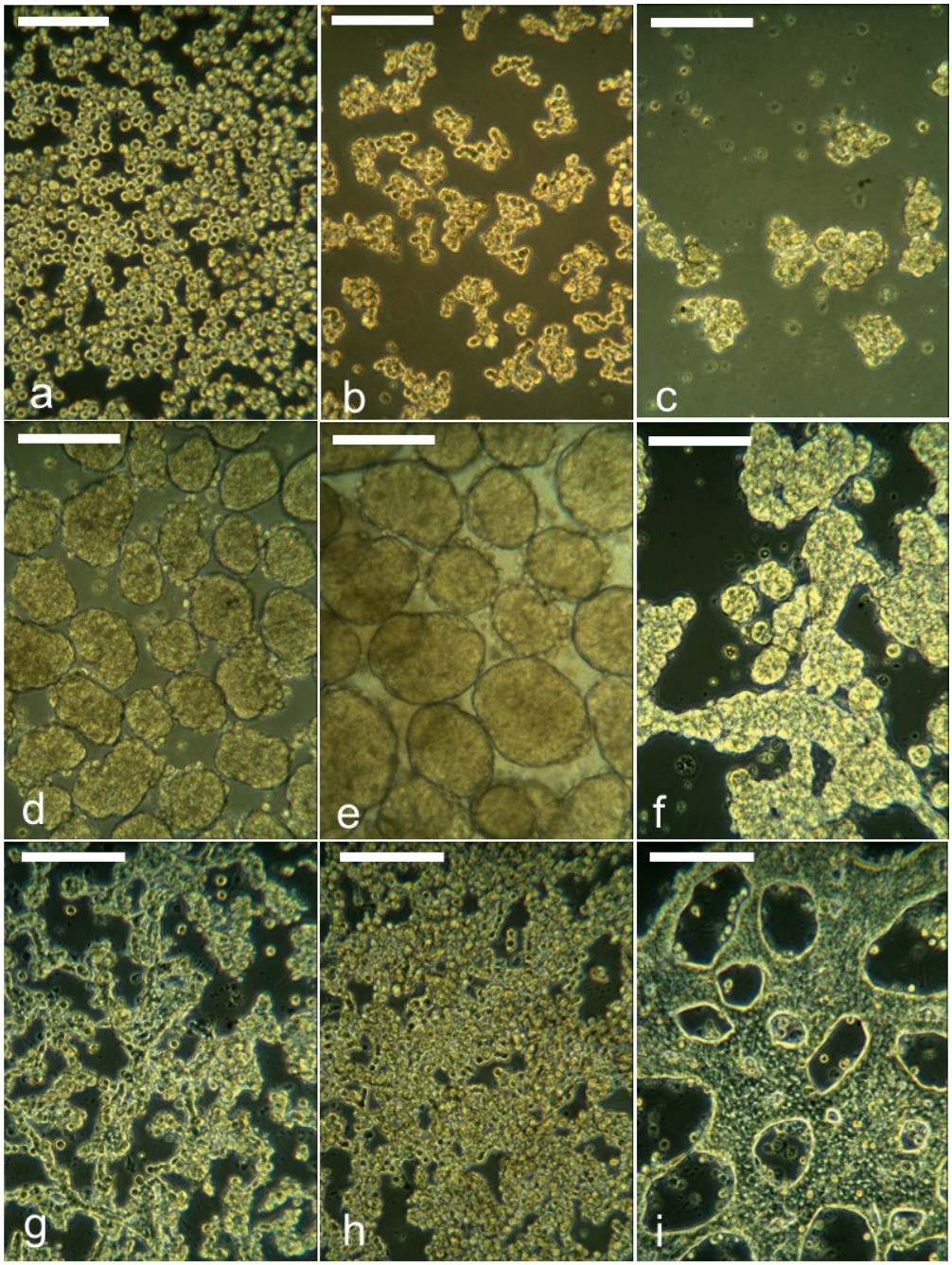


Figure 3.2 3-D and 2-D cultures. (a) Freshly seeded single cells; (b) aggregates at 24 h; (c) 3 d; (d) 4 d and (e) spheroids at 8 d, all supplemented with 10% FBS. (f) Monolayer cultures at 3 d supplemented with 5% SR and (g) in serum-free media after 2 d. (h) Addition of 1 % FBS had noticeable effects on monolayer cultures with well-defined cell-cell contacts observed from 24 h and a total culture period of 8 d maintained (i). Scale bar = 100 μm. Images taken at 100 X magnification.

Spheroids produced from cells seeded at 1 x 10^6 cells ml^{-1} had a more consistent size and structure than those cultures seeded at 2 x 10^6 cells ml^{-1}, with fewer single cells remaining in the culture medium and after 8 d exhibited a tight, 'ball-like' structure (Fig. 3.2e).

Preliminary experiments where spheroids were formed on a rotating platform at room temperature (21 ºC) resulted in clumping after 4 - 5 d of culture. Initial problems with cell culture incubators and subsequent temperature control resulted in maintenance of fish cultures at 18 ºC. Successful spheroid formation was observed when cells were maintained at this temperature. However, beyond 16 d individual spheroids began to clump together in culture (Fig. 3.3a). No clumping was observed when spheroids were maintained at 15 ºC.

The effect of rotation speed (50–85 RPM) on hepatocyte aggregation was tested based on previous studies (Flouriot et al. 1993; Walker et al. 2000; Xu et al. 2003a). During the initial aggregation process, rotation speeds > 70 RPM and a small rotation orbit (10 mm) resulted in the formation of irregular-shaped aggregates (Fig. 3.3b-c). Slower rotation speeds (50–65 RPM) caused a degree of clumping between developing spheroids regardless of orbit diameter (Fig. 3.3d). A consistent rotation speed of 70 RPM implemented at initial seeding; a rotation orbit of 19 mm; an incubating temperature of 15 ºC and an accurately maintained well volume (3 ml) was deemed optimum for aggregation and maintenance of developing spheroids.

Lower seeding densities produced smaller, weaker aggregates after the same time, with noticeably higher single cell populations. The highest seeding density (4 x 10^6

cells ml^{-1}) was deemed to be over populated with cells, producing large, rough clumps of cellular aggregates. Cells that were cultured in 2% SR, 5% SR, 2% trout serum, 5% trout serum and serum-free conditions did not form spheroids with a noticeable deterioration beyond 24 h, where upon all cells had aggregated into a large, single clump. Spheroids cultured with additional 2% and 5% FBS showed a slower rate of aggregation, exhibiting non-uniform aggregates after 8 d in culture. The formation of spheroids was similar in both DMEM:F12 and L-15 medium.

For spheroid formation between 11 and 21 d, culture medium replacement was extended to every 5 d. Beyond 21 d medium was changed every 7 d. After 21 d, FBS was removed from the culture media, as the morphological integrity of spheroids remained tight. Rotation speeds were increased to and remained at 80 RPM from 8 d to eliminate clumping of spheroids.

3.3.2.2 2-D (monolayer) culture

Cultures grown as monolayers exhibited good attachment after 24 h at all seeding densities. Cultures seeded at higher cell densities (1-4 x 10^6 cells ml^{-1}) exhibited the most prolonged growth with the addition of 10% FBS. Monolayers cultured with 2 and 5% SR formed aggregated clumps after 3 d (Fig. 3.2f) and monolayers had peeled away from the well base. In serum-free conditions, monolayers grew well over a 2 d period, with noticeable cell-cell contacts (Fig. 3.2g) however deterioration of cultures was evident after 3 d with large numbers of detached cells present. The addition of 10% FBS had very noticeable effects on the cultures with well-defined cell-cell contacts from 24 h (Fig. 3.2h) and a culture period of 8 d was maintained in both culture conditions (DMEM:F12 and L-15) (Fig. 3.2i).

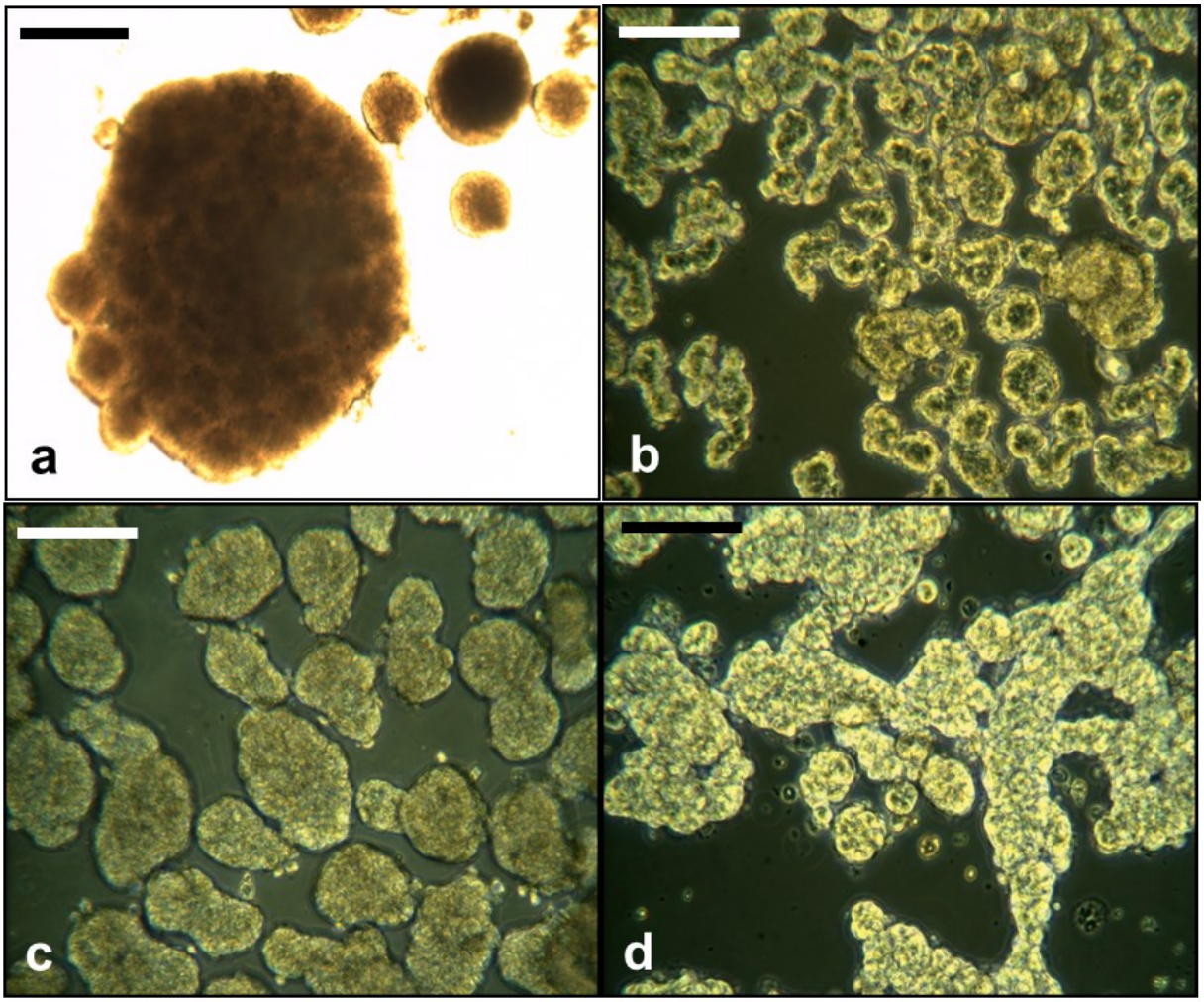


Figure 3.3 Effects of temperature and rotation speed / orbit on developing spheroids. (a) Large, multi-spheroid clump after 16 d in culture incubated at 18 ºC. Scale bar = 50 µm, image taken at 40 X magnification. (b) Irregular-shaped 4 d and (c) 6 d spheroids rotated at 80 RPM / 10 mm orbit. (d) Spheroids at 8 d rotated at 50 RPM. Scale bar (b-d) = 100 µm, images taken at 100 X magnification.

3.4 Discussion and conclusions

Cell isolation procedures and subsequent culture parameters can significantly affect the formation and functionality of *in vitro* hepatic cultures (Lin et al. 1995; Niwa et al.

1996; Juillerate et al. 1997). This method development study has demonstrated the importance of optimising these parameters (i.e. fish size, perfusion periods, enzyme concentrations, purified hepatic suspensions, serum-supplemented culture media, culture set-up and maintenance) to produce viable cell yields and for successful spheroid formation. Moreover, a thoroughly validated method development will give greater confidence in the use of a 'novel' *in vitro* fish model. To date, conventional models (i.e. fresh hepatocytes and monolayer cultures) are commonly used for fish *in vitro* studies.

Fish of various size (100 - 5000 g) (Bailey et al. 1982) have been used for obtaining hepatocytes, however the use of smaller fish (100 - 300 g) used for *in situ* liver perfusions is well-documented in the literature (Flouriot et al. 1993; Lipsky et al. 1986; Pesonen et al. 1992). Poorly perfused livers and low cellular yields obtained from larger fish (800 - 1800 g) in this study, suggest an increase in the physiological and biochemical stress on cells as a result of prolonged perfusion and enzymatic digestion periods. Large livers from older donor fish may be more resilient to such perfusion techniques; therefore a prolonged and more concentrated enzymatic digestion period would explain a reduction in cellular viability. Parameters such as fish sex, strain, maturity and dietary intake may also directly affect the physiology of the liver and therefore perfusion yields (Baksi and Frazier 1990), however investigation of these parameters was beyond the scope of this study. Cell yields produced in this experiment are considerably higher than those demonstrated in previous studies (Klaunig et al. 1985; Baksi and Fraser 1990), thus highlighting the successful isolation of hepatic trout cells at high densities through the use of an accurately refined harvesting procedure.

Purified hepatic suspensions combined with gyratory-mediated culture techniques have demonstrated successful aggregate formation in fish previously (Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996). In mammalian studies, however, un-purified, whole liver cell suspensions can successfully re-aggregate into spheroids (Walker et al. 2000; Ma et al. 2003; Xu et al. 2003a) suggesting that enzymatic cell isolation procedures may damage sensitive non-parenchymal cell types from fish liver, which may explain why un-purified cell suspensions do not re-aggregate under rotational shaking.

Serum-supplemented culture media vastly improved the aggregation process of developing spheroids (Flouriot et al. 1993), on which formation and growth of hepatocytes in particular are dependent (Pannevis and Houlihan 1992; Garmanchuk et al. 2010). Monolayer cultures were also successfully maintained for 8 d in serum-supplemented media, exhibiting good cell-cell contacts after 24 h in culture. Interestingly, the use of trout serum inhibited spheroid formation, causing a high-degree of clumping after 24 h in culture. Previous studies found pre-frozen (-20 ºC) trout serum to be more efficient in promoting attachment of trout hepatocytes than FBS (Kocal et al. 1988). In trout gill cell culture, both fresh and frozen trout serum had an inhibitory effect on cell attachment, where frozen serum appeared to be directly toxic to the cells (Part et al. 1993). It could therefore be that the serum preparation utilised in this experiment requires further characterisation. The use of trout serum for culturing primary liver cells would be considered as a ‘Refinement’ tool when considering alternative methods to whole animal studies, as serum can be taken from the same donor fish used for cell isolation and would not rely on the use

of expensive mammalian sera. Further investigations into the effect of freshly purified trout serum on spheroid formation are therefore required.

No obvious differences were observed between cultures grown in different media compositions. The use of L-15 media, supplemented with 10% FBS over a more complex mixture of DMEM:F12 with added buffering agents, offers a more simplistic and convenient culture recipe that can be replicated with less variation between media preparations. L-15 is buffered by phosphates and free-base amino acids and the production of acidic metabolites is reduced by replacing glucose with galactose and sodium pyruvate (Freshney 2010). The addition of an increased concentration of sodium pyruvate (5 mM) in L-15 medium is primarily to facilitate the use of fish cell cultures in CO_2-free environments.

The routine maintenance of cultures, in terms of regular, fresh media changes was imperative for maintaining both 3-D and 2-D cultures over-time. Spheroid cultures whose media was only initially replaced after 3 d, allowing the cells time to aggregate, rapidly changed in appearance after a further 24 h in culture. Developing spheroids will have a high demand on extra-cellular nutrients from the medium, of which depletion may inhibit formation. Therefore it is recommended that a medium change after 24 h in culture, with care not to remove any part of the cell suspension, be included to optimise culture conditions and aggregation times.

An effect on the morphology and longevity of spheroids in culture was clearly evident when utilising different incubation temperatures. The use of a more physiologically relevant temperature specific to rainbow trout (15 ºC) demonstrated efficient hepatocyte aggregation and liver spheroid formation and subsequent and prolonged

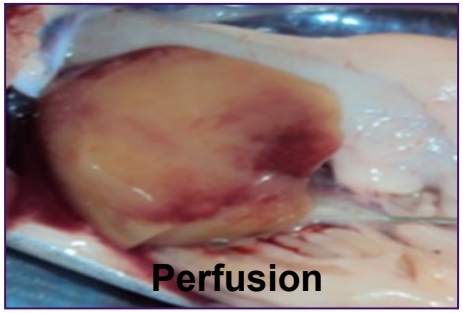
maintenance in culture (≤ 80 d). This temperature was also indicative of the water temperature used for the husbandry and maintenance of fish, highlighting the importance of maintaining physiologically relevant conditions when conducting primary tissue culture studies. Similar temperatures have been used in other studies involving aggregate / spheroid formation (Flouriot et al. 1993; Uchea et al. 2013), both demonstrating prolonged culture periods of liver spheroids.

Gyratory-mediated methods for trout hepatocytes differ significantly to those designed for generating rat hepatic spheroids, where higher rotation speeds are required to induce liver cell aggregation (Walker et al. 2000). This suggests that the difference in overall hepatocyte size between rats (24.1 ± 0.1 μm in diameter) (Katayama et al. 2001) and fish (10.9 ± 0.9 μm in diameter) (Baron et al. 2012) dictates the rotational forces required to keep cells in an optimal suspension state to allow aggregation to take place, which is also significantly affected by the rotator orbit (larger orbit resulting in more efficient aggregation). The difference in size of hepatocytes between species also seems to control the size of mature spheroids as those formed from rat liver are significantly larger (Ma et al. 2003).

A summary of *in situ* perfusion and *in vitro* culture parameters to generate viable 3-D hepatic cultures are outlined in Figure 3.4. Although smaller fish (Group C) are recommended, larger fish (Group B) can also be used to produce larger cellular yields for more cell-demanding experiments, but care must be taken to ensure good cell viability is achieved. An average fish liver in this study could generate ~86 x 10^6 cells, which could produce ~29 individual cultures (6-well) at the seeding densities outlined in this method. The benefit to the improvements in cell yield in this

experiment highlights the importance of refinement particularly for *in vitro* toxicological assays, and as a result, a greater number of assays could be conducted from an individual fish allowing for a more economical use of the organ. The work described in the following experimental chapters involves the use of these optimised and validated cultures to investigate liver-specific functions such as morphological maturation; changes in albumin, glucose and LDH profiles; protein content and EROD activity and to investigate the competency of spheroids to metabolise environmentally relevant chemicals including pharmaceuticals.

80 – 150 g



1 ml min^{-1} flow rate / 33 min / 0.1% enzyme

Cell viability (%)	**≥ 85**
pH	**7.40**
Temperature (°C)	**18.0**
Rotation speed (RPM)	**70**
Media	**L-15 + 10% FBS**
Seeding density (cells ml^{-1})	**1×10^{6}**
Media changes	**~50% after 24 h then every 48 h**
Coated plates	**2.5% pHEMA**

Figure 3.4 Summary diagram of *in situ* perfusion and *in vitro* culture parameters for the generation of 3D hepatic spheroids.

CHAPTER 4

Relative characterisation of morphological, biochemical and metabolic organ-functionality in 2-D and 3-D liver cell culture models: Are 3-D liver spheroids more physiologically relevant?

4.1 Introduction

Primary cells of fish origin in particular express many of the differentiated cellular structures and functions of their source tissues (Dowling and Mothersill 2001; Segner and Cravedi 2001), making them suitable as *in vitro* alternatives for short-term, acute toxicological studies. However as mentioned previously, drawbacks to conventional methodologies utilised in environmental toxicological studies (fresh hepatocytes, 2-D monolayers, microsomal preparations) such as short culture periods and loss of organotypic functionality, limit their use in chronic toxicity and bio-accumulation studies, as these conventional culture methods do not fully represent the growth conditions or 3-D architecture of *in vivo* tissues (Baron et al. 2012).

The physiology of *in vitro* hepatocyte models in fish, rainbow trout in particular, has been well-documented suggesting those biochemical-physiological parameters such as glucose regulation (Weber and Shanghavi 2000); amino acid utilisation (French et al. 1981); albumin synthesis (Klaunig et al. 1985); and mitochondrial health (Bains and Kennedy 2004) are useful end-points for determining the basal and chemically-exposed functional-status of *in vitro* fish hepatocyte systems. Limited previous work has also demonstrated the use of 3-D aggregate culture systems for characterising levels of estrogen receptor and vitellogenin (Vg) mRNAs in rainbow trout with comparisons to 2-D systems, that has been described as a promising *in vitro* model to investigate the bio-transformation pathways in fish and their regulation by endogenous and exogenous compounds (Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996).

Therefore, in order to support the hypothesis that 3-D fish liver spheroids exhibit more physiologically relevant, *in vivo*-like functional characteristics compared to conventional 2-D monolayer systems, this study aimed to characterise baseline bio-transformation activity and morphological and biochemical organ-functionality of 3-D spheroids and compare the responses with that of 2-D monolayer cultures.

4.2 Materials and Methods

4.2.1 Primary cell culture

Plates of spheroids were prepared from rainbow trout (wet weight: 168.10 ± 68.10 g) as outlined previously (Section *2.5.1*). These were placed at 18 ºC in a refrigerated incubator (MaxQ 4000) on an orbital shaking platform set at a constant rotation speed of 70 RPM. Plates of monolayer cultures were incubated statically at 18 ºC in the same incubator as per Section *2.5* and as demonstrated previously (Flouriot et al. 1993).

4.2.2 Cultures and batches

4.2.2.1 Morphological characterisation

4.2.2.1.1 Size measurements

Diameters of individual spheroids (n = 20) were measured from three individual cultures from three individual animals. The same three wells were used at each respective time point (day of formation).

4.2.2.1.2 Microscopy

Images of spheroids and monolayers were acquired from individual wells of cultures as per sections *2.7.2 – 2.7.6.*

4.2.2.2 Biochemical characterisation

Each biochemical endpoint was tested in six individual cultures from three individual animals, resulting in a total of eighteen replicates for each culture treatment. Culture treatments compared in this study were (a) 8 d spheroids (mature); (b) 2 d aggregates (immature) and (c) 2 d monolayers (functional). The selection of bio-chemical endpoints (i.e. protein, albumin, glucose, LDH) was based on previous mammalian studies investigating liver spheroid functionality (Walker et al. 2000; Ma et al. 2003; Xu et al. 2003a) and performed as per Sections *2.8.1 – 2.8.5*). Protein per hepatocyte values were measured from six individual cultures for each culture treatment from an individual fish as per Section *2.8.2.*

4.2.2.3 EROD activity

The production of resorufin was measured in six individual cultures from two individual animals (as per Section *2.8.6*), resulting in a total of twelve replicates for each test concentration of β-napthoflavone (BNF, 0 and 0.36 µM). BNF was chosen as a positive control based on previous EROD induction studies using trout hepatocytes (Pesonen et al. 1992; Cravedi et al. 1996). Culture treatments compared were (a) 8 d spheroids (100 and 500 spheroids well^{-1}) and (b) 2 d monolayers (250,000 and 500,000 cells well^{-1}).

4.3 Results

4.3.1 Morphological maturation

Single hepatocytes maintained under constant gyratory-mediated culture conditions re-aggregated to form small, irregular, aggregate clusters after 24 h in culture (Fig. 4.1a). Over a period of 2 – 4 d these smaller aggregates joined together to form larger aggregates, exhibiting a more spherical-like shape, with an average diameter of 53 ± 12 μm (*n* = 180) at 4 d (Fig. 4.1d). After 6 – 8 d in culture the diameter of the aggregates had increased (67 ± 13 and 83 ± 14 μm, respectively; *n* = 180) and the shape became more stable forming neat, regular liver spheroids with a defined, tight outer surface that could be routinely formed in our laboratory under the proposed culture conditions to 8 d old (Fig. 4.1f).

Hepatocytes collected from 105.70 ± 18.50 g trout were 10.90 ± 0.90 μm in diameter. Mammalian hepatocytes are reported to be typically much larger. For example, those of a similar sized rat are 24.10 ± 0.10 μm in diameter and spheroids produced from this measure 200 ± 25 μm in diameter upon reaching morphological maturity (Ma et al. 2003). Hepatocytes isolated from smaller rainbow trout (105.7 ± 18.5 g) tended to form neater and more spherical spheroids compared to those formed from larger trout (230.4 ± 29.1 g). The number of hepatocytes per individual aggregate or spheroid also increased with maturity with a ~50% doubling of cell number in the transition from aggregate to spheroid (Table 4.1). Spheroids maintained their neat, spherical structure ≤ 5 weeks in culture, but began to display darker patches of potentially necrotic cells towards the inner region of the spheroid (Fig. 4.1h). In on-going characterisation studies using this protocol, large primary hepatic spheroids

have been maintained for ≤ 80 d, however nearly the entire inner and part of the outer region of the spheroid appears to have become necrotic (Fig. 4.1i).

Table 4.1 Size measurements of spheroids. Values are expressed as mean ± SD.

Day	Diameter (μm)	Volume (x10^5 μm^3)	Cells $spheroid^{-1}$
4	53 ± 12	9 ± 6	127 ± 90
6	67 ± 13	18 ± 10	252 ± 147
8	83 ± 14	32 ± 17	457 ± 247

The SEM analysis further illustrates the morphological maturation process of single cells → aggregates → spheroids. Aggregates in the early stages of spheroid formation (24 h post-seeding) where only a few individual cells have joined, exhibit weak cell–cell contacts where well-defined single cells are clearly visible (Fig. 4.2a). After a further 24 h in culture the aggregation between individual cells increases, forming chains of cells (Fig 4.2b). Boundaries between individual hepatocyte contacts become less well defined in 3 - 4 d old spheroids (Fig. 4.2c-d) as more hepatocytes fuse together, forming an outer layer. Spheroids at 4 d appear to form a more 'ball-like' shape characteristic. A smooth outer surface and tight cell–cell contacts are now clearly visible in 8 - 10 d old spheroids (Fig. 4.2e-f). A small degree of 'blebbing' can be seen on the outer surface of the spheroid structure (Fig. 4.2f) which forms as a result of the cryo-preservation process.

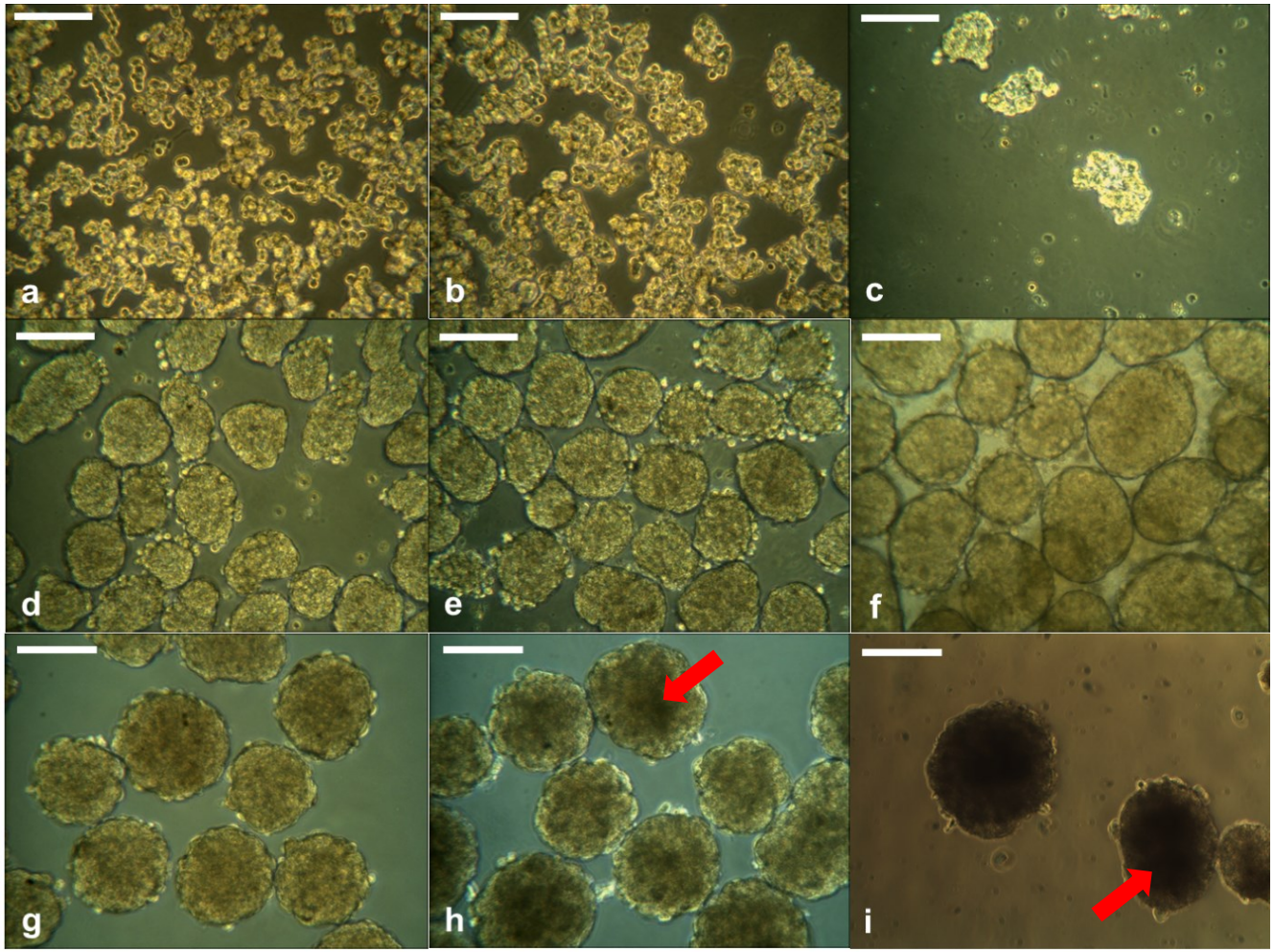


Figure 4.1 Light microscopic images taken during spheroid formation process. (a) 24 h; (b) 2 d; (c) 3 d; (d) 4 d; (e) 6 d; (f) 8 d; (g) 21 d; (h) 35 d and (i) 80 d. Scale bar = 100 µm. Red arrows indicate areas of potential necrosis.

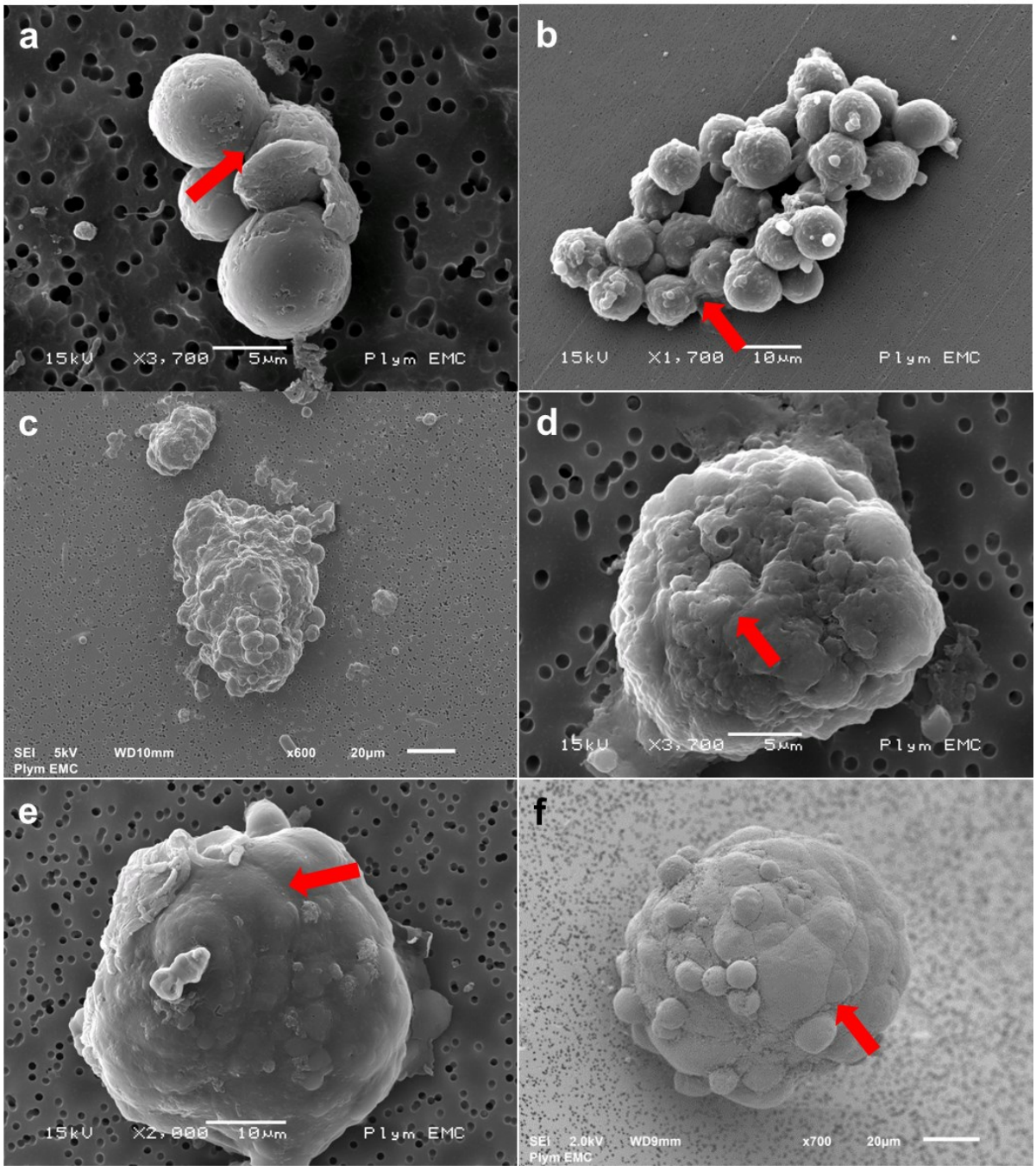


Figure 4.2 SEM images taken during the spheroid formation process. (a) 24; (b) 2 d; (c) 3 d; (d) 4 d; (e) 8 d and (f) 10 d. Spheroids were fixed via chemical fixation (a, b, d and e) and cryo-fixation (c and f). Red arrows indicate contact points between individual cells. Variable magnifications with scale bar.

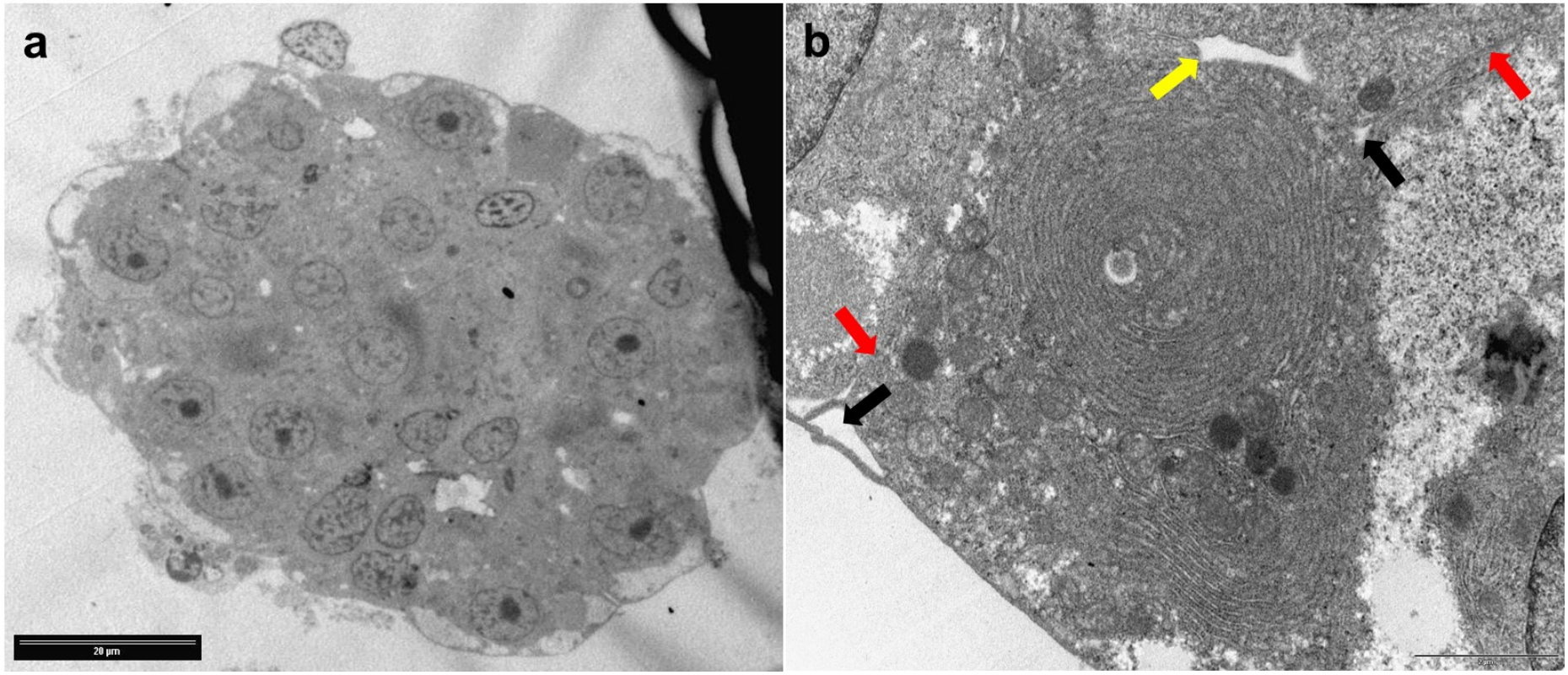


Figure 4.3 TEM images of an 8 d old spheroid. Red arrows indicate cell boundaries between cells; black arrows indicate formation of tight-junctions and yellow arrow indicates bile canaliculi formation. Variable magnifications with scale bar.

The TEM analysis illustrates the development of the micro-environment within a spheroid. The arrangement of individual hepatocytes as well as a distinct outer surface (Fig 4.3a); the formation of tight-junctions between cells and the appearance of bile canaliculi (Fig. 4.3b) are clearly visible within the tight spheroidal structure. Therefore, aggregating hepatocytes can be morphologically classified as 'mature' spheroids after 6 - 8 d in culture and those during 1 - 5 d of aggregation classified as 'immature spheroids'.

In monolayer cultures, single hepatocytes attached to the well surface within 1 - 2 h and after 24 h had begun to regroup into rows of cell networks (Fig. 4.4a). After 48 h in culture tightly-packed rows of hepatocytes were visible (Fig. 4.4b). This tight, neat structure soon began to disaggregate and after 8 d in culture, hepatocytes had begun to detach from the surface (Fig. 4.4c). Preliminary experiments demonstrated a decrease in liver functionality of monolayer hepatocytes particularly between 4–8 days, which is well documented in mammalian studies (Walker et al. 2000). They were therefore classified as 'functional' between 2–4 days in culture.

4.3.2 Cell-cell adhesion development

Single hepatocytes were chemically-fixed after 1 h post isolation. The appearance of F-actin plaques can be clearly seen on both single cells and between cells that have begun the hepatocyte aggregation process (Fig. 4.5a). After 24 h of hepatocyte re-aggregation, F-actin stain between cells can be seen as cells begin to form tight connections between each other (Fig. 4.5b). As the spheroid maturation process continues, a layer of F-actin stain can be clearly seen on the spheroid surface and the stain between cells is now visible within the spheroidal structure (Fig. 4.5c).

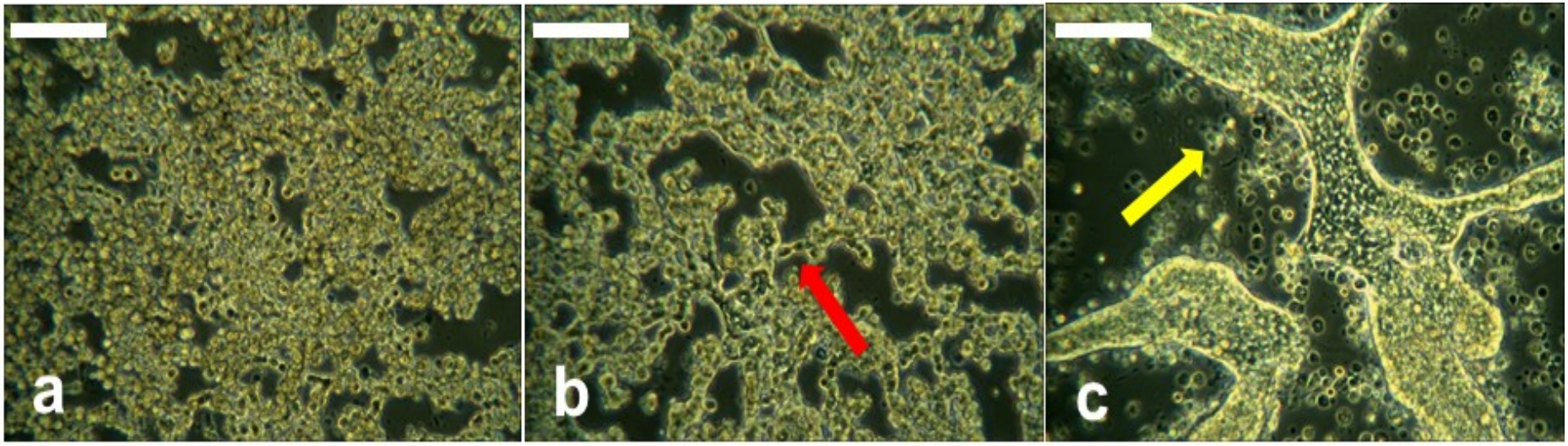


Figure 4.4 Light microscopic images taken during monolayer formation process. (a) 24 h; (b) 2 d and (c) 8 d. Red arrow shows tight-rows of hepatocytes. Yellow arrow shows individual hepatocytes that have detached from the main body of the monolayer.

After 8 d in culture, the shape and size of mature spheroids stabilise with a more regular appearance than aggregates at earlier time points, with obvious adhesion binding between cells (Fig. 4.5d)

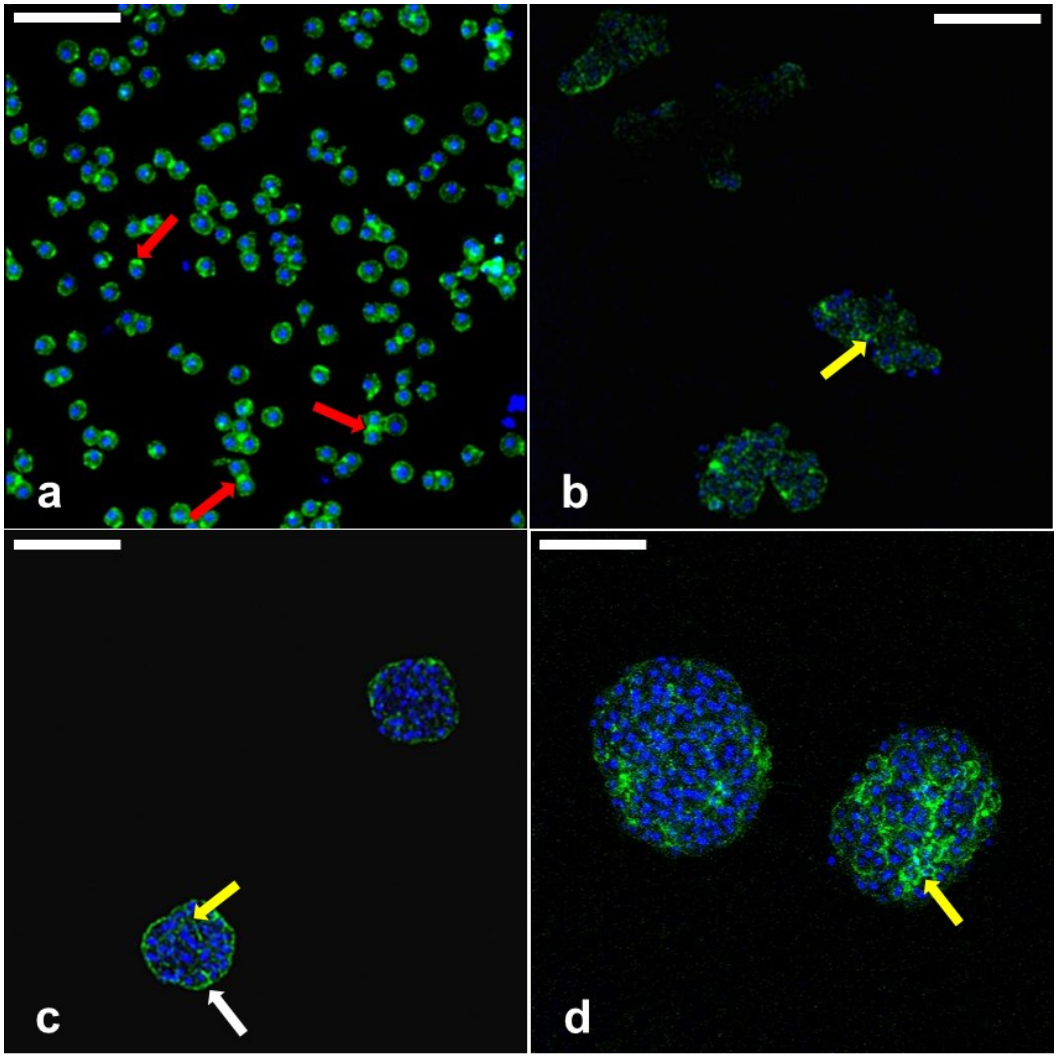


Figure 4.5 Confocal images of spheroid cultures stained with (i) DAPI (blue) and (ii) Alexa flour 488 phalloidin (green). (a) fresh hepatocytes; (b) 1 d; (c) 4 d and (d) 8 d old spheroids. Red arrows indicate F-actin plaques between newly connected, individual cells; yellow arrows indicate F-actin cell-cell adhesion development within spheroids and white arrow indicates development on the spheroid surface. Scale bar = 50 μm.

4.3.3 Biochemical characterisation

4.3.3.1 Protein

Each of the values of the biochemical parameters was normalised against total protein per well. Total protein content of aggregate and spheroid cultures decreased during the maturation process due to removal of dead or clumped hepatic tissue (i.e. less cells per well). Protein per hepatocyte was constant between culture treatments except in fish three, where a significant decrease in protein per hepatocyte of mature spheroid cultures was observed when compared to both monolayer and immature spheroid cultures ($P < 0.01$) (Fig. 4.6).

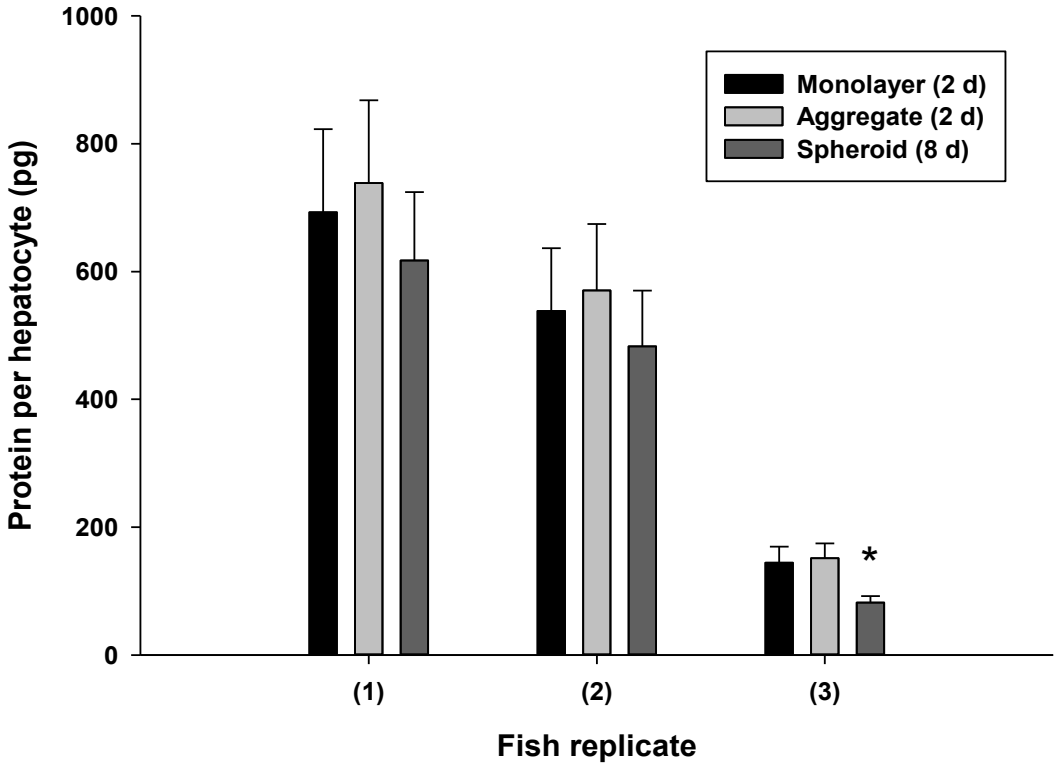


Figure 4.6 Protein per hepatocyte values of hepatocyte cultures from three individual fish experiments. Values are expressed as mean ± SD. * $P < 0.01$, compared with the value of monolayer and aggregate cultures (Fish replicate 3).

4.3.3.2 Albumin

The rate of albumin secretion of both monolayers and liver spheroids is shown in Fig. 4.7a. A significant increase in albumin secretion was observed during liver spheroid formation ($P < 0.01$) and both immature and mature spheroids had a significantly higher secretion rate than that in monolayer cultures ($P < 0.01$), with an approximate five-fold increase observed between monolayers and mature spheroids. No difference in the level of intracellular albumin was observed between mature and immature spheroids, the highest of which was observed in monolayer cultures which was significantly higher than in spheroids ($P < 0.01$) (Fig. 4.7b).

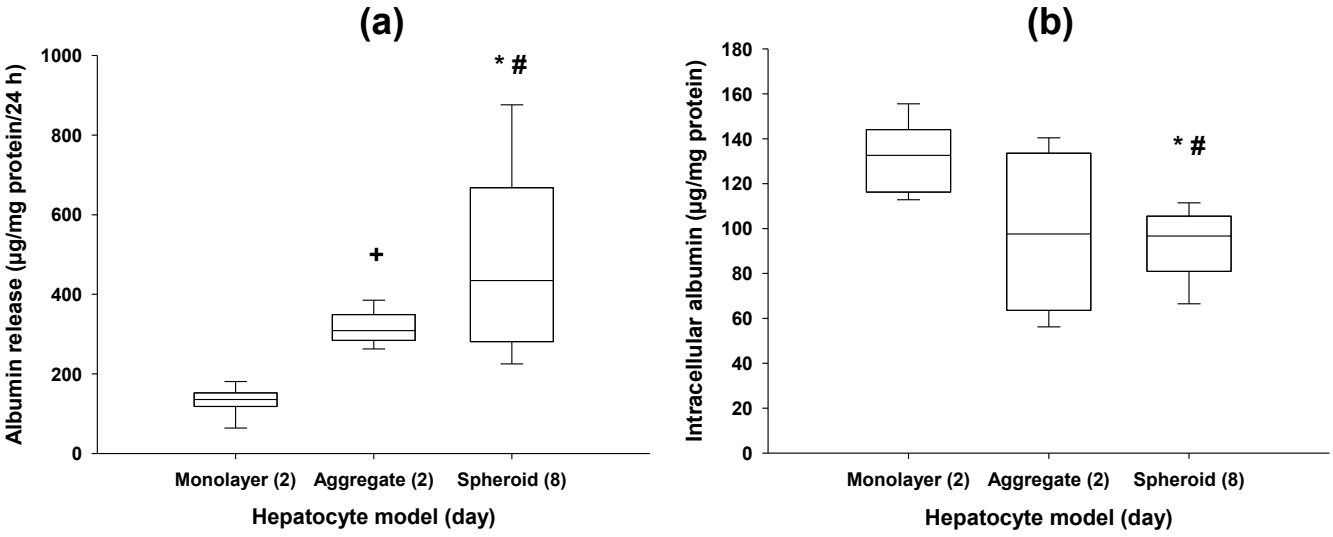


Figure 4.7 Intra & extracellular albumin during spheroid maturation compared with monolayer cultures. (a) Albumin release; (b) intracellular albumin. Values expressed as median values/$q1$-$q3$. * $P < 0.01$, compared with the value of monolayer cultures. # $P < 0.01$, compared with the value of immature aggregates. + $P < 0.01$, compared with the value of monolayer cultures.

4.3.3.3 Glucose

The change in intracellular and extra-cellular glucose in monolayers and during spheroid formation is shown in Fig. 4.8a. Glucose secretion by mature spheroids was significantly higher than that in monolayers ($P < 0.01$) and immature spheroids ($P < 0.01$). No difference was observed between monolayer and immature spheroid cultures. Intracellular glucose was constant during spheroid formation (Fig. 4.8a) but was significantly lower than that observed in monolayer cultures ($P < 0.05$).

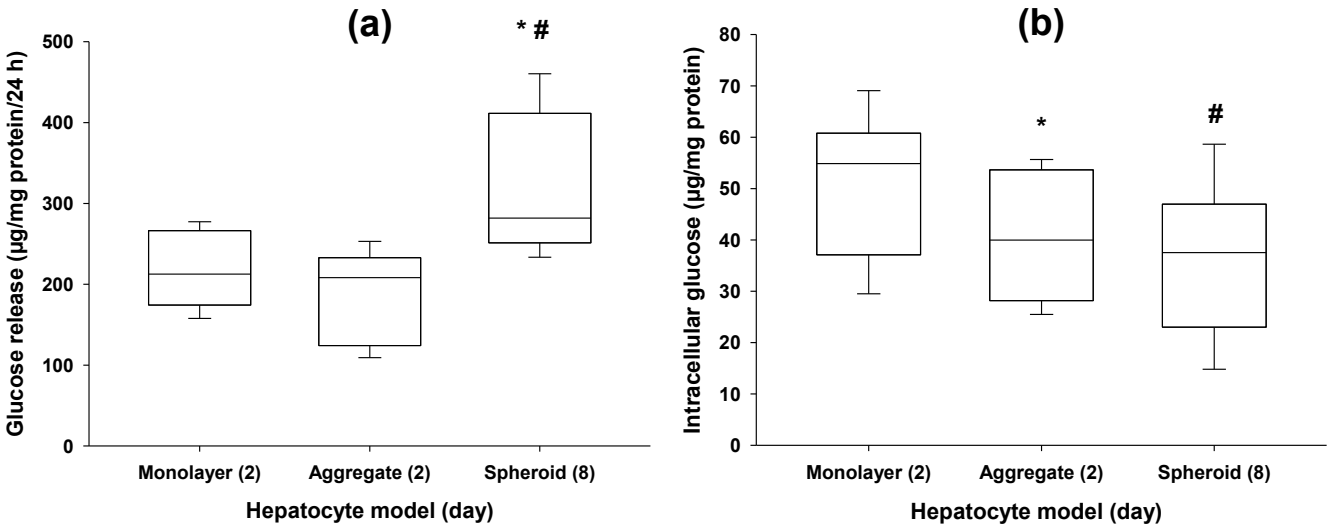


Figure 4.8 Intra & extracellular glucose during spheroid maturation compared with monolayer cultures. (a) Glucose release; (b) intracellular glucose. Values expressed as median/*q*1-*q*3. * $P < 0.05$, compared with the value of monolayer cultures. # $P < 0.01$, compared with the value of monolayer cultures.

4.3.3.4 LDH

Changes in intracellular and extracellular LDH in monolayers and spheroids are shown in Fig. 4.9a. As spheroids matured the release of LDH into the surrounding culture media decreased significantly ($P < 0.01$). The highest amount of LDH release was observed in monolayer cultures which were significantly higher than that in spheroids, both immature and mature ($P < 0.01$). The highest amount of intracellular LDH was observed in mature spheroids (Fig. 4.9b) which was approximately seven-fold higher than that in immature spheroids and monolayers ($P < 0.01$). No difference was observed between monolayers and immature spheroids.

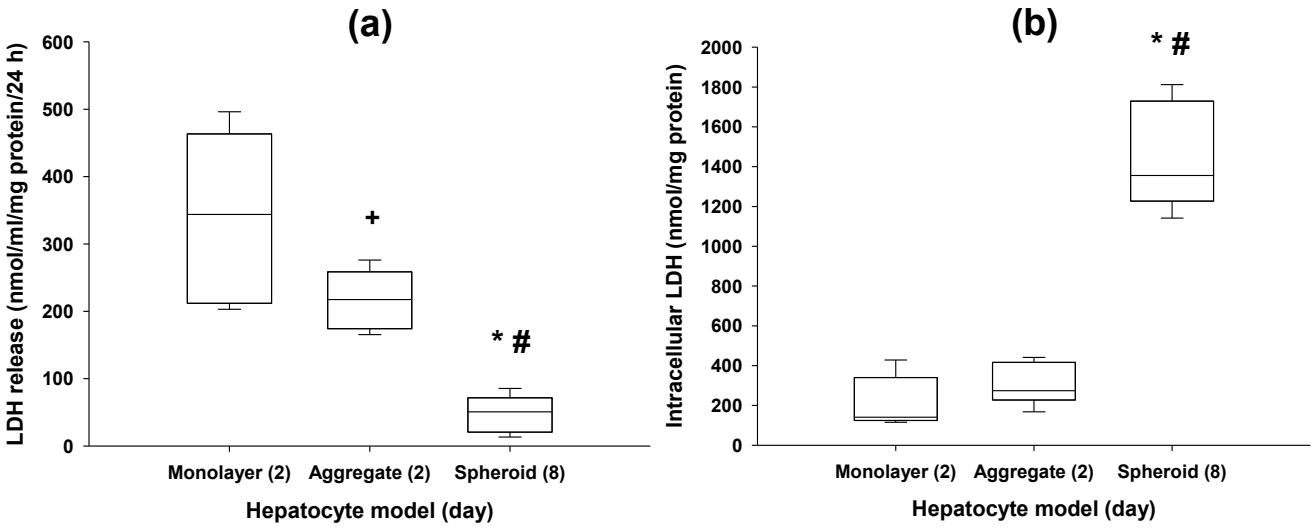


Figure 4.9 Intra & extracellular LDH during spheroid maturation compared with monolayer cultures. (a) LDH release; (b) intracellular LDH. Values expressed as median/$q1$-$q3$. * $P < 0.01$, compared with the value of monolayer cultures. # $P < 0.01$, compared with the value of immature aggregates. + $P < 0.01$, compared with the value of monolayer cultures.

4.3.4 EROD activity

The rate of resorufin production was measured utilising a live cell EROD assay (Fig. 4.10) and subsequent EROD activity calculated for monolayer and spheroid cultures (Fig 4.11). After 48 h exposure to 0.36 µM BNF, both culture types showed significantly higher EROD activity compared to controls ($P < 0.01$) at all seeding densities. Exposed monolayers seeded at 250,000 cells well^{-1} showed a significantly higher EROD induction rate than those seeded at 500,000 cells well^{-1} ($P < 0.01$; Fig. 4.11a). There was no significant effect of spheroid seeding density on EROD activity (Fig. 4.11b).

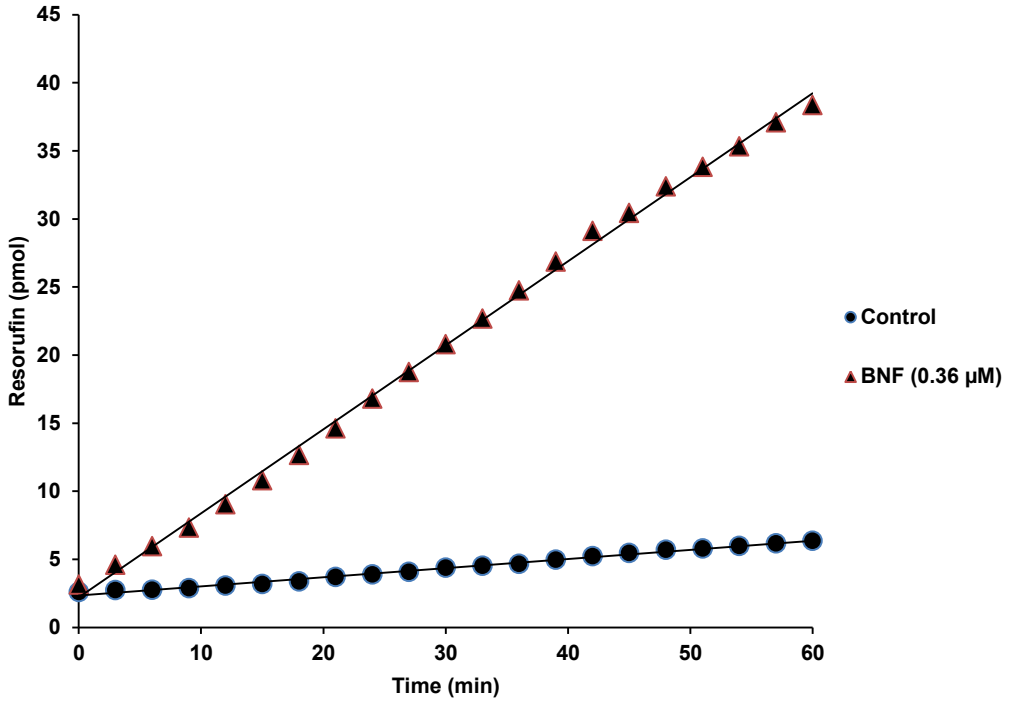


Figure 4.10 Resorufin productions over time from 8 d spheroid cultures (n = 6) utilising a live cell EROD assay (described in Section 2.8.6). The curves are representative of both control and BNF-induced cells from two independent experiments (n = 2 fish). Reactions were generally close to linear for the entire 60 min kinetic measurement period.

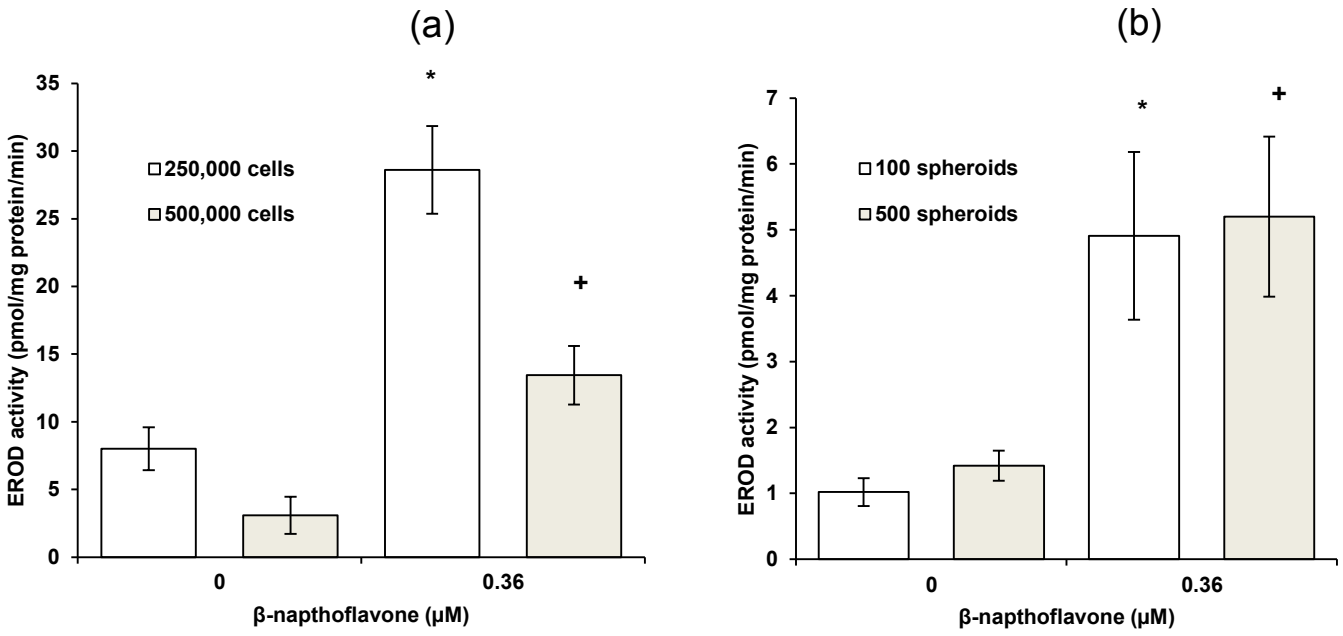


Figure 4.11 EROD activities in monolayer and spheroid cultures after 48 h exposure to 0.36 μM β-napthoflavone. (a) Monolayer cultures seeded at 250,000 and 500,000 cells well^{-1}; (b) spheroid cultures seeded at 100 and 500 spheroids well^{-1}. Values expressed as mean ± SD. * $^{+}$ $P < 0.01$ compared with respective controls.

4.4 Discussion and conclusions

Hepatocyte spheroid formation was divided into two stages in this study according to the morphological formation process: immature and mature (Ma et al. 2003). Immature spheroids (1 - 5 d) under-go the transition from single cells → irregular aggregates → morphologically stable spheroids. Mature spheroids (≥ 6 d) exhibit a relatively stable and regular shape, with an increase in size due to the fusion of smaller spheroids. The classification of fish hepatocyte spheroids based on morphological maturity (Hansen et al. 1998) is important new information, particularly when determining functional status during and after spheroid formation. Between 10

- 16 d in culture, spheroids continued to increase in size with the fusion of mature spheroids. Beyond 16 d and following an increase in culture rotation speed (70 → 80 RPM), the fusion process ceased and spheroids were maintained in culture for a period of ≤ 80 d. By not intervening in the spheroid-fusion process by increasing the culture rotation speed, mature spheroids would continue to fuse together to eventually form a very large, single, multi-spheroid structure. The appearance of tight-junctions and boundaries between individual cells in the ultra-structure of mature spheroids is similar to those determined previously in rat liver spheroids (Ma et al. 2003). The appearance of bile canniculi suggest that fish liver spheroids may the retain normal-liver function of hepatocyte bile secretion into the bile duct, which has been shown to contain both metabolised and un-metabolised non-steroidal anti-inflammatory drugs in adult rainbow trout (Kallio et al. 2010).

The size increase in liver spheroids (≤ 16 d) is more likely to be related to spheroid-fusion rather than proliferation, which is characteristic of cell-line, derived hepatic spheroids (Liu et al. 2007). After 16 d in culture, it was observed that spheroids maintained a constant size and shape, supporting the concept that cells in 3-D cultures undergo growth arrest and cell survival which are regulated by an array of signalling pathways involved in 3-D cell-cell communications, which differ from those in 2-D monolayer cultures (Bates et al. 2000). Continued iterative optimisation of culture parameters has prolonged the integrity and survival of fish hepatocyte spheroids, which would render them a useful tool for chronic toxicity, bioaccumulation and biotransformation studies.

The spheroid maturation process can be further characterised by assessing the kinetics of F-actin plaque formation in the cell-cell adhesion process. Understanding liver cell-cell connections after isolation procedures, or even in response to injury and during regeneration processes, will allow not only the development of new approaches in studying biological functions of cell membrane in repair, liver diseases and exploring new therapies for liver injury or disease but will also aid the establishment of new methods for developing tissue engineering and three-dimensional cell models (Xu et al. 2013). Actin constitutes ~10% of the total cytoplasmic protein, existing either as a globular monomer (G-actin) or a filament form polymer (F-actin) (Raven 1999; Lambrechts et al. 2004) and is an important adhesion molecule involved in cell connection processes. F-actin in particular, is involved in cell movement or migration, cell-cell binding and signal transduction between cells (Zigmond 1996; Tzanakakis et al. 2001; Rombouts et al. 2002). Observations obtained here demonstrate strong staining of F-actin between cells and tight cell-cell connections as spheroids mature. This demonstrates the process of isolated cells forming F-actin plaques, making cell-cell connections and developing adhesion binding between cells, reflecting the whole process of freshly isolated hepatocytes re-establishing cell connections after enzymatic digestion and a membrane function performing adaptive changes from cells to re-establish contacts, as demonstrated previously (Xu et al. 2013).

The amount of total protein of hepatocytes in each well can indirectly reflect the total viable cell number. Total protein per well decreased during the spheroid maturation process. In common with mammalian studies (Ma et al. 2003), this is likely to be reflected by loss of cells due to the enzymatic isolation process which was observed

during culture maintenance, where only viable cells re-aggregated during spheroid formation and as a consequence, dead or clumped groups of cells were removed from the culture well. In one of the individual fish experiments, protein per hepatocyte decreased during the transition from immature to mature spheroids which could suggest a decrease in the viability of spheroids during maturation. However, this contrasts with (a) a stable amount of protein per hepatocyte between culture treatments in the other two individual fish experiments and (b) increased levels of albumin synthesis and significantly lower levels of LDH leakage, which suggests a good degree of functionality and membrane integrity.

The data derived for protein per hepatocyte for fish three could have arisen for a number of reasons. It is possible that this batch of aggregated hepatic spheroids utilised intracellular amino acids as sources of energy, such as the conversion to glucose and/or CO_2 during cellular respiration (French et al. 1981) for more energy demanding cells, as may be expected within a functioning spheroid. In addition, as this particular fish was taken from a larger-sized population of animals (~250-350 g) compared to the other two smaller animals (~100-150 g), there may be a correlation with the hepatosomatic index i.e. as liver size increases the utilisation of amino acids as endogenous energy sources increases, which may be replicated in tissue cultures that more closely resemble whole organs. However, as no evidence in the literature exists that could support this hypothesis; further characterisation of hepatosomatic index / protein content relationships in both *in vivo* and *in vitro* models is required.

Furthermore, changes in the protein content of liver tissue are affected primarily through changes in the rate of intracellular protein degradation in mammals

(Sommercorn and Swick 1981), which is responsive to both gross insult, such as isolation procedures and to physiological stimuli e.g. starvation and repletion (Garlick et al. 1973; Conde and Scornik 1976). Studies have demonstrated that mammalian hepatocytes in primary monolayer culture recover from the trauma of cellular isolation as evidenced by active amino acid transport (Kletzien et al. 1976) and higher rates of protein synthesis, after only a short time in culture (Pariza et al. 1975), in comparison to freshly isolated hepatocytes. As protein per cell was higher in both monolayer and aggregate cultures in this study, one would expect to see this recovery in a mature eight day spheroid (as observed in the other two fish experiments).

It has also been shown that the rate of hepatic protein breakdown in isolated hepatocytes is related inversely to the extracellular amino acid concentration (Sommercon and Swick 1981). As protein turnover is energetically expensive, an absence or a reduction in the supply of exogenous amino acids (i.e. from the medium) could limit endogenous protein degradation to the point where a supply of amino acids for protein synthesis in sufficient amounts would have survival value for the tissue, such as during periods of starvation (Sommercon and Swick 1981). In rainbow trout, liver protein synthesis and protein degradation are controlled primarily by feeding and fasting events, with degradation at its highest when fish are deprived of food for prolonged periods (4-6 d) (McMillan and Houlihan 1992). In the current study, the medium contained extracellular amino acids and levels were replenished at each medium change. Therefore, it is possible that 3-D spheroid cultures obtained from larger fish require a higher extracellular (i.e. medium based) amino acid concentrations than those available in the basal culture medium to meet higher

energy demands. This potential replication *in vitro* is worth consideration when utilising total protein content to normalise against other biochemical parameters.

Gluconeogenesis is the major pathway responsible for glucose production in primary cultures of trout hepatocytes (Mommsen et al. 1999). In mammals, the liver plays an important role in maintaining blood glucose concentration and is also the major organ responsible for converting galactose into glucose and synthesizing glucose from other substances such as pyruvic acid and amino acids (Zubay 1988). The present study demonstrated that fish hepatocyte spheroids may retain the similar function to rat liver spheroids of converting galactose to glucose (Xu et al. 2002; Ma et al. 2003), as the media recipe used in this study contained galactose but no glucose or gluconeogenic controlling hormones. Damage to mitochondria can affect many functions of liver cells, including galactose transformation, gluconeogenesis and glucose secretion, suggesting that glucose secretion may reflect a general state of energy metabolism in spheroids (Xu et al. 2003a).

A higher rate of glucose secretion and a reduction in the amount of intracellular glucose as observed between monolayers and spheroids in this study may reflect significant differences in the metabolic state of 2-D and 3-D hepatocyte models in fish, although a distinct lack in the scientific literature of glucose studies in 2-D cultures for fish makes this difficult to confirm. The secretion of glucose in mammalian and non-mammalian *in vitro* cultured hepatocytes has been shown to be far lower than that *in vivo* (Weber and Shanghavi 2000; Xu et al. 2003a), suggesting that *in vitro* studies have less physiological importance when investigating liver specific function. Mature spheroids however, may reflect similar levels of *in vivo*

glucose production in fish as previously demonstrated in organotypic liver slice models (Morata et al. 1982), suggesting their usefulness as a more *in vivo*–like model.

Albumin synthesis is a useful marker for the assessment of liver specific function. Culture conditions in particular seem to play a key role in the synthesis of albumin in rat hepatocytes (Hamilton et al. 2001). Albumin synthesis was significantly higher in both developing and mature spheroids compared to monolayer cultures in this study, which could suggest the need for albumin proteins during more complex hepatocyte re-aggregation. Fish hepatocytes cultured in serum-supplemented media exhibit better cell–cell contacts and longevity in culture which was confirmed here (Klaunig et al. 1985; Kocal et al. 1988). Hepatocytes cultured in collagen matrix configurations have been shown to maintain a high albumin secretion rate for prolonged periods (Dunn et al. 1989). The spheroid culture used in this study utilises a medium-based extra-cellular matrix (ECM) which seems to stimulate albumin synthesis, suggesting that rotary-mediated suspension culturing of spheroids could offer a simple alternative and more physiologically-relevant method to conventional ECM-based spheroid culture methods for fish.

The use of LDH as a marker for chemical induced cytotoxicity is well supported in the literature (Pesonen and Andersson 1992; Bains and Kennedy 2004). It can also be used to assess the basal viability in non-chemical induced hepatocytes as indicators of cell membrane integrity. This is particularly useful when assessing hepatocyte aggregation as previously mentioned, it is possible to elucidate the viable state of spheroids when effects of total protein loss are perhaps not clear. In rat

hepatocyte spheroids, the *in vivo*-like 3-D environment permits to prolong hepatocyte viability *in vitro* (Tong et al. 1992). There was an inverse relationship between LDH leakage and intracellular LDH in the present study. With particular reference to mature spheroids, a low level of basal LDH leakage suggests that membrane integrity within spheroids is maintained. High intra-spheroidal levels of LDH could suggest a large deposit of the enzyme within the spheroids complex 3D structure, perhaps in the central region of the spheroid. The tight structure of multiple individual cells, combined with the increased diameter of the spheroid structure compared to smaller, poorly-formed aggregates could inhibit release of the enzyme. This seems unlikely, however, as neither glucose nor albumin release were inhibited and similar patterns of higher, but stable intracellular LDH have been observed previously in mammalian liver spheroids, particularly during the 'mature' phase of the spheroid maturation process (6-15 d) where other functional and biochemical properties (e.g. cellular Ɣ-GT activity, glucose activity and albumin secretion) also remained stable (Ma et al. 2003).

It is also likely that the higher level of LDH release in monolayer and aggregates after 2 d in culture could be explained by the initial cell isolation process (i.e. a degree of membrane integrity loss in freshly isolated cells as a result of the enzymatic dissociation process). As cells resume cell-cell contacts and a stable histological environment is achieved in mature spheroids, most functional and biochemical properties are stabilised or recover for a period of time (Ma et al. 2003). Therefore, high intracellular LDH could be used as an indicator of stable spheroid functionality, however, further characterisation of the enzyme's concentration in older spheroids (>8 d) would need to be determined to support this.

Measurement of EROD as a biomarker for CYP1A activity has been widely used within fish studies (Carlsson et al. 1999; Whyte et al. 2000; Jonsson et al. 2006; Bartram et al. 2012; Uchea et al. 2013), as it provides evidence of receptor-mediated induction of CYP1A and thus has been used for inference of CYP1A activity and potential metabolism. Previous studies have demonstrated significant induction of EROD in both fish monolayer and mature liver aggregates after exposure to the putative chemotherapeutic agent BNF (Cravedi et al. 1996). Liver aggregates have been suggested as a suitable alternative model for the *in vitro* assessment of metabolism and bioaccumulation potential of chemicals when compared to currently utilised microsomal and S9 systems, shortly lived freshly isolated cells and de-differentiated monolayer cultures (Uchea et al. 2013). Application of conventional EROD assays that utilise S9 systems to primary hepatocyte cultures suffers from some disadvantages. In particular, methods utilised for the harvesting of cells, preparation of subcellular fractions and for the analytical detection of resorufin formation requires high cell numbers per sample that can limit the number of replicates and treatments that can be performed from one cell isolation (Kennedy et al. 1993; Behrens et al. 1998). They also require the routine addition of an auxiliary NADPH generating system which is present in intact cell systems due to endogenous cofactor production (Behrens et al. 1998).

This study utilised a 'live-cell' EROD assay, which allows the rapid and sensitive measurement of EROD activity from cells directly within microtiter plates i.e. the release of product (resorufin) from intact cells resulting from CYP1A1-mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) (Whyte et al. 2000; Semino et al. 2003; Liu et al. 2007). The addition of substrates to inhibit sulfate and

glucuronide conjugation which can lead to intracellular metabolism of resorufin formed in the EROD reaction, thus leading to an underestimation of catalytic activity is also not required. Studies measuring EROD activity in cells from the fish cell line PLHC-1 (Hahn et al. 1996) did not require inhibition of phase II conjugation or DT-diaphorase, unlike studies with mammalian hepatocytes, where the addition of salicylamide to inhibt sufate and glucuruonide conjugation, and of dicoumarol to inhibit DT-diaphorase catalysed reduction of resorufin was found to be necessary (Donato et al. 1992; Lubinski et al. 1994). Behrens et al (1998) also demonstrated that the addition of salicylamide, β-glucuronidase and arylsulfatase to assay medium was not necessary to inhibit the conversion of formed resorufin to low-fluorescent conjugates in primary fish hepatocyte cultures. Cytoplasmic DT-diaphorase can lead to a significant underestimation of EROD activity which can be remedied with the addition of the substrate dicoumarol (Behrens et al. 1998). To eliminate underestimation of EROD activity in the present study, dicoumarol was added to the assay medium (18 μM, final well concentration).

This method was adapted from previous studies that incorporated live measurement of EROD activity in avian cells (Kennedy et al. 1993); permanent fish cell lines (Hahn et al. 1995; Clemons et al. 1996; Hahn et al. 1996) and primary cultures of fish hepatocytes (Behrens et al. 1998) to measure EROD activity in 3-D spheroid hepatocyte cultures, that has been previously demonstrated in studies utilising spheroids derived from mammalian cell types (Liu et al. 2007). This is, to our knowledge, the first time EROD activity has been measured in this way in 3-D liver cultures generated from fish tissue.

A variation in EROD induction between monolayer and spheroid cultures was observed with spheroids demonstrating a significantly lower induction rate, perhaps due to the resilience of the spheroid surface structure to the test chemical, or a lack of penetration of the chemical throughout the spheroid., which may also explain a slight lag period (1-2 min) at the start of some reaction periods. Previous investigations support this finding, showing that rat liver spheroids appear to be less sensitive than monolayer cultures when exposed to the chemical methotrexate (Walker et al. 2000). Equally, it may be argued that spheroids are the more sensitive model to chemical exposure, where complex histo-architecture and maintenance of important cellular functions (Xu et al. 2003; Liu et al. 2007; Baron et al. 2012) such as cytochrome P450-1A1 activity is replicated more closely to *in vivo* levels (Uchea et al. 2013). No difference was seen in activity between spheroid seeding densities, yet a decrease was measured as monolayer seeding density increased. This may be due to substrate (7-ER) depletion as seeding density increases or, with respect to the higher spheroid seeding density (500 spheroids well^{-1}), an inhibition of EROD induction due to a decrease in culture viability if spheroids begin to clump.

Further to our work on primary liver tissue, it is worth considering the use of established fish cell lines in generating spheroids for alternative ecotoxicity / metabolism assessment. We have successfully formed spheroids from both rainbow trout gonad (RTG-2) and rainbow trout liver (RTL-W1) cell lines in our laboratory, with the latter of particular interest in offering a complete alternative to animal-based toxicity studies. Established liver cell lines such as RTL-W1 demonstrate a degree of cytochrome P450-dependent monooxygenase activity (Lee et al. 1993) under 2-D

conditions, but would require further characterisation to determine morphological, metabolic and biochemical functionality as spheroidal aggregates.

In conclusion, trout spheroids take ~6 – 8 d to reach maturity whereupon they would be suitable for use in acute toxicological tests and can survive in culture for a period of ~80 days, an important criterion for both chronic toxicity and bio-accumulation tests. Mature trout liver spheroids appear inherently more variable in size than comparable mammalian 3-D tissue cultures; however their morphological, biochemical and metabolic markers suggest that their condition and function can provide more realistic organotypic responses than conventional *in vitro* models and provide a new testing platform for assessing xenobiotic metabolism.

CHAPTER 5

In vitro bio-transformation of pharmaceuticals in 3-D hepatic fish spheroids

5.1 Introduction

Due to their potential for inducing both short and long-term biological effects in aquatic organisms, with particular concern as to their potential to bio-accumulate in tissue, the study of pharmaceuticals and there implications for environmental risk assessment have gained increasing attention. An increasing number of studies have demonstrated biological responses to pharmaceuticals in a range of aquatic species, but these have generally been reported from experiments where concentrations were above both environmentally-relevant and human-therapeutic ranges (Corcoran et al. 2010). As pharmaceuticals are designed to act on specific enzyme pathways and transporters in target organs (Hutchinson 2008), and that these are often conserved across vertebrate phyla, it is possible that inducible effects seen in human target organs may also induce similar effects in non-target organisms such as fish (Gunnarsson et al. 2008) at these lower concentrations.

With this in mind, the continued presence of pharmaceuticals in the aquatic environment gives rise to the potential risk of bio-accumulation as a result of absorption, distribution, metabolism, and excretion (ADME) processes within the aquatic organism. Since the United Nations Stockholm Convention on persistent organic pollutants (POPs) was adopted in 2001, there has been significant activity concerning the assessment of persistent, bio-accumulative and toxic (PBT) substances worldwide (Scholtz et al. 2013). In addition, there have been extensive calls from regulations such as REACh to avoid unnecessary testing on animals, instead utlising existing information from standard and non-standard methods, *in vitro* methods, *in silico* methods, read-across, and weight-of-evidence in an

integrated testing strategy for assessing the bio-accumulative potential of a substance (ECHA 2011).

The use of *in vitro* techniques in bio-accumulation assessment is beginning to receive attention, particularly to measure the metabolism of pharmaceuticals and to use this information as a key parameter in combination with existing models (Scholtz et al. 2013). *In vitro* liver model systems such as sub-cellular fractions, fresh hepatocyte suspensions and 2-D monolayer cultures are convenient and useful candidates for determining metabolism rates of pharmaceuticals, but as mentioned previously, there lack of cellular complexity / organelle composition and rapid loss of organotypic functionality respectively, limit their use in understanding the mechanistic actions concerning the regulation systems of bio-transformation enzyme activities (Cravedi et al. 1996). Here we report the use of 3-D fish liver spheroids to determine metabolic rate constants and half-life's for a range of environmentally relevant pharmaceuticals that are currently receiving attention for their biological effects on fish.

5.2 Materials and Methods

5.2.1 Spheroid culture

Plates of spheroids were prepared from rainbow trout (wet weight: 115.96 ± 21.67 g) as outlined previously (Section *2.5.1*). These were placed at 15 ºC in a temperature-controlled laboratory on an orbital shaking platform set at a constant rotation speed of 70 RPM. Penicillin-Streptomycin (5,000 U ml^{-1}) and amphotericin B (250 µg ml^{-1}) were added to the culture medium (1% v/v).

5.2.2 Preparation of test chemicals

Where appropriate, test chemicals were first dissolved in DMSO and diluted to a final concentration of 200 µg l^{-1} (0.2% DMSO) in L-15 medium. The final well concentration of test chemicals was 100 µg L^{-1} (0.1% DMSO) based on maximum tolerated concentrations (MTCs). Test concentrations and solvent controls were prepared fresh on the day of exposure. Test chemicals that carried a salt weight were accounted for when calculating exposure concentrations, therefore a final well concentration of 100 µg l^{-1} refers to parent compound minus counter ion.

5.2.3 Morphological integrity assessment

Morphological integrity of spheroids was assessed under an inverted light microscope to determine their suitability for exposure as per Section *2.7.*

5.2.4 Cultures and batches

5.2.4.1 WST-1 assay

Spheroid viability was measured in six individual cultures for each test concentration from at least two individual animals as per Section *2.9.1*. Solvent controls, positive controls (1% Triton X-100) and blanks were run in triplicate wells. This technique requires neither washing nor harvesting of cells and the complete assay from micro-culture to data analysis can be performed in the same micro-plate. In contrast to conventional viability assays incorporating the reduction of the tetrazolium dye 3-(4,5-dimethyliazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the WST-1 reagent is more stable, yields water-soluble formazan cleavage products that can be measured without an additional solubilisation step, is a ready-to-use solution that can

be stored for several months without significant degradation. Previous studies have demonstrated its application to 3-D spheroid viability investigations (Liu et al. 2007).

5.2.4.2 Substrate depletion assay

Each 96-well micro plate was divided in two and spheroids prepared from two separate fish were assigned to their respective section of the plate i.e. each exposure was run in two separate fish in parallel. Each micro-plate row (*n* = 12 wells) was allocated a time-point. Test chemical depletion was measured in six individual cultures from at least two individual animals as per Sections *2.10.1 – 2.10.2.* Solvent controls and blanks were run in triplicate wells. A plate layout for a typical exposure is shown in Figure 5.1.

5.2.4.3 Protein assay

Total protein of spheroid cultures (100 spheroids well^{-1}) was measured in six individual cultures from seven different animals to assess variability between seeding batches as per Sections *2.8.2.*

5.2.4.4 Calculation of rate constant (*k*) and half-life ($t_{1/2}$)

The depletion of parent chemical from the culture medium was determined by plotting measured concentration of test chemical (µg l^{-1}) vs. incubation time (h). The rate constant (*k*; h^{-1}) was calculated by non-linear regression analysis (Sigma Plot 12.5), using a two parameter, exponential decay equation ($y = ae^{-bx}$). The half-life ($t_{1/2}$) for the exponential decay of the parent chemical was calculated using the rate constant in the following equation ($t_{1/2} = \frac{ln2}{k}$).

		1	2	3	4	5	6	7	8	9	10	11	12	Time Point (h)
Fish 1	**A**	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	0
	B	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	1
	C	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	4
	D	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	24
Fish 2	**E**	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	0
	F	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	1
	G	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	4
	H	Control	Control	Control	Blank	Blank	Blank	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	Spheroid Sample	24

Figure 5.1 Plate layout for a LOP metabolism assay (24 h exposure). **Control** = spheroids without test chemical; **Blank** = test chemical without spheroids; **Spheroid Sample** = Spheroids with test chemical.

5.3 Results

5.3.1 LOP assay validation

5.3.1.1 Antibiotic / antifungal mixture in culture media

The addition of a penicillin / streptomycin and amphotericin B mixture to the culture medium during spheroid formation was used so as to reduce the risk of culture contamination while spheroids were cultured outside of strict, aseptic incubator conditions. The temperature controlled laboratory was thoroughly cleaned with 70% ethanol prior to culturing and preventive protocols put in place to reduce contamination risk (entry restriction to the laboratory; cultures situated away from door entry and regular top-surface sterilisation). No difference in the morphological appearance of spheroids was observed over the 8 d culturing period when compared to those cultured without an antibiotic / antifungal mixture.

5.3.1.2 Micro-plate seeding optimisation

Total protein content of spheroid cultures (100 spheroids well^{-1}; n = 6) was measured in preparations from individual fish (n = 7) to assess the variability of spheroid seeding between experiments (Fig. 5.2). No significant difference was observed between animals.

5.3.1.3 Spheroid viability

Preliminary MTC assays were performed for all test chemicals in separate micro-plates of spheroids. Viability of spheroids was determined by (a) morphological assessment and (b) WST-1 assay. No dissociation or clumping of spheroids or viability loss was observed with any test compounds except cyclophosphamide,

which resulted in a degree of spheroid dissociation (Fig. 5.3) and a decrease in viability (15.50 ± 1.20% loss compared to control; $P < 0.05$) at 320 µg l^{-1}.

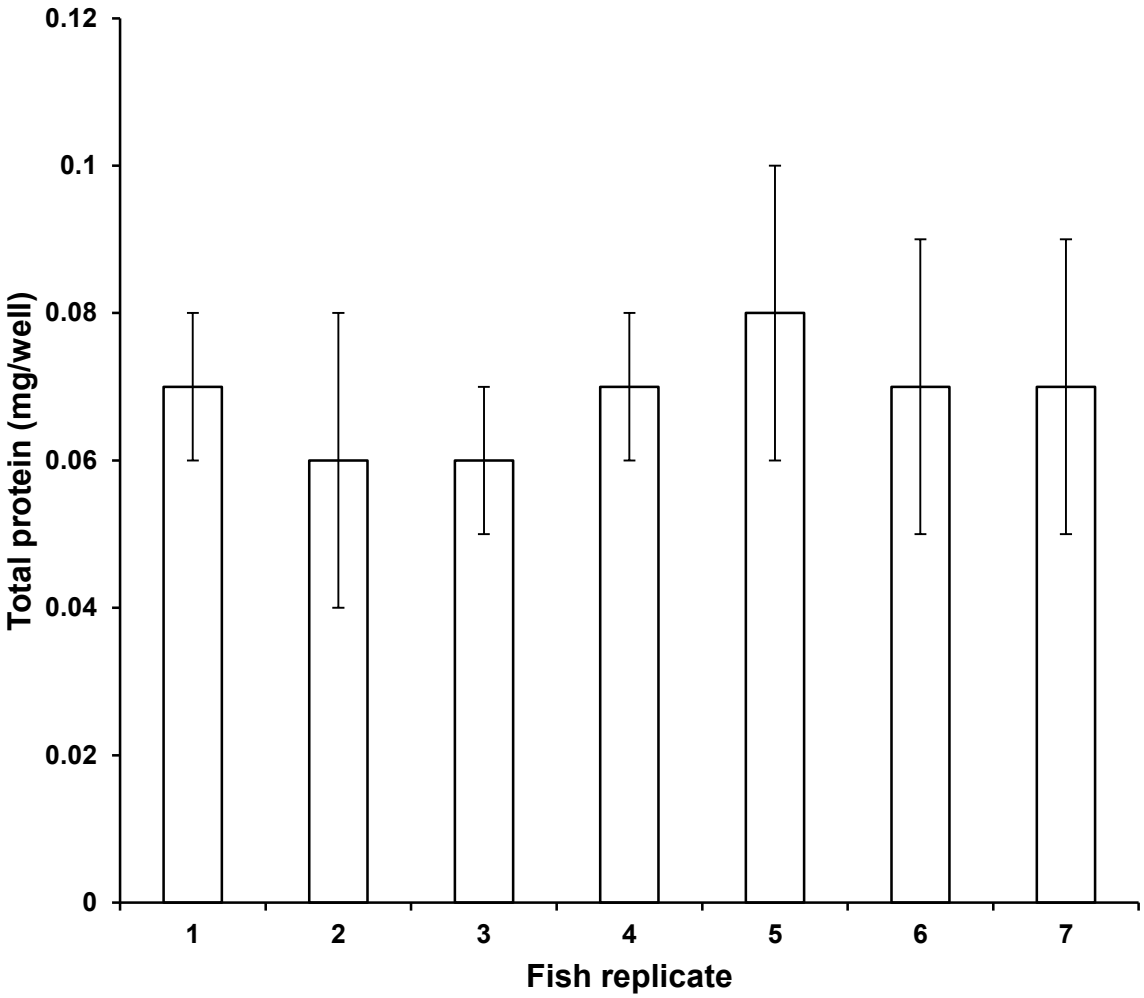


Figure 5.2 Total protein in spheroid cultures seeded at 100 spheroids $well^{-1}$ from seven individual fish replicate experiments. Values expressed as mean ± SD.

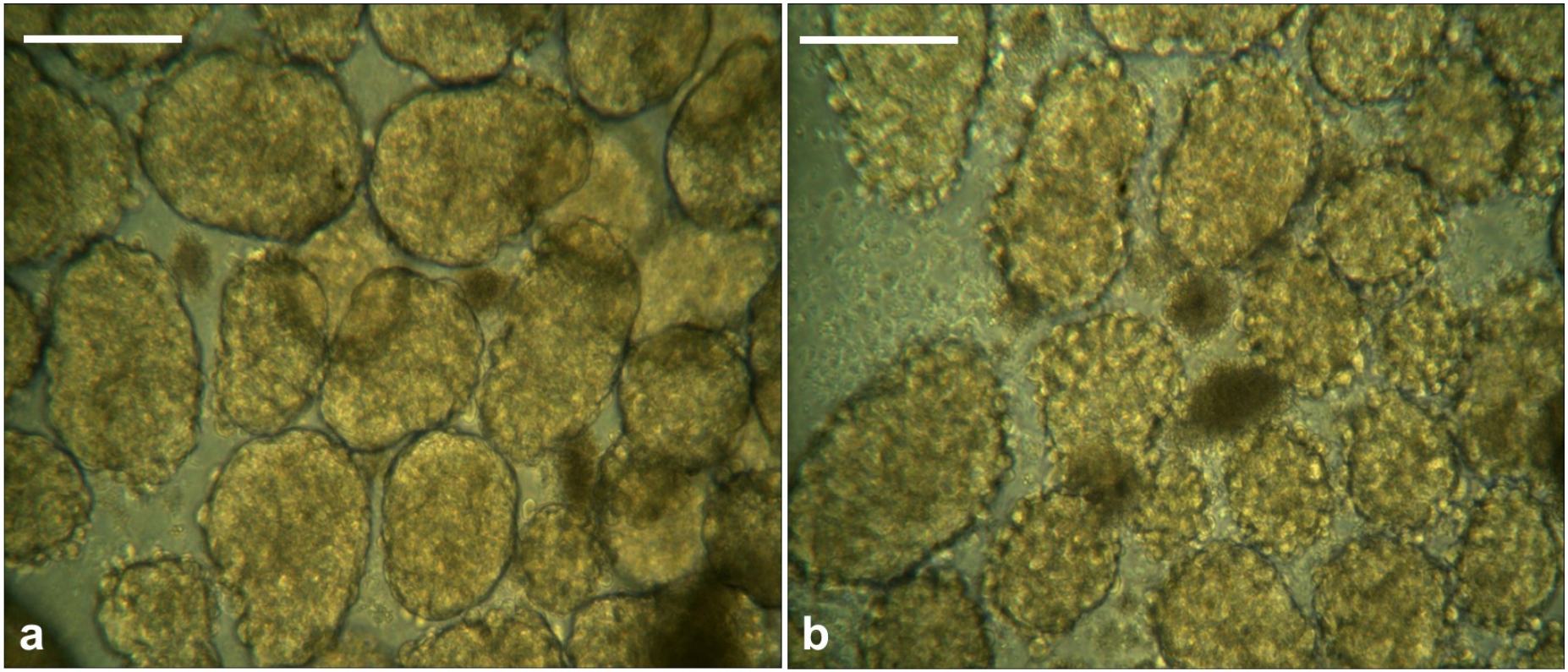


Figure 5.3 Images of spheroids taken after 48 h exposure to 320 µg l^{-1} cyclophosphamide monohydrate. (a) Control spheroids and (b) exposed spheroids**.** Scale bar = 50 µm.

5.3.1.4 Seeding density optimisation

The effect of spheroid seeding density (100 and 500 spheroids well^{-1}) on substrate depletion was determined using a metabolised (diclofenac) and non-metabolised (fluoxetine) test chemical. No significant difference in the rate constant (*k*) was observed between seeding densities in either chemical (Table 5.1).

Table 5.1 Rate constants (*k*; h^{-1}) of diclofenac and fluoxetine in trout liver spheroids seeded at different densities (100 and 500 spheroids well^{-1}). Values expressed as mean ± SD.

Test chemical	Fish (*n*)	Spheroids well^{-1}	Depletion rate constant (*k*; h^{-1})
Diclofenac	2	100	0.025 ± 0.005
		500	0.026 ± 0.004
Fluoxetine	2	100	No metabolism
		500	No metabolism

5.3.1.5 Propranolol exposures (positive control)

Substrate depletion of propranolol was measured in individual fish (*n* = 12) over 24 h. Concentrations of propranolol were recorded initially at six time points (*n* = 2 individual fish) to determine substrate depletion kinetics and rate constants were determined using exponential decay curve-fit analysis (non-linear regression; Fig 5.4). Substrate depletion of propranolol was measured in all twelve individual fish experiments (*k*: 0.022 ± 0.01; %*CV*: 46.4; Table 5.2). No effect of propranolol (≤320

μg l^{-1}) was observed on either the morphological integrity or viability of spheroids in all experiments.

5.3.1.6 Pooled culture exposures

To consider the effect of single fish vs. mixed fish experimental approach (Table 5.2), rate constants for pooled cultures from four individual fish (*k*: 0.0131) were compared with individual fish experiments (*k*: 0.020 ± 0.011; %*CV*: 52.3; *n* = 4 fish)

5.3.2 Rate constant (*k*) and half-life ($t_{1/2}$) of test chemical metabolism

A description of the test chemicals, rate constants and half-life's is shown in Table 5.3. Test chemicals were chosen based on (a) their reported presence in the aquatic environment at environmentally-relevant levels and/or (b) their metabolism and bio-accumulation characteristics taken from published human, mammalian and fish data.

5.3.2.1 Metabolised test chemicals

The degree of substrate depletion was similar in both the NSAID drugs diclofenac (*k*: 0.021 ± 0.008; %*CV*: 38.7; *n* = 4 fish) and phenylbutazone (*k*: 0.015 ± 0.006; %*CV*: 38.7; *n* = 3 fish). The antipsychotic drug thioridazine was also metabolised by liver spheroid cultures (*k*: 0.014 ± 0.007; %*CV*: 48.5; *n* = 5 fish).

Individual fish experiment	Time (h)			Rate constant (k; h^{-1})
	0	**4**	**24**	
1	99 ± 5	86 ± 6	76 ± 5	0.010
2	101 ± 4	86 ± 8	64 ±9	0.017
3	95 ± 4	103 ± 6	61 ± 10	0.020
4	99 ± 7	59 ± 4	32 ± 11	0.045
5	97 ± 2	90 ± 4	61 ± 5	0.019
6	97 ± 3	78 ± 4	49 ± 12	0.027
7	100 ± 4	94 ± 4	66 ± 4	0.018
8	97 ± 4	75 ± 7	44 ± 5	0.032
9	97 ± 3	95 ± 7	66 ± 4	0.017
10	97 ± 4	84 ± 7	48 ± 6	0.029
11	101 ± 3	90 ± 4	50 ± 7	0.029
12	101 ± 5	97 ± 4	85 ± 5	0.007
Average	**98 ± 2**	**86 ± 12**	**59 ± 15**	**0.022 ± 0.010**
Pooled	100 ± 4	91 ± 6	72 ± 7	0.0131

Table 5.2 Concentrations of propranolol (μg l^{-1}) measured over time with calculated rate constants (k; h^{-1}) for liver spheroid cultures from individual fish experiments. Values at each time-point (μg l^{-1}, expressed as mean ± SD; n = 6 wells from each individual experiment (n = 12)). Fish 9-12 were used to prepare 'pooled' cultures.

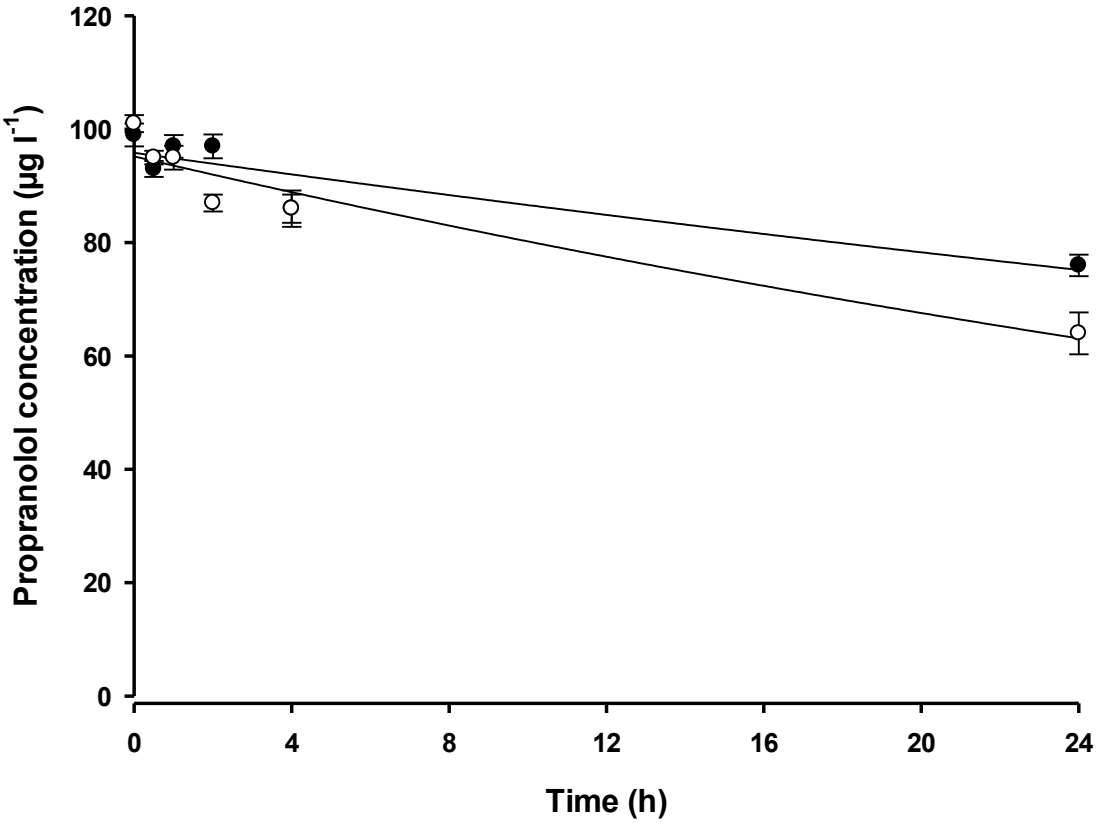


Figure 5.4 Substrate depletion of propranolol by trout liver spheroid cultures. Closed circles denote cultures from fish one; open circles denote cultures from fish two (*n* = 6 at each time point). Values are mean ± SE. Substrate depletion kinetics determined using two-parameter, exponential decay curve-fit analysis (non-linear regression; Sigma Plot v12.5).

Table 5.3 Test chemical rate constants and half-life's in trout liver spheroid cultures. † Ranked according to the Biopharmaceutics Drug Disposition Classification System (BDDCS) (Benet et al. 2011) where **1** = High solubility / extensive metabolism; **2** = Low solubility / extensive metabolism; **3** = High solubility / poor metabolism. Values expressed as mean ± SD.

Chemical	Classification †	Category	Mechanism of action	Log K_{ow} (pH)	% excreted unchanged in human urine †	Rate constant (k; h^{-1})	Half-life ($t_{1/2}$)
Propranolol HCl	1	Non-selective beta blocker	Antagonist of β_1 and β_2 adrenergic receptors	0.72 (7.0)	0.25	0.020 ± 0.010	39.4 ± 23.9
Diclofenac sodium salt	1	NSAID	COX inhibitor	4.02 (7.0)	0.5	0.021 ± 0.008	39.2 ± 22.4
Thioridazine HCl	1	Antipsychotic	D_2 dopamine receptor antagonist	5.90 (?)	-	0.014 ± 0.007	60.1 ± 24.7
Phenylbutazone	1	NSAID	COX inhibitor	3.16 (7.4)	1	0.015 ± 0.006	51.0 ± 22.9
Metoprolol succinate	1	Selective beta blocker	Antagonist of β_1 receptor	0.09 (7.4)	10	-	-
Fluoxetine HCl	1	SSRI	Agonist of σ1 receptor	4.64 (7.4)	1.25	-	-
Cyclophosphamide monohydrate	1	Nitrogen mustard alkylating agent	Adds alkyl groups to GUA residues	-	6.5	-	-
Diazepam	1	Benzodiazepine anxiolytic	Enhances GABA (CNS depression)	2.70 (7.0)	0.5	-	-
Carbamazepine	2	Anticonvulsant	Stabilizes inactivated state of voltage-gated sodium channels	2.47 (7.0)	0.5	-	-
Atenolol	3	Selective beta blocker	Antagonist of β_1 receptor	0.02 (7.4)	94	-	-

5.3.2.2 Non-metabolised test chemicals

In comparison to propranolol, the β-blockers atenolol (*n* = 4 fish) and metoprolol (*n* = 4 fish) demonstrated no evidence of substrate depletion, which was evident ≤48 h (atenolol) and ≤72 h (metoprolol). Fish liver spheroids also showed no measurable metabolism of the serotonin re-uptake inhibitor (SSRI) fluoxetine, nor was any production of its primary metabolite, norfluoxetine detected in any of the fish replicates (*n* = 4). No substrate depletion was measured in any experiments with carbamazepine, cyclophosphamide and diazepam (*n* = 2 fish).

5.3.2.3 Non-detected test chemicals

An additional six test chemicals (4-acetamidophenol, caffeine, nicotine, phenobarbital, ibuprofen and naproxen could not be detected using the LC-MS/MS method used here (*n* = 2 fish).

5.4 Discussion and conclusions

A preliminary period of method development was carried out to optimise both culture condition and micro-plate seeding densities. The addition of an antibiotic / fungicide mixture was implemented during this study so as to reduce the risk of potential contamination that may arise from culturing in a non-sterile environment (temperature controlled laboratory). No effect on the spheroid formation process was observed on cultures containing antibiotic / antifungal mixtures and as metabolism was measured routinely in a range of test chemicals, there does not seem to have been an effect on bio-transformation. Therefore, spheroid culturing may be

undertaken without the use of a specialised cooled incubator as long as temperature-controlled facilities can be accurately controlled.

As mentioned previously, the total protein of hepatic cultures in each well can indirectly reflect the total viable cell number (Baron et al. 2012). A seeding method was developed whereby spheroids could be 'loaded' into micro-plates quickly using a multi-channel pipettor, thereby reducing the variability that would arise from loading wells singly. Total protein per well was stable between replicate experiments (i.e. per fish) suggesting that this method could be utilised towards developing a more rapid screen for measuring total protein content between spheroid preparations and its integration into developing a more high-throughput substrate depletion assay. It also demonstrates the low level of variability in protein content of spheroid cultures between individual fish, a parameter which can influence greatly metabolism rates in S9 methods (Johanning et al. 2012).

This study also demonstrated that the seeding density of spheroids did not affect the substrate depltion rate as demonstrated previously with the EROD activity of BNF-induced spheroids (Section *4.3.4*). Assessment of morphological integrity, in particular the degree of spheroid-clumping, was observed and found not to be a factor on spheroid viability when spheroids were seeded in 96-wells at higher densities (~500 well^{-1}), which may have resulted as a combination of acute exposure periods with non-toxic test chemicals at pharmacologically relevant concentrations. Therefore as fewer spheroids are required per replicate well and a single fish can generate a large number of spheroids, it is possible to develop this assay to analyse

large numbers of test chemicals or concentrations and time courses (Baron et al. 2012).

Propranolol metabolism is well documented in mammals (Bourne 1981; Walle and Walle 1985; Masabuchi et al. 1994; Imamura et al. 2002), particularly in humans, where cytochrome P4501A2 (CYP1A2) catalyses side chain N-desisopropylation of propranolol, accounting for 32 - 50% of the total metabolism of the drug (Masabuchi et al. 1994). Aromatic hydroxylation (second major pathway) of propranolol is catalysed mainly by polymorphic CYP2D6. Propranolol exposure under both *in vitro* and *in vivo* conditions has demonstrated comparable hepatic biotransformation enzyme activity (EROD) induction rates in rainbow trout previously (Bartram et al. 2012), suggesting that similar enzyme isoforms to CYP1A2 present in fish (i.e. CYP1A1) may represent an alternative piscine pathway for propranolol metabolism. Effective propranolol metabolism rates have also been demonstrated in both liver and gill S9 fractions prepared from rainbow trout and channel catfish (*Ictalurus punctatus*) (Gomez et al. 2010; Connors et al. 2013). Phase I metabolites of propranolol have also been measured in 2-D primary trout hepatocyte cultures (seven out of ten normally found in human 2-D hepatic cultures, a mixture of identified and un-identified; Owen et al. *unpublished*).

An acute exposure period (4 h) may be sufficient to measure metabolism of propranolol, yet fish liver spheroids seem competent in continued metabolism of the drug for a period ≤ 24 h. With respect to rates of CYP induction measured previously in trout hepatocytes (Bartram et al. 2012) and in keeping with previous studies that have utilised more representative pharmacological concentrations (Smith et al. 2010;

Connors et al. 2013), we validated this assay within the human therapeutic dose range for propranolol (Bartram et al. 2012). Variability between individual rate constants is inevitably as a result of inherent differences between fish, particularly in terms of their physiology and genetic makeup, which could be driving different approaches to the metabolism of propranolol i.e. modulation between the three major pathways (Bartram et al. 2012). It is unlikely that potential differences in the viability and integrity of spheroids would have an effect on metabolism as significant substrate depletion of propranolol was measurable in all individual experiments and morphological integrity was rigorously assessed before each exposure. In addition, no effect of propranolol was observed on spheroid viability (≤ 320 µg l^{-1}).

Furthermore, the substrate depletion rate for pooled cultures from four individual fish was compared with those of individual fish spheroid cultures to consider the effect of single fish vs. mixed fish experimental approach. It is apparent that the substrate depletion rate of fish twelve was 2-3-fold lower than that of the other individuals, which is likely to be reflected in the lower depletion rate and high variability of the mixed fish cultures. If fish twelve was removed from the analysis (reduced to n = 3 fish) it is likely that the depletion rates obtained from both single and mixed fish experiments would be similar. It is therefore concluded that either single or mixed fish culture approach could be used for the determination of substrate depletion, with the latter approach particularly suited for high spheroid number experiments (i.e. chronic experiments with multiple doses over several time-points). Our choice of single fish replicates in this experiment was to highlight the importance of the fact that differences between individual animals and likely differences within each animal over time with endogenous metabolism cycles, could potentially affect the rate of

xenobiotic metabolism (Bartram et al. 2013). As there was (a) no effect of propranolol concentration on spheroid viability; (b) measurable substrate depletion of propranolol in each individual experiment; and (c) with consideration to propranolol's low lipophilicity (log D_{ow} = 1.2; pH 7.2), rapid uptake across the gills and low toxicity *in vivo (*Owen et al. 2009*)*, a concentration of 100 µg l^{-1} and an exposure period of 24 h were chosen as suitable parameters for subsequent chemical exposures.

Besides the current study and two others (Gomez et al. 2010; Connors et al. 2013), nothing is currently published on the metabolism and pharmacokinetics of β-blockers in fish (no comparative studies for atenolol or metoprolol; Owen et al. 2007), therefore we have no choice but to rely on extrapolated metabolism / pharmacokinetic data from mammalian studies to aid in our prediction of biotransformation rates in fish. Although their chemical structures share a number of similarities, the variation around the aromatic ring leads to a number of pharmacokinetic differences between different β-blockers. These include rate of uptake and bio-concentration, lipid solubility, degree and rate of first-pass metabolism in the liver, binding to plasma proteins, half-life, and renal clearance of the drug and/or its metabolites (Owen et al. 2007).

In mammals, hydrophobic β-blockers such as propranolol and metoprolol undergo extensive Phase I hepatic metabolism with ~90% of the parent excreted by the kidneys as metabolites in the urine (Walle and Walle 1985; Benet et al. 2011). Hydrophilic atenolol does not undergo such extensive metabolism and is excreted predominantly as the parent compound (Bourne 1981) with 94% excreted unchanged in human urine (Benet et al. 2011). Similarly to propranolol, metoprolol is

metabolised and eliminated by several oxidation pathways, the major of which via O-demethylation and further oxidation to a carboxylic acid metabolite that accounts for ~65% of the dose (Lennard et al. 1982). Metabolism of propranolol is affected by genetic polymorphism for both CYP1A (mephenytoin hydroxylation) and CYP2D6 (debrisoquine hydroxylation) isozymes in the liver (Ward et al. 1989; Yoshimoto et al. 1995). Metoprolol metabolism in particular is significantly affected by debrisoquine hydroxylation polymorphism (Ward et al. 1989). Fish are not known to possess the debrisoquine isozyme (CYP2D6) which could explain the lack of metabolism demonstrated in this study.

This approach of studying the effects of pharmaceuticals in the environment via the "read-across" of mammalian toxicity and detoxification systems is important when such little data exists on these systems in fish (Huggett et al. 2004; Owen et al. 2007; Gunnarsson et al .2008; Winter et al. 2010; Bartram et al. 2013). It would be of further interest to understand why metoprolol is not well metabolised in fish despite its extensive metabolism in humans. This may be linked to the absence of a CYP2D6-type isoform in fish or that metabolism of metoprolol occurs via the third major pathway (N-dealkylation) which accounts for < 10% of the dose in humans (Murthy et al. 1990).

Fluoxetine is a highly prescribed model SSRI that is used to assess the impacts of antidepressants on aquatic organisms (Smith et al. 2010). As the fish serotonin neuroendocrine system has been shown to be analogous to that of mammals (Gould et al. 2007), it is acceptable to assume that fish may be susceptible to the same adverse effects and thus demonstrate the same biological responses associated with

SSRI exposure in mammals (Smith et al. 2010). In particular, alterations in physiological function (Foran et al. 2004; Morando et al. 2009) and behaviour (Stanley et al. 2007) have been observed in a range of species. With respect to biotransformation, recent studies have demonstrated that Japanese medaka (*Oryzias latipes*) are capable of *in vivo* metabolism of fluoxetine and production of its major metabolite, norfluoxetine, and where a moderate bioaccumulation factor (< 200) and a relatively long half-life of fluoxetine (9.4 d) are observed (Nakamura et al. 2008; Paterson et al. 2008). With respect to the "read-across' approach, human studies (Heimke and Hartter 2000) suggest that the metabolic conversion of fluoxetine is significantly slower in fish.

To date, two other studies have investigated the *in vitro* hepatic metabolism of fluoxetine in fish (Smith et al. 2010; Connors et al. 2013). It was observed that incubation of fluoxetine at therapeutic dose with fish liver microsomal fractions obtained from rainbow trout, demonstrated poor levels of metabolism with often undetectable production rates of norfluoxetine. Metabolism was only evident after pre-incubation with carbamazepine, which induces CYPs responsible for fluoxetine metabolism in mammals, yet production of norfluoxetine remained low and variable between species, suggesting that norfluoxetine is unlikely to be the primary fluoxetine metabolite in fish. The lack of fluoxetine metabolism and production of norfluoxetine in this study could be explained again by the lack of CYP2C and 2D homologs in fish. In humans, fluoxetine is primarily metabolised into norfluoxetine through demethylation by CYP2D6 and to a lesser extent by CYPs 2C9, 2C19 and 3A4 (Smith et al. 2010). This low level of metabolism detected in these two *in vitro* studies suggests that fish bio-accumulate fluoxetine *in vivo* (Nakamura et al. 2008).

Metabolism of diclofenac has been demonstrated previously *in vitro* with rainbow trout liver S9 fractions (Connors et al. 2013). Both larval zebrafish (*Danio rerio*) (Alderton et al. 2010) and juvenile rainbow trout (Mehinto et al. 2010; Lahti et al. 2011) show diclofenac metabolism *in vivo* with measurable levels of phase I and II metabolites. Metabolites and un-metabolised diclofenac have also been detected in the bile of adult rainbow trout (Kallio et al. 2010), although large variations in both up-take and metabolism were observed between individual animals. Like propranolol, diclofenac is substrate for more than one human CYP, including CYP1A2 and 2D6 (Connors et al. 2013). The metabolism of these two test chemicals in the present study further supports the hypothesis that fish may lack a mammalian-like CYP2D6 isoform, with the predominant level of metabolism controlled by the human homologous CYP1A1 and 1A2. A measured bio-concentration factor of < 10 (Memmert et al. 2013) and repeatedly-measured metabolism in this study, suggests that diclofenac may not demonstrate a relevant bio-accumulation risk in fish.

Recent reports of trace levels of the drug phenylbutazone in meat products for human consumption have raised concerns over its ability to bio-accumulate in tissues and for its potential toxicological side effects. Phenylbutazone has been demonstrated to be extensively metabolised in both humans (Aarbakke et al. 1977); horses (Lees and Toutain 2013) and fish (present study). Reported low levels of accumulation in horse tissue (< 1/1000 of a therapeutic does in man) and its classification of 'non-carcinogenic' (Lees and Toutain 2013) in conjunction with significant hepatic biotransformation in fish, suggests that the drug is unlikely to bio-accumulate and therefore may not pose a public health issue. Thioridazine, a prototype drug of phenothiazine neuroleptics of the piperidine-type, is a substrate

and an inhibitor of polymorphic CYP2D6 in humans (Daniel et al. 2000). Metabolism of this test chemical in any aquatic species has not been previously reported, which makes prediction of specific CYPs controlling its metabolism in fish, difficult.

No measurable metabolism of carbamazepine in fish liver S9 fractions, a substrate for CYP3A4 in humans, has been described recently (Connors et al. 2013). These authors also suggest that there are differences in CYP3A specificity between trout and mammals that could lead to an absence of CYP3A4-like activity in trout, and that trout and other fish species may metabolise some, but not all, mammalian CYP3A substrates utilizing enzymes from other CYP families (e.g. CYP1A). Propranolol positive controls were run alongside these test chemicals and metabolism in these spheroid sets was detectable, highlighting confidence in the analytical method. It may be assumed therefore, that these test chemicals are not metabolised *in vitro* but may be accumulated in tissue.

In addition to the ten test chemicals that were analysed for substrate depletion, an additional six (4-acetamidophenol, caffeine, nicotine and phenobarbital – class 1; ibuprofen, naproxen – class 2; Benet et al. 2011) were unable to be detected by the LC-MS/MS method used here. This highlights the importance of developing the appropriate analytical method (GC-MS; LC-MS/MS; HPLC) for each particular test chemical (Johanning et al. 2012). To re-analyse those chemicals that were not detectable with LC-MS/MS with a GC-MS analytical method was not within the scope of this study due to timescale and costs, but does highlight the need for multiple analytical methods when determining depletion rates for a range of test chemicals, that can in turn be used to produce a more robust, repeatable metabolism assay.

In conclusion, this study reports the method development for an alternative, *in vitro* substrate depletion assay to determine depletion rate constants and half-life's of environmentally-relevant pharmaceuticals. The assay was characterised to optimise both culture and exposure conditions and validated with the non-selective β-blocker propranolol, to establish a 'positive control' test chemical. The measurement of depletion rates in environmentally-relevant pharmaceuticals was compared with datasets from published scientific literature, both *in vitro* and *in vivo* in fish and 'read-across' to mammalian studies. With further characterisation and consideration of the assessment of ADME processes in the organism (Owen et al. 2007), there is the potential to utilise spheroids as an alternative *in vitro* bio-transformation model to better understand the bio-accumulation potential of pharmaceuticals, particularly those which may be considered to be persistent in the environment.

CHAPTER 6

General discussion

6.1 Global calls for *in vitro* alternatives

There have been significant calls for the development of robust *in vitro* technologies to support regulatory ecotoxicology globally. For example in Europe there are demands from the regulatory directives and guidance documents (controlling the use of chemical and biological products) for the development of alternative methods (e.g. REACh (REACH 2009); European Medicines Agency (EMA 2006) and plant protection products directive (PPPR 2009). Similar regulatory developments have also taken place in the US (Voluntary EPA-High Production Volume Challenge Programme) and in Japan (TTREC-Toxicity Testing Reports for Environmental Chemicals (Schrattenholz and Klemm 2006). It is therefore not surprising that an important vision for toxicological studies in the 21st century is to replace the currently used animal tests with *in vitro* systems to gain insights of the mechanisms of actions of environmental toxicants to protect both human and environmental health.

Compared to mammalian toxicology, however, *in vitro* technologies for fish seem to be less well developed, perhaps because of the perceived difficulty of establishing these 3-D cultures for fish cells, or perhaps because traditionally it has been relatively simple to expose fish directly to the compound of interest. Longer lived and metabolically more realistic fish cell models could allow the development of new predictive screens that would take the potential for regulatory alternatives beyond the current compromise of short-term cultures in the weight of evidence assessments (Scholtz et al. 2013), and provide a platform for further alternatives to *in vivo* fish studies in ecotoxicology (Baron et al. 2012) (Fig. 6.1).

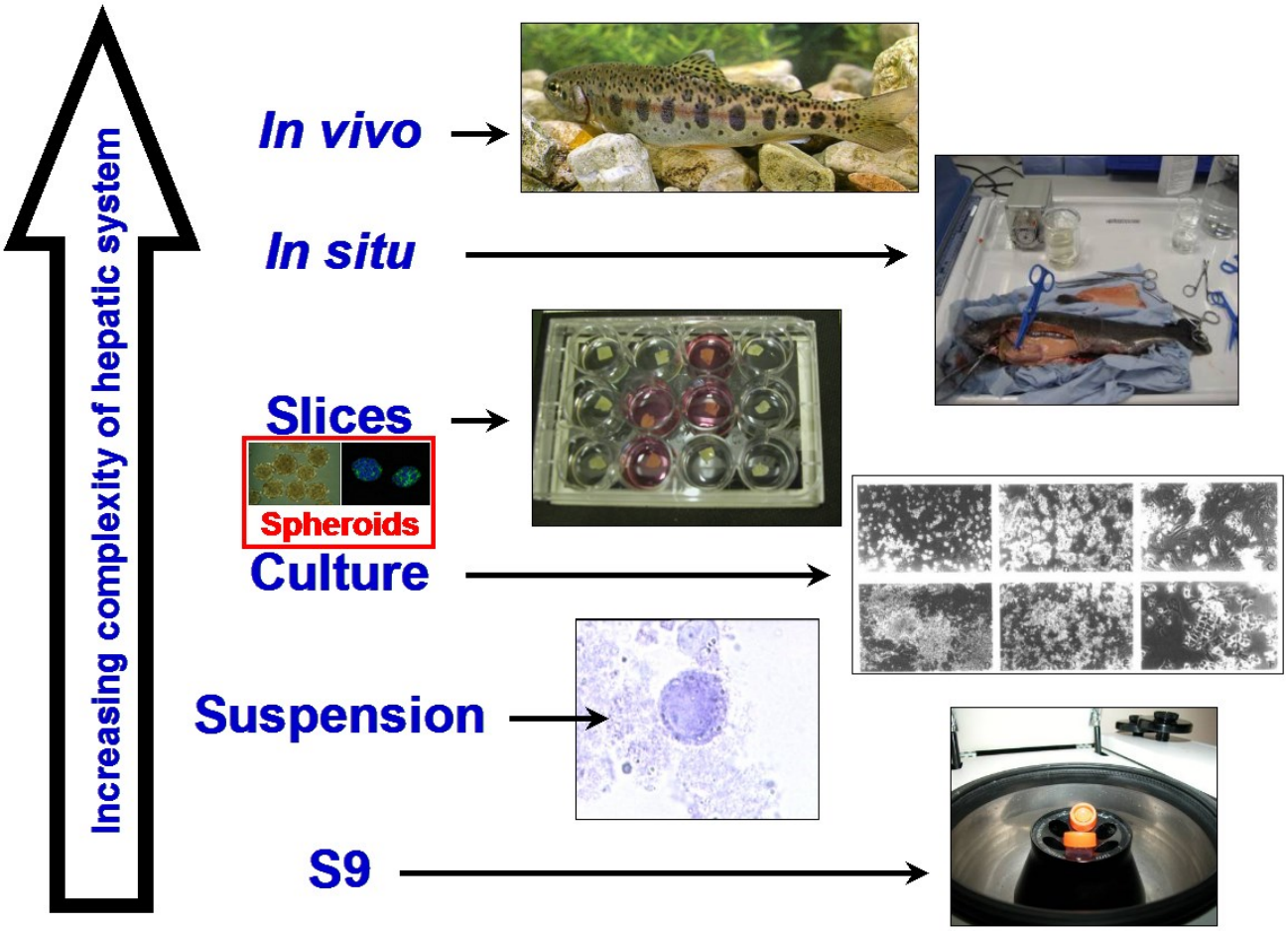


Figure 6.1 A representation of the increasing complexity of *in vitro* hepatic systems in fish. Suspensions and culture systems sit just above S9 preparations but under more *in vivo*-like liver slice and *in situ* exposures. Images acquired from studies undertaken at Brixham Environmental Laboratory (Brixham, UK).

6.2 Importance of *in vitro* testing strategies

The suitability of *in vitro* techniques as an alternative screening tool to whole animal *in vivo* studies has large implications on future ecotoxicity studies. The potential to replace current fish tests that require large numbers of animals (i.e. OECD 203: Fish, Acute Toxicity Test and OECD 305: Bio-concentration, Flow-through Fish Test) with a more high-throughput, economical, ethical and more predictive *in vitro* model, that

will accommodate the large number of chemicals and discovery pharmaceuticals that need testing under new EU legislation, is an exciting and much needed forward movement in xenobiotic testing. The use of spheroids could act as an additional stage in a tiered testing strategy to complement conventional *in vitro* techniques, if good correlation between 3-D *in vitro* and whole animal *in vivo* data is confirmed; which could offer a more predictive *in vitro* model, without the need for large-scale animal *in vivo* tests.

In addition, the use of more advanced *in vitro* assays in ecotoxicological studies provides the opportunity for extrapolation from *in vitro* to *in vivo* systems and generates more closely related information on biological responses at relatively high levels of biological organisation (Castano et al. 2003). This is important, given that non-animal techniques are likely to dominate 21st century toxicology investigations compliant with the 3Rs principles (Seidle and Stephens 2009) and following increased pressure from new EU regulation, REACh (EU regulation no:1907/2006) adding thousands of new chemicals each year for toxic evaluation (van Hemmen 2009). In particular under such regulation, it is likely that many man-made chemicals could be tested for their potential to bio-accumulate and enrich the organisms through different routes of exposures, either aqueous or dietary.

6.3 Application of 3-D liver spheroids

The findings from this study supported by those from the primary literature and with particular reference to the 3Rs principles, demonstrate that the application of 3-D cell culture to fish ecotoxicity studies has many advantages. Firstly, trout hepatocytes can be readily isolated via our optimised enzymatic digestion method, producing

large hepatic cellular yields with high cellular viability. This methodology underwent extensive optimisation experiments throughout the study to produce a standard operating procedure (SOP) for the routine formation of hepatic fish spheroids. Since a single fish can provide a large number of spheroids, there is the benefit of a more economical use of the organ (Refinement) with the potential to develop assays for large numbers of test chemicals, or concentrations and time courses, which can reduce the overall number of fish required for testing (Reduction). 3-D spheroids are generated under very simplistic gyratory-mediated conditions that are relatively inexpensive to create and maintain, need little specialist equipment and do not require large fish-housing facilities and technical personnel (Fig. 6.2).

Spheroids take ~6 – 8 d to reach maturity whereupon they can mimic the cyto-architecture of tissues, exhibiting many important cellular characteristics including tight cell-cell connections, refined extracellular ECMs, formation of tight-junctions and bile canniculi and maintenance of important cell adhesion molecules (F-actin). They also exhibit maintenance of organ specific functions (glucose, albumin and LDH release) and bio-transformation capability (EROD activity, parent compound metabolism), important characteristics for quantifying xenobiotic metabolism that is limited in S9 preparations and conventional fresh hepatocyte and 2-D monolayer cultures (Ma et al. 2003; Baron et al. 2012; Uchea et al. 2013; Xu et al. 2013). These parameters suggest that they could be a useful alternative tool for investigating both the acute toxicity and metabolism of test chemicals and as the fish themselves are not exposed to the toxicant before death, there is an additional benefit in the reduced requirement for large quantities of the test compound (Refinement) (Baron et al. 2012).

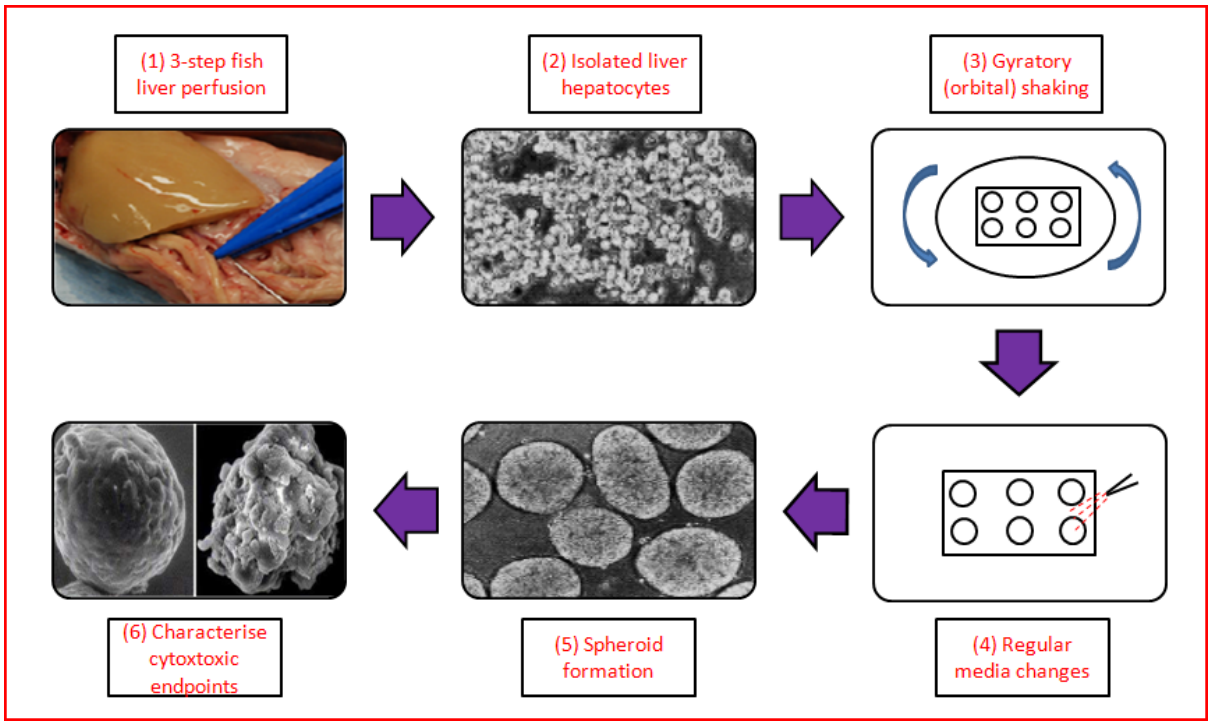


Figure 6.2 Simplified flow diagram demonstrating procedural stages of spheroid formation. Methodology is relatively simple, cost-effective, utilises inexpensive equipment and can be performed completely in a small laboratory. Images 2 and 5 taken from Flouriot et al. 1993.

These spheroids have also been shown to survive in culture for a period ≤ 80 d, an important criterion for both the chronic toxicity and bio-accumulation assessment of test chemicals, particularly those considered to be persistent in the environment. However, further characterisation of their organ-specific functions over prolonged culture periods needs to be investigated in order to fully support their use for these testing methods. They may therefore represent a better alternative to exposing large numbers of fish to toxicants in order to study the biochemical and bio-transformation response (Replacement) (Baron et al. 2012).

Despite the characteristics of spheroids offering obvious advantages and wider applications for chemical toxicity and metabolic assessment, there are limitations to the model that should be considered. As demonstrated in this study and in other complementary mammalian spheroid studies (Ma et al. 2003), size variation between individual spheroids does arise when cultured under gyratory-mediated conditions. Despite the relatively simplistic nature of the method to generate large quantities of spheroids, the rotational forces applied cannot precisely control the size of individual spheroids (i.e. < 15% variation in diameter). This is particularly important for experiments designed to test the therapeutic effects of drugs in growth assays (i.e. tumour growth), where a hanging drop type methodology that can generate individual spheroids within a narrow size distribution may be more applicable (Kelm et al. 2003). Equally, a uniform size in spheroids could aid a better prediction of drug penetration in, and distribution throughout the tissue model, which are important parameters for more accurate PBTK modelling in both mammals and fish. With this information in mind it is also worth considering that differences in the size of livers between individual, size-matched animals may still exist. Therefore, the size variation between liver spheroids formed under gyratory-mediated conditions may reflect similar size variations *in vivo*.

With respect to the metabolic capacity of 3-D spheroids, this study has demonstrated, with the use of environmentally relevant and hepatic-metabolised pharmaceuticals, that this model system retains a baseline bio-transformation capability for some of these compounds, that compares with both *in vitro and in vivo* data sourced from the literature and may offer a suitable alternative to conventional *in vitro* models (S9, fresh hepatocytes and 2-D monolayers) that are limited by lack of cellular complexity

and organelle composition (S9) and rapid loss or organ-specific functionality (fresh hepatocytes; 2-D monolayers) (Cravedi et al. 1996; Baron et al. 2012; Uchea et al. 2013). There are some questions, however, that remain over the predictability of the model for determining bio-transformation rates from a range of pharmaceutical classes, which may be addressed by combining the substrate depletion method outlined here with molecular biomarkers (i.e. expression of specific cytochrome P450 genes) to determine the specific enzymatic pathways with which these pharmaceuticals are, or are not metabolised, or that fish may or may not possess compared to mammalian systems. Pharmaceuticals not metabolised in this study may need combining with CYP-inducing compounds e.g. β-napthoflavone to determine metabolic activity, which in combination with gene and protein expression techniques, could assist in the identification of which cytochrome P450 enzymes are responsible for metabolising specific pharmaceuticals.

Pharmaceuticals of bio-accumulative concern e.g. SSRI's such as fluoxetine, sertraline and paroxetine that do not display metabolism in *in vitro* studies but have been detected in parent and metabolite form in fish tissue collected from the field (Chu and Metcalfe 2007; Ramirez et al. 2009; Fick et al. 2010 Du et al. 2012), urgently require a better understanding of their metabolism in a range of *in vitro* fish models to compare with *in vivo* assessments of metabolism. In addition, the measurement of chemical uptake and product analysis (metabolite formation) in addition to substrate depletion measurements would provide further understanding with respect to the mechanistic action, bioaccumulation potential and specific toxic effects of these chemicals. It is also stressed that a combination of analytical techniques be used (i.e. GC-MS and LC-MS/MS) to optimise methods for particular

compound groups to determine metabolism in those pharmaceuticals that could not be detected by the proposed LC-MS/MS analytical method.

The majority of current alternative, preliminary accumulation assessments rely on QSAR- and logK_{ow}-based estimates for fish (Arnot and Gobas 2006) that only incorporate estimated bio-transformation rates (Scholtz et al. 2013). While the measurement of metabolism in the current study represents a forward-step in improving these current assessments, it must be stressed that the measurement of metabolism alone is not a complete assessment of the bio-accumulation potential of a test chemical and must consider the impact of adsorption, distribution, metabolism and excretion (ADME) processes in the organism (Owen et al. 2007) in order to provide a more-comprehensive PBTK model for fish. These considerations can include the measurement of uptake of the chemical into and its distribution within the spheroid and whether or not this affects the rate of metabolism (i.e. measurement of uptake into the surface cells or penetration throughout the spheroid). Measurement of both phase I and phase II bio-transformation processes, subsequent metabolite identification and elimination of parent compound and metabolites via the kidney can identify the complexity by which spheroids can bio-transform test chemicals and the potential toxicity to the fish of each component.

6.4 Future work: co-culture of 3-D *in vitro* organotypic models

It is, however, to be realised that the bio-accumulation potential and toxicity of chemicals are tissue and/or organ specific phenomena. In this context therefore, one may hypothesise that by combining tissues from multiple organs of a fish under *in vitro* conditions in co-culture as 3-D structures, could represent a more functional *in*

vitro fish model for assessing the bio-accumulation potential as well as toxicity of test chemicals which would allow for a more direct comparison with *in vivo* testing approaches. One such approach is the combination of a gill, gut and liver 3-D tissue *in vitro* model, whereby multiple cell types can be extracted simultaneously from a single donor animal. Measurement of uptake and metabolism in 3-D branchial tissue could act as a "first pass" metabolic pathway for some compounds as demonstrated by previous studies (Jonsson et al. 2006; Bartram et al. 2012). Dietary exposure of test chemicals whereby aqueous exposures are not suitable for certain groups of test chemicals ($\log K_{ow}$ >5.5) (Scholtz et al. 2013) could identify uptake and metabolism rates in 3-D fish gut tissue (Fig. 6.3). In combination with 3-D liver spheroids that are long lived (≤ 80 d) there is the potential to represent a direct chronic model comparable with the current OECD *in vivo* methods used for bioaccumulation assessment (OECD 305: Bio-concentration, Flow-through Fish Test). Information gained on the pharmacokinetics of test chemicals is a layer of detail absent from current regulatory testing and such an approach could provide additional ADME information of chemicals, which is often absent from single culture systems, especially from 2-D monolayer culture-based studies.

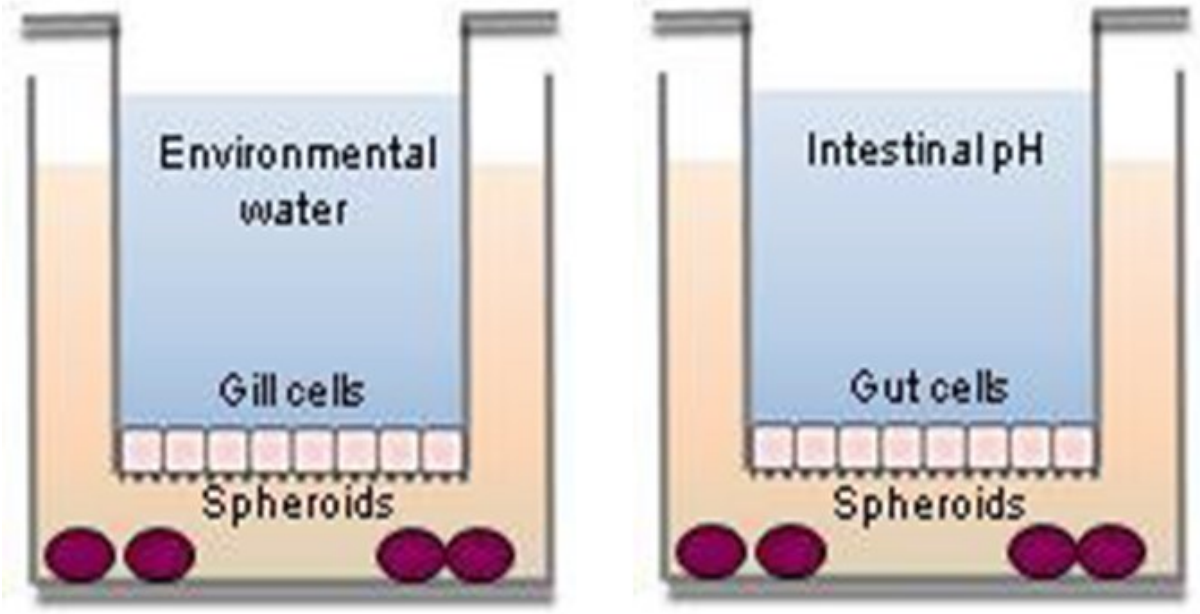


Figure 6.3 Conceptual model for the development of 'virtual fish' using cells of different tissue origin. Diagram of two wells from a 24-well plate containing spheroids (pink shapes) in physiological media: in close proximity to a suspended basket containing gill or gut cells and test compounds, alone or in mixture. The model could measure uptake of a chemical from the water into the gill cells, transport into the media, uptake into the spheroids, metabolism and the manufacture of metabolites, excretion rates back across the gills into the water and kinetics for each. Similarly for the dietary model gut cells could be used, using intestinal pH media to simulate dietary exposures.

In recent years attempts have been made to establish relative sensitivity of fish and mammalian cells of different origin for a range of different toxicological endpoints under *in vitro* conditions (Castano and Gomez-Lechon 2005; Raisuddin and Jha 2004; Papis et al. 2011). Such studies are mainly aimed to verify if fish and mammalian cells could equally predict the toxicological endpoints and whether the

huge amount of toxicological information available for mammalian studies could be translated for aquatic species, in particular for fish. The limited available information pertaining to the relative sensitivity of fish and mammalian cells produce contrasting results. Whilst some studies suggest that fish and mammalian cells show similar basal cytotoxicity for a range of chemicals (Castano and Gomez-Lechon 2005), others suggest that fish cells are more sensitive for a range of cytotoxic and genotoxic parameters (Raisuddin and Jha 2004; Papis et al. 2011). Whilst there are many fundamental similarities between fish and mammalian cells, fish cells have many specific characteristics including higher tolerance to osmolality, slow growth and cellular-turnover rate at lower temperature and perhaps low DNA repair capacity (Castano et al. 2003; Kienzler et al. 2012). The relative comparison of fish and mammalian cells for both tissue and organ specific accumulation as well as for toxicological endpoints needs further elaboration for both a human and environmental health point of view.

In recent decades, development in so called 'omics' technologies have also advanced the development of alternatives to *in vivo* toxicity testing (Bhogal et al. 2005). These technologies (transcriptomics, proteomics and metabolomics) are based on the assumption that physiological, pharmacological and toxicological events would change the protein composition and activities of particular cells or tissues and therefore elucidate the mechanisms of toxicity. Whilst adopting 'omics' approaches using aquatic models including fish under *in vivo* conditions are increasingly being used for hazard and environmental risk assessment (Cossins and Crawford 2005; Craft et al. 2010; Katsiadaki et al. 2010; Santos et al. 2010; Van Aggelen et al. 2010; Fent and Sumpter 2011; Villeneuve et al. 2011); use of these

technologies for 3-D spheroid fish models is lacking. In common with human 3-D models, where microarray technologies are being applied to evaluate biological responses for chronic and acute exposures (Mezentsev and Amundson, 2011), it will be interesting to verify how the 'omics' responses under 3-D and *in vivo* conditions compare following exposure to different contaminants. Such an approach could further strengthen the adoption of 3-D models for environmental hazard and risk assessment.

In addition, it is also emerging that interaction of chemicals with biomolecules such as DNA could also induce epigenetic effects (e.g. via DNA methylation) and such epigenetic effects have been correlated with specific DNA methylation patterns in tumours of fish collected from contaminated sites (Burchiel et al. 2001; Mirbahai et al. 2011; Vandegehuchte and Janssen 2013). Such modifications could be manifested trans-generationally with detrimental effects on individuals and populations. Therefore, further development of 3-D cultures and adoption of various experimental approaches could further facilitate and elaborate the molecular understanding of the interactions between chemicals and biomolecules within the whole organism. It is believed therefore that these methods, combined with those carried out in the current study are an important step towards further developing alternative *in vitro* tools for fish ecotoxicological studies.

APPENDICES

Appendix I: List of perfusion reagents and solution preparation

1. Calcium / Magnesium-free HBSS:

- To 400 ml of stock calcium & magnesium-free HBSS add:
  - 0.175 g $NaHCO_3$
  - 0.336 g EDTA
- Dissolve via magnetic stirring for approximately 10 minutes.
- pH of the final solution adjusted to 7.4 with the addition of 2M NaOH.
- Final solution made up to a final volume of 500 ml, sterile filter through a 0.22 µm syringe filter and store at 4^{o}C for ≤ 1 month.

2. Collagenase D digestion Buffer:

- To 40 ml of stock HBSS (+ calcium & magnesium) add:
  - 0.018 g $NaHCO_3$
  - 0.050 g Collagenase D
- Dissolve via magnetic stirring for approximately 5 min.
- pH of the final solution adjusted to 7.40 with the addition of 2M NaOH.
- Final solution made up to a final volume of 50 ml, sterile filter through a 0.22 µm syringe filter and store at 4^{o}C for ≤ 7 d.

3. DMEM:F12 nutrient mixture media:

- To 450 ml of stock DMEM:F12 media add:
  - 1.720 g TES
  - 0.504 g $NaHCO_3$
- Dissolve via magnetic stirring for approximately 5 min.
- pH of the final solution adjusted to 7.4 with the addition of 2M NaOH.

- Final solution made up to a final volume of 500 ml, sterile filter through a 0.22 µm syringe filter and store at 4°C for ≤ one month.
- NOTE: allow for correct v:v ratio in media recipe if serum is required.

4. L-15 media:

- pH of final solution (500 ml) adjusted to 7.4 with the addition of 2M NaOH.
- Sterile filter through a 0.22 µm syringe filter and store at 4°C for ≤ one month.
- NOTE: allow for correct v:v ratio in media recipe if serum is required.

5. pHEMA solution:

- Measure 90 ml ethanol and 10 ml de-ionised water in a volumetric cylinder and transfer to a 250 ml glass conical flask then add:

  - 2.500 g pHEMA
- Seal the flask with Parafilm® and dissolve the pHEMA on a magnetic stirrer for 5-6 h at room temperature (DO NOT heat the solution). The pHEMA takes a long time to dissolve, so maintain constant stirring if required.
- Sterile filter the solution through a 0.22 µm syringe filter and collect into 2 x 50 ml sterile centrifuge tubes and store for ≤ 6 months.

Appendix II: Perfusion and enzymatic digestion pump calibration

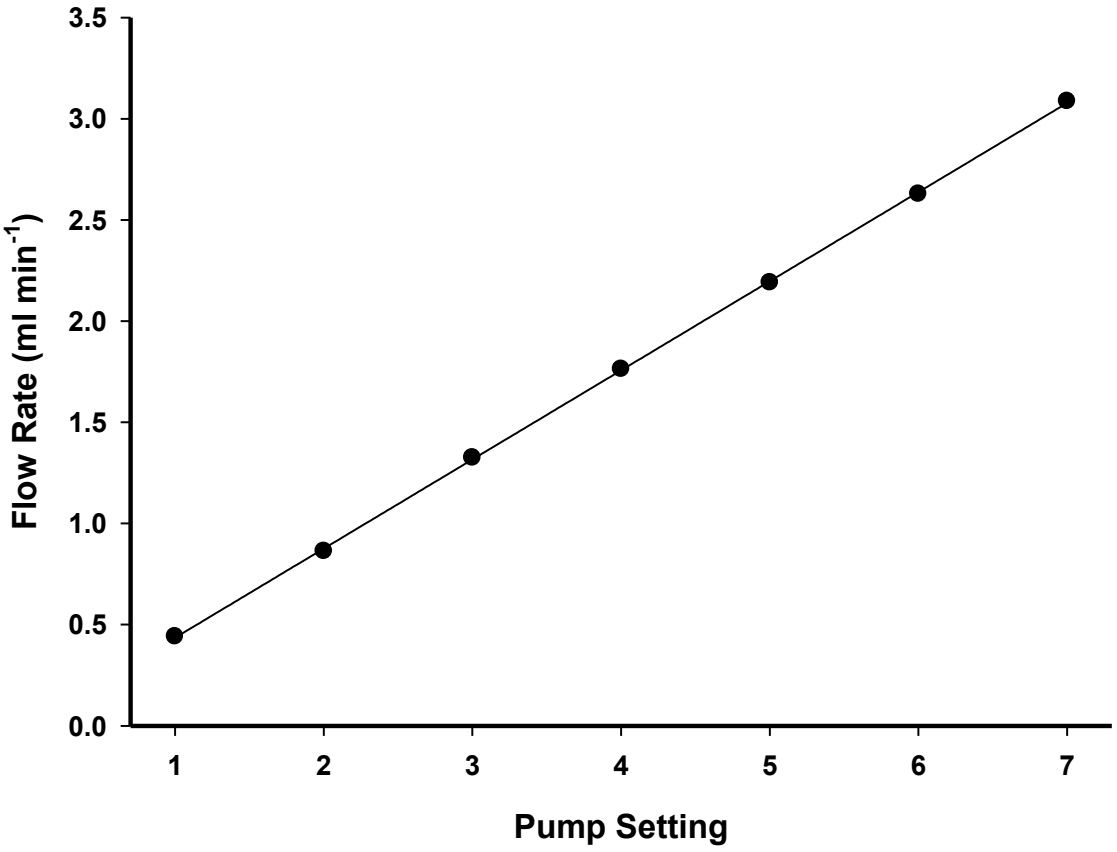


Figure A1 Calibration of Watson Marlow® peristaltic pump for *in situ* rainbow trout liver perfusion. Flow rate = (0.44 x pump setting) + 0.01; R = 0.99; *n* = 4.

Appendix III: Publications, presentations and awards

Publications:

Matthew G. Baron, Wendy M. Purcell, Simon S. Jackson, Stewart F. Owen, Awadhesh N. Jha (2012) Towards a more representative *in vitro* method for fish ecotoxicology: morphological and biochemical characterisation of three-dimensional spheroidal hepatocytes. Ecotoxicology 21 (8):2419-2429

Platform presentations:

Development of 3-D fish hepatocyte spheroid cultures as an *in vitro* tool for environmental chemical toxicity. SETAC Europe 22nd Annual Meeting / 6th SETAC World Congress, Berlin, 24th May 2012.

3-D spheroidal hepatocytes as an alternative *in vitro* model in fish ecotoxicoloy. 1st European Conference on the Replacement, Reduction and Refinement of Animal Experiments in Ecotoxicology, Dubenforf, 28th June 2012.

3-D spheroidal fish hepatocytes as an alternative *in vitro* model for environmental toxicology. The UKEMS Annual Meeting, Swansea, 18th July 2012.

A 3-D spheroidal fish liver model alternative to better undsrstand bio-accumulation in ecotoxicology. SETAC Europe 23rd Annual Meeting, Glasgow, 16th May 2013.

Poster presentations:

Potential application of 3-D fish hepatocyte spheroids as an environmental bio-sensing tool. 2nd International Conference in Bio-sensing Technology, Amsterdam, 15th May 2011.

3-D fish hepatocyte spheroid culture: an alternative *in vitro* tool for environmental chemical toxicity. Implementing the $3R_s$ in behavioural and physiological research: A joint meeting of ASAB, SEB and the $NC3R_s$, London, 4th July 2013.

A 3-D spheroidal fish liver model alternative to better undsrstand bio-accumulation in ecotoxicology. Joint Annual Meeting of the Ecotoxicology Research and Innovation Centre (Plymouth University) and Society of Environmental Toxicology and Chemistry UK Branch, 9th September 2013.

Awards:

Developing *in vitro* alternatives to fish bio-accumulations studies in ecotoxicology. UK $3R_s$ Award Winner 2012 (Aztrazeneca and MedImmune).

Developing *in vitro* alternatives to fish bio-accumulations studies in ecotoxicology. Global $3R_s$ Award Highly Commended 2012 (Aztrazeneca and MedImmune).

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