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Gill cell culture systems as models for aquatic environmental monitoring

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Abstract

A vast number of chemicals require environmental safety assessments for market authorisation. To ensure acceptable water quality, effluents and natural waters are monitored for their potential harmful effects. Tests for market authorisation and environmental monitoring usually involve the use of large numbers of organisms and, for ethical, cost and logistic reasons, there is a drive to develop alternative methods that can predict toxicity to fish without the need to expose any animals. There is therefore a great interest in the potential to use cultured fish cells in chemical toxicity testing. This review summarises the advances made in the area and focusses in particular on a system of cultured fish gill cells grown into an epithelium that permits direct treatment with water samples.

Introduction

The industrial revolution caused a rapid rise in use of raw materials and urbanisation as the populace moved to the cities for employment. Since this time, there has been a continuous increase in living standards that to a large part has been fuelled by innovations within the chemical and pharmaceutical industry. Life expectancy has increased due to great advances in medical practices and effective drugs against many fatal diseases. The increase in life expectancy has seen the population of the world grow, reaching 7 billion in 2012 and to feed this population there has been great advances in agriculture productivity partly via the development of pesticides and nitrate/phosphate based fertilisers. These activities have altered the geochemical cycling of elements, increasing or decreasing concentrations in earth system compartments and increasing global distribution (Doney, 2010). Anthropogenic activities have left a cumulative and lasting impression on the biosphere - so much so that geologist have termed the current epoch the anthropocene (Zalasiewicz et al 2010).

The increase in agricultural and industrial production and consumption of raw materials produces vast amounts of waste that enters the aquatic ecosystem. An acknowledgement of the decline in environmental quality due to contaminants has led to the development of environmental quality standards (EQS) in many countries and to assess if these standards are being adhered to many jurisdiction also have a programme of waste water effluent testing (WET) and/or biomonitoring. The EQS are derived from toxicity tests that use numerous organisms per compound, and to set standards several species are tested. In the USA approximately 3 million fish are used in WET procedures (reported in Tanneberger et al 2013). There is a move towards reducing the number of animals used in research and toxicology studies and there are a number of international initiatives aimed at investigating the 3Rs, reduction, replacement and refinement, in animal research (for example see http://www.nc3rs.org.uk/). Within the context of the need to determine EQS for new materials

and re-evaluating environmental risk posed by products already on the market under EU Registration, Evaluation, Authorisation and restriction of new CHemicals (REACH) regulations there is a desire to identify alternative methods for evaluating contaminant risk and hazards to help define better environmental regulations and for use for biomonitoring. A number of excellent reviews are available assessing the use of cell lines for toxicity testing (Bols et al 2005, Castano et al 2003, Segner, 2004, Schirmer, 2006). In this article we will review current primary gill cell culture techniques and the use of the cultured epithelium as a surrogate for an intact gill, in this context we will focus on the use of this system for regulatory and environmental monitoring and briefly compare this system to cell line alternatives.

The primary gill cell culture

The gill epithelium is a complex tissue comprising of a number of different cell types such as pavement, mitochondrial-rich and mucous cells and accounting for 50% of the body surface area (Wood, 2001). The cuboidal and squamous pavement cells make up most of the respiratory surface, interspersed with ovoid mucous cells (aka goblet cells) and the larger and columnar mitochondria-rich cells (aka chloride cells or ionocytes), which have several subtypes (Galvez et al 2002) and are important for inorganic ion transport. The gill is multifunctional, being the site of oxygen uptake and metabolic waste excretion, as well as ion uptake or extrusion (Evans 2005). It is constantly bathed in water and thus is also one of the first organs to be affected by water borne contaminants and is a major site of toxicant uptake. The importance of gill epithelium for fish health means that there is great interest in understanding branchial physiological processes and the response to toxicants and has seen a number of researchers look for suitable models to investigate branchial function (Wood et al 2002).

77 Pärt and colleagues (Pärt et al 1993) pioneered the development of a culture technique of
78 rainbow trout gill cells in multiwell dishes. The techniques have also been developed for
79 other species including freshwater tilapia, goldfish, puffer fish and Japanese eels, as well as
80 the marine fish Sea bass (see Table 1 for references). It is not until collaboration with Wood
81 in 1997 (Wood and Pärt, 1997) that the gill cells were cultured on permeable supports and
82 shown to generate a polarised epithelium that can withstand the application of water to the
83 apical surface (see Figure 1). At that time the seeding onto permeable supports was from a
84 single fish, and cells were initially cultured in a flask, trypsinised and then seeded onto the
85 insert, referred to single seeded inserts (SSI), this preparation contained only respiratory
86 pavement cells (Wood and Pärt 1997; Fletcher et al 2000). Direct seeding cells onto inserts
87 (SSDI) occasionally produced viable confluent inserts, but also lacked the mitochondrial rich
88 cells (Wood et al 2002) . In 2000 Fletcher and colleagues developed a novel double seeded
89 technique (double seeded inserts, DSI), where cells from one fish are seeded directly onto
90 inserts and after 24hrs the cells are washed and a cell preparation from a second fish are
91 placed on top. The reason for this is that the first seeding appears to provide a scaffold on
92 which mitochondrial rich cells are able to adhere and flourish (Figure 1). A tight epithelium is
93 formed in 6 – 14 days and as a pavement cells:mitochondrial rich cell ratio (85:15) similar to
94 that observed *in vivo* (Walker et al 2007).
95
96
97 The formation of tight junction between cells results in an increase in transepithelial
98 resistance (TER). TER of >1 kOhms is reached with SSI from rainbow trout, goldfish and
99 tilapia in symmetrical condition (L15 on both sides) after 6-14 days culture (Table 2). If the
100 apical medium is replaced with sterile water the TER increases reaching >10 kOhms. The
101 extent of TER formation is batch and season dependent. In general the TER for DSI
102 preparations are a lot higher in symmetrical conditions reaching~30 kOhms if compared to
103 values measured from SSI, and there is little change when water is added to the apical
104 compartment (Table 2). The exceedingly high TER is reflected in extremely low permeability

to ^{3}H-PEG-4000 (Table 2. In symmetrical conditions the transepithelial potential is positive (see Table 2) and is generated by either anion extrusion or cation uptake (Wood et al 2002). On exposure to water the TEP becomes negative (see Table 2) and is attributed to higher passive permeability to Na^+ and Cl^-. The tightness of the epithelium, changes in electrical potential across the membrane under different culture condition and evidence that the cell inserts respond differently whether water is added to the apical or basolateral compartment (a far greater increase in TERs when water is added apically) indicates a polarised epithelium grown (Wood et al 2002).

The initial premise of developing a culture system from the gill was to analyse active ion transporting properties and the use of this cultured epithelium for physiological studies has been reviewed by Wood et al (2002). The criterion for active ion uptake is a disagreement between the measured fluxs (apical → basolateral vs. basolateral → apical) and that predicted based on the Ussing flux ratio equation (Wood et al 2002). Under symmetrical condition (L15 in both apical and basolateral compartments) rainbow trout or tilapia SSI, that lack the mitochondrial rich cells show similar unidirectional influx and efflux rates with slight deviations from the Ussing flux ratio indicating non-diffusive uptake (Wood et al 1997; Gilmour et al 1998; Kelly and Wood, 2002a). In asymmetrical conditions there is evidence for a small active Cl^- influx (apical → basolateral) and an active efflux (basolateral → apical) of Na^+, (Wood and Pärt 1997; Kelly and Wood 2002a). The DSI react similarly to the SSI in response to asymmetrical conditions, but the active components of the Na^+ and Cl^- movements across the epithelium are only a very small percentage of the total movement of these ions (Wood and Pärt 1997; Gilmour et al 1998). In DSI, which contains mitochondrial rich cells there is an active influx of Ca^{2+} from water in asymmetrical condition, but there is also a large passive efflux (Fletcher et al 2000; Walker et al 2007). In an attempt to improve the response of the SSI or DSI to freshwater (e.g. an increase in active Na^+ or Cl^- influx) a number of studies have treated the cells with hormones, cortisol, thyroid hormone (T3) and prolactin, known to be involved in regulating ion transport in intact fish. However, these

133 treatments have had very little effect on active influx rates in asymmetrical conditions either
134 with SSI or DSI (Gilmour et al 1998; Kelly and Wood 2001a, 2002a,b) and suggests that
135 another stimulus is required to activate active ion uptake processes in these cells.
136 Interestingly, T3 on SSI and DSI and prolactin on DSI stimulates the activity of Na^+/K^+-
137 ATPase activity, an enzyme associated with the basolateral membrane and involved in the
138 transfer of Na^+ from the cell to the circulation in freshwater fishes (Kelly and Wood, 2001a,
139 2002a). Treatment of SSI or DSI with cortisol cause an increase in TER and a decrease in
140 membrane permeability, that is reflected in lower unidirectional Na^+ and Cl^- fluxes (Kelly and
141 Wood, 2002b). Kelly and colleagues have extensively studied the effect of hormones on
142 membrane permeability and tight junction formation in rainbow trout, goldfish and tilapia
143 membrane cell cultures (Chaositis et al 2010, 2011a,b; Kelly and Chaostis, 2011). They
144 have shown that hormones influence membrane permeability and integrity via the induction
145 of the expression of claudins, ZO-1 and occludin proteins that are integral in tight junction
146 formation (e.g. Günzel and Yu, 2013). The expression of these proteins decreases
147 membrane permeability and is an explanation where these hormones may decrease ion
148 efflux. The SSDI, SSI and DSI membranes from a number of species have been important
149 in identifying factors that influence membrane permeability and passive ion and ammonia
150 fluxes (Table 1). But, further work is required to identify the stimulus necessary to induce
151 active Na^+ and Cl^- uptake if this *in vitro* model is to be used to gain a better understanding of
152 ionoregulation in freshwater fish.
153
154 **Regulations – Environmental Risks**
155
156 Cell lines *vs.* Primary cells
157 Both cell lines and primary cell cultures have their advantages and disadvantages. Cell lines
158 are relatively easy to maintain, handle and can in theory be cultured indefinitely. But, re-
159 culturing over time may results in the cells losing some of the morphological and
160 physiological characteristics of the tissue from which they were first isolated. However, Lee

et al (2009) have reported the presence of pavement cells, mitochondrial rich cells and goblet cells in a cell line derived from the gills of rainbow trout (RTgill-W1). In contrast, the protocols for isolating primary cells are more complex and cells can only be kept for a limited period. However, in contrast to cell lines, primary cells generally maintain the physiological characteristics of the parent tissue better than cell lines.

Toxicity Tests

Acute toxicity tests (e.g. OECD test guidelines 203) identify the concentrations of contaminants that cause mortality, and the potency of a toxicant to cause mortality is often referred to as the LC or LD 10, 20 or 50 or LD50 (lethal concentration or dose that kills 10, 20, 50% of the population). Similarly, the No Observed Effect Concentration (NOEC) is the highest concentration tested without an effect and the Lowest Observed Effect Concentration (LOEC) the lowest concentration tested where mortality was observed. Chronic toxicity tests (e.g. OECD 229) can include mortality during a longer time of exposure, but also evaluate growth and/or reproduction as an endpoint from which EC or ED (effect concentration or dose) can be calculated. To assess the safety of a chemical to the environment toxicity tests are conducted on a limited number of organisms, typically a prokaryote, a plant, an invertebrate and a vertebrate (e.g. standard organisms in a battery of tests may include zebrafish to represent fish, *Daphnia magna*, to represent aquatic invertebrates and *Selenastram* to represent freshwater algae) and in well defined water. How jurisdictions use these data to set permissible environmental water quality standard to protect aquatic life vary slightly from region to region. But, the objective is to ensure that a concentration of a chemical does not exceed a certain threshold to cause mortality to none or only a few species. Basing water quality standards on values that cause 50% mortality to a species may appear ludicrous, but environmental risk assessments typically involve application of different safety factors, which may be very high if the assessment is based on acute LC50 data from only one or a few species and much lower if chronic toxicity data are available for a number of species. If data are abundant, the environmental risk assessment

189 can be further refined by building a species sensitivity distribution in which a statistical or
190 empirical distribution function is used to fit the proportion of species affected (e.g. NOEC,
191 LC50, EC50) as a function of stressor concentration or dose. From a species sensitivity
192 distribution plot it can then be possible to determine what percentage of species are affected
193 by any given concentration of the chemical. Depending on the quality of the data available,
194 e.g. whether it is acute or chronic data, whether the SSD plots are representative of a large
195 range of species and taxa, a further assessment (or safety) factor (e.g. 10 or 100 fold
196 decrease) is applied. This approach to environmental risk assessment is a good way of
197 ranking the hazard of chemicals, but a number of issues have been raised about the use of
198 results from laboratory toxicity tests to derive meaningful environmental water quality
199 standards and consequently, EQS is often under- or overprotective. Firstly, the tests are
200 often performed on organisms that have been cultured in the laboratory for a number of
201 generations and do not represent those in the wild. Secondly, the tests are performed in
202 well-defined clean water that are not representative of natural water. Thirdly, tests are
203 performed on single compounds, but seldom are organisms exposed to only one compound
204 - though a number of studies are now evaluating contaminant mixtures. Fourthly, the tests
205 are often performed on single species and do not take into account the complex interaction
206 within an ecosystem. The use of cell-cultures instead of animals in toxicity tests may not
207 necessarily solve this problem, but cell cultures have the potential to provide high-throughput
208 screens to allow more in-depth investigation of chemicals that are more likely to be
209 problematic, and they may also be used in biomonitoring (see below) which serves to detect
210 if chemicals in natural waters have effects on biota.
211
212 All new chemical products have to undergo environmental risk assessment, which includes
213 toxicity tests. For example, the OECD 203 acute fish test prescribed the use of between 42
214 and 60 fish per tests. There is a move towards reducing the use of animals in research and
215 toxicity testing and there are initiatives worldwide to investigate alternative methods that
216 replace, reduce or refine (3Rs) the use of animals in research. *In vitro* techniques offer an

alternative and a number of studies have compared the in vitro cytotoxicity of compounds to fish cell lines and compared this to in vivo acute toxicity (reviewed in Segner, 2004 and Schirmer, 2006). If acute toxicity is due to cytotoxicity, usually due to disruption to membrane integrity, then the in vitro results should be a good predictor of those obtained in vivo. Results often show a relatively good linear relationship, (with r^2 values of between 0.64 and 0.98 reported in Segner, 2004) and appear to be useful in ranking hazardous compounds, but more often than not fish cell culture systems underestimates the toxicity by up to 3 orders of magnitude (Table 3; See also reviews by Segner et al 2004; Schirmer, 2006). This makes extrapolation of in vitro results for environmental risk assessment difficult because it is difficult to derive a standard that is considered to be safe. A possible explanation for this disparity is that the mechanism behind what kills an organism is not always the same as that killing a cell. To overcome this a number of ideas have been proposed that uses cells derived from organs that are the site of toxicity and increasing the sensitivity by modifying the cell culture conditions to better mimic those *in vivo* (Schirmer, 2006). In a recent study, Tanneberger et al (2013) used RTgill-W1 cell line, derived from rainbow trout gill cells to screen 35 compounds of differing modes-of-action. They integrates 3 measures of cell cytotoxicity, Alamar Blue as a measure of metabolic activity, 5-carboxyflourescein diacetate acetoxymethyl ester (CFDA-AM) as a measure of cell membrane integrity and Neutral red as a measure of lysosomal membrane integrity. The data showed an improvement in predicted toxicity with only a 5-fold difference between EC50 and LC50 values for the compounds tests. Of those, the AChE inhibitors showed an even better correlation between EC50 and LC50 of 1.

Two major projects have evaluated the use of the primary gill cell culture system as an alternative for toxicity screening. Sandbacka and colleagues (1999) and Lilius and colleagues (1995) compared the responses of gills cells in primary culture, suspension or on membrane supports to reference chemicals from the multicentre evaluation of in vitro cytotoxicity (MEIC) project (Bondesson et al 1989). As a measure of cell viability cells in

culture or suspension where incubated with calcein-AM; the accumulated intracellular calcein-acetoxymethyl ester (AM) is cleaved by esterases resulting in increased fluorescence, thus if a chemical causes cell death there is a reduction in fluorescence. However, a confounding factor in this measure is that calcein-AM is also a substrate for ABC transporters that are present in culture gill cells (Fisher et al 2011) and will, if active, export the compound from the cell. Transepithelial resistance (TER) was used as a measure of membrane integrity in cell culture on inserts. The cells culture and suspension showed similar EC50 values for the compounds compared and a conclusion from the study was that gill cell suspensions could be used in a testing protocol to assess chemical hazards (Table 3). The results for the cells cultured on inserts were less encouraging, because TER is extremely variable between inserts. In addition, TER may not be a very reliable or sensitive endpoint for cell viability. Seldom have we observed a significant reduction in TER for DSI inserts exposed to metals resuspended in water in the laboratory (Walker, 2008) or to natural waters contaminated with metals (Minghetti et al unpublished results) for 24 hrs. Likewise, Zhou et al (2006) observed no change in TER on exposure to AhR agonist toxicants, thus, other endpoints maybe more appropriate as a measure of a toxic response.

In addition to these cell viability tests as a means of ranking hazard the primary gill cells can be used to assess the branchial uptake and metabolism of xenobiotics. This can provides a better understanding of the toxicokinetics of compounds as they cross the gill epithelium. Gill cells contain the phase one enzyme cytochrome P4501A, whose activity can be measured as 7-ethoxyresorufin-O-deethylase (EROD), and . Zhou et al (2006) compared the response of Nile tilapia DSI and primary hepatocyte cultures to 1,3,7,8-tetrachlorodibenzo-p-diozin (TCDD), benzo(a)pyrene (BaP), polychlorinated biphenyl (PCB) mixture (Aroclor 1254) and polybrominated diphenyl ether (PBDE) mixture (DE71). Both hepatocytes and DSI exhibited a good dose-response curve to TCDD, BaP and PCBs, but not PBDEs generating 24h EC50 values in the 10^{-6} to 10^{-9}M range. These results are as predicted because TCDD, BaP and several congeners in Aroclor 1254 are strong Ah Receptor agonists whereas the PBDEs in

DE71 are not. The increase in EROD was more rapid in DSI than in hepatocytes with induction being observed within 3hrs following exposure. Thus, EROD activity is probably not a good marker for PBDE toxicity, but it may be possible to devise surrogate toxicity endpoints to different classes of stressors using the power of genomics. Expression microarray analysis on exposed DSI has revealed unique transcript profiles that identify on- and off-target effects to different classes of compounds (e.g. diclofenac, clofibrate, ethinyl oestradiol, fluoxetine and triclosan), as well as to mixtures of EU priority substance (Schnell, Bury & Hogstrand unpublished data).

In the laboratory, the DSI responds in a similar way to intact animals to metals. For example, silver at environmentally relevant concentrations inhibits whole body Na^+ influx, an adverse outcome pathway (AOP) of acute metal toxicity to freshwater fish, and in exactly the same water conditions induces the expression of two isoforms of the cysteine rich metal binding protein metallothionein (MT-A and MT-B) in DSI (Table 3; Walker et al 2008). Altering water chemistry (dissolved organic carbon, chloride and sodium) alleviates whole organism toxicity and reduces DSI MT-A and B expression in a similar way (Walker et al 2008). Expression of these genes was induced by other metals Cd, Zn, and Pb (Walker et al 2008) and other genes known to be involved in metal transport (ZIP1, ZnT1) and antioxidant defence (GST, G6PD) were also elevated in response to Ag, Cu, Cd and Zn (Walker et al 2008). The pesticides atrazine and pentachlorophenol (PCP) did not induce MT expression, but in contrast paraquat and irgarol caused a moderate induction of both MTA and MTB, indicative of intracellular zinc release (Walker et al 2008). The metal-regulatory transcription factor 1 (MTF1) is an important factor mediating the response of a cell to metals and acts as an intracellular sensor of zinc (Colvin et al 2010). The zinc-MTF1 complex interacts with metal response elements (MRE) in the promoter of metal responsive genes. For example, the promoter region of rainbow trout MT-A possess 6 MREs 4 of which are arranged in tandem (Olsson et al, 1995) and MT-B possesses 4 MREs (Samson and Gedamu, 1995). Metals may displace Zn from metal binding sites within the cell increasing intracellular free zinc and

inducing MT expression. Other compounds, such as triclosan (Tamura et al., 2012) and potentially paraquat and irgarol (Walker et al., 2008), can cause an increase in the intracellular concentration of free zinc(II) and illicit gene expression via the MTF1 pathway.

Bioconcentration factors

EU REACH legislation requires companies to provide data on the environmental impact of substance that are produced in excess of 1 tonne. A regulatory requirement is to provide data on the persistence, bioaccumulative and toxicological (PBT) properties of compounds. The OECD 305 Fish Bioconcentration Factor test (BCF) assesses bioaccumulation of a compound from the water. The original test used 108 fish (de Wolf et al 2007) per chemical, but the OECD305 guidelines have recently been revised and the numbers of fish used per test has been reduced to 80 (OECD305). It was initially estimated that 30,000 chemicals may require re-evaluation, but this maybe a vast underestimate and the number of substance registered with the European Chemicals Agency (ECHA) by the 1st December 2008 deadline was 146,000 (Gubbels-van Hal and Pelkmans, 2009). Bioaccumulation test for each of these compounds would use a worryingly high number of animals and money, with current EU environmental testing market valued in excess of £500million. To reduce animal numbers and cost under REACH legislation BCF testing is only required for substances whose production exceeds 100 tonnes, or if the physiochemical properties suggest a lipophilic compound (logP >3), and further refinements to this methodology and reductions in numbers used have been suggested (de Wolf et al 2007). Based on an estimated 30,000 chemicals that requires re-evaluation ECHA estimates there are in excess of 1000 chemicals to be tested by OECD 305, which conservatively equates to 80,000 fish but this is now likely to be in excess of 380,000 given the 146,000 chemicals registered with ECHA (Gubbels-van Hal and Pelkmans, 2009). This will have a significant impact on the number of fish used for scientific research in the UK; Home Office statistics show that in 2011, 563,905,155 fish were used for scientific procedures, and 76,350 in toxicological

procedure. However, it must also be remembered that REACH is only applicable to the EU and other regions such as the US and Japan have their own requirements and evolving legislation. Worldwide there is a move towards a reduction in animals used in experiments thus reliable alternatives to animal toxicity tests, bioaccumulation studies and environmental monitoring are urgently needed.

Replacing the number of animals used in BCFs with *in* vitro and *in silico* methods would be desirable. Quantitative structure–activity relationship models (QSAR models) can be used to predict the potential of a chemical to bioaccumulate and bioconcentrate. However, QSAR models are only as good as the database on which they are built and without real experimental data on structurally related chemicals BCF calculations can be inaccurate. Experimental data on accumulation can potentially be generated with cell cultures, but identifying suitable cell culture endpoints is difficult because the current OECD 305 BCF test requires a period of accumulation to steady-state followed by a lengthy depurations period. With respect to uptake the cultured gill cells grown on semipermeable supports offers an alternative to whole animal studies. The cultured gill epithelium expresses a number of tight junctions proteins, such as the claudins and ZO-1 that enables the cells to forms a tight epithelium with a transepithelial electrical resistance in excess of 20KOhms (e.g Chasiotis et al 2010; Chasiotis and Kellt, 2011a; Kolosoc and Kelly, 2013). The claudins are a large superfamily of proteins that are anchored in the membrane and facilitate paracellular transport. However, the majority of bioaccumulation of organic compounds is likely transcellular. Usually, the uptake is directly proportionate the compound's log K_{ow} (octanol/water partition coefficient), a measure of lipohilicity. A number of studies have used parallel artificial membrane permeability assays (PAMPA) to determine the relationship between log K_{ow} and partitioning across a membrane. However, these artificial membranes lack transport proteins. A number of mammalian studies have identified active transport of drugs across membranes (Sugano et al 2010; Dobson and Kell, 2008) and indicated that the uptake rate of all compounds will be summation of that via passive diffusion or carrier

mediated processes. Recent, studies in our laboratory have shown that DSI preparations can be used as a surrogate for pharmaceutical uptake from the water into fish, in particular for propranolol (Stott et al unpublished data). Interestingly in concentration equilibrium transport assays (CETA) a proportion of uptake of propranolol from the apical to basolateral membrane was greater, demonstrating uptake against a concentration gradient. There are a number of solute carrier transport proteins (members of the SLCO1, SLCO2, SLC15, SLC22 and SLC47 families; reviewed by Dobson and Kell, 2008) that have been shown to transport drugs and a number of homologs to these transporters have been sequenced from fish species or identified in fish genomes (Meier-Abt et al. 2005; Romano et al. 2006; Verri et al. 2012). The expression pattern and regulation, as well as functional characterisation of these fish proteins is required to establish their ability to transport drugs and other xenobiotics across the gills from the water, as well as, interestingly, their natural physiological role.

The gills possess many of the enzymes involved in xenobiotic metabolism and transport proteins for the export of these metabolites. The transporters that mediate import of xenobiotics are collectively referred to as Phase 0 proteins (Figure 2). Lipophilic xenobiotic chemicals are then biotransformed to make them hydrophilic and easier to excrete. Biotransformation starts with Phase I enzymes, which often involves the family of cytochrome P450 enzyme that catalyse the mono-oxygenation reaction of a wide range of natural and synthetic compounds (Uno et al. 2012). Phase II reactions involves enzymes that catalyse sulfonation (sulfontransferases, SULT), acetylation (N -acetyltransferases), methylation (methyltransferases), glutathione conjugation (glutathione-s-transferase) and glucuronidation (glucuronidation) of the polar moieties of products from Phase I reactions. The xenobiotic conjugates from Phase II are exported from the cell by a family of proteins called the ATP binding cassette (ABC) transporters and there are 8 ABC transporter families in fish (Lončar et al. 2010). These transporters are involved in the Phase III detoxification process, extruding xenobiotics and their metabolites from the cell. Abcb1 (Pgp) is capable of exporting non-metabolised xenobiotics, whereas abcb11, abcc1-5, also known as the

multidrug resistant associated protein (MRP) and abcg2 export products of phase II metabolism (Xu et al. 2005). In the primary gill cells the cultured branchial epithelium possess the CYP450A1 enzymes necessary for xenobiotic biotransformation (Carlsson et al 1997, Zhou et al 2006; Leguen et al 2000; Jönsson et al 2006) and in the RT-gill-W1 cell line there is a high expression of abcc1, -2, -3 and -5 (Fischer et al 2011). However, in another study, only abcc3 was detected in gill tissues (Lončar et al 2010). The extent to which the primary gill cells retain functional ABC transporters remains to be ascertained. But the uptake and export of calcein-AM, a substrate for a number of the abc's (Fischer et al 2011), by cultured gill cells would suggest that these proteins are functional (Sandbacka et al 1999). This is significant if DSI are to be used to assess the toxicological significance of xenobiotic exposure via the water, because the biotransformation of xenobiotics as they cross the branchial will significantly alter their fate (Weisbrod et al 2009). However, the actual xenobiotic biotransforming properties of DSI cells and their capacity to transport these metabolites need to be determined.

Once a xenobiotic, or its metabolite, has crossed the branchial epithelium it enters the circulatory system. The liver is the main organ involved in xenobiotic metabolism and elimination in fish (Hinton et al 2001) and to a lesser extend the kidney (Larsen and Perkins, 2001). The use of piscine hepatocyte cultures for the study of xenobiotic metabolism has been extensively reviewed (Segner and Cravedi, 2001) and its use as an in vitro screen for fish bioaccumulation discussed (Weisbroid et al 2009). Isolated hepatocytes cultured on plates can last for up to 8 days (Segner, 1998) and possess phase I and II enzymes (Cravedi et al 2001). The metabolising profiles from cultures match those in vivo (Nishimoto et al 1992; Cravedi et al 2001), however, the metabolising activity declines as the culture ages (Cravedi et al 2001). Recent advances in piscine hepatocyte culture techniques have seen the development of a protocol to generate hepatocyte spheroids (Baron et al 2012). This technique has been adopted from mammalian studies where spheroids culture better reflect the architecture of the liver and retain the native organ's metabolising activities over a

longer period (Liu et al 2007), and build on early work that cultured piscine liver aggregates for up to 30 days (Flouriot et al 1993). The spheroid cultures take between 6 – 8 days to mature, but are viable for over a month making them suitable for toxicological studies (Baron et al 2012). Future developments whereby the DSI and liver spheroids are combined have the potential to be useful tool to assess waterborne xenobiotic uptake and internal biotransformation.

Biomonitoring

Sediment extracts

Risk assessment of sediments is a challenging task as sediments contain mixtures of pollutants of high complexity. Traditionally chemical analysis has been carried out to evaluate the risks of potentially contaminated sediments. However, chemical analysis alone does not provide any information about potential hazards to organisms as it is not realistic to identify and measure concentrations of all toxicants. Moreover, combined effects of pollutants in benthic organisms and their bioavailability are not considered (Chapman, 2007). On the other hand, *in vitro* bioassays using cultured cells have been shown to be very useful tools in order to characterize the environmental quality of sediments as they allow an estimation of the total biological activity of chemicals. Often, a combination of both, *in vitro* bioassay and chemical analysis are needed for the identification of substances causing an observed effect and their potential source (Brack and Schirmer 2003; Kinani et al 2010). However, there are several problems when assessing the quality of sediments. Sediment samples have to be processed to get them into a form which allows *in vitro* exposure (e.g. reconstitution of powdered culture media with sediment elutriates, concentrated organic sediments extracts etc.) (Davoren et al 2005). Due to this processing, difficulties arise when trying to associate observed effects with bioavailability of contaminants present in the sediments.

Despite this, several studies have shown the usefulness of fish cell cultures as screening tools for an evaluation of the risk arising from environmental samples. Davoren and colleagues (2005) assessed three different fish cell lines and their potential to serve as tools for cytotoxicity testing of estuarine sediment aqueous elutriates. The outcome was that out of the three tested cell lines, RTG-2, had the highest potential to serve as such a tool for screening of aqueous phases in terms of tolerating osmotic stress but further tests regarding sensitivity are required. The study by Kinani et al. (2010) showed that the hepatoma fish cell line PLHC-1 is a suitable model system to detect dixoin-like compounds in sediments which was also demonstrated in a study by Schnell et al. (2013) where controls sites could be clearly teased a part from contaminated sites. Strmac and Braunbeck (2000) exposed isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) to native river waters and sediment extracts from two small river systems. Significant differences in terms of toxic burden between both rivers as well as between free water phase and sediment were detected applying different endpoints focusing on cytological and biochemical changes. These examples illustrate how useful cell cultures systems-permanent cell lines and primary cells- can be to characterize the environmental quality of sediments.

Water samples

In the US over 3 million fish are used for waste effluent toxicity (WET) testing to assess whether end-of-pipe effluent affects the ability of organisms to survive grow and reproduce (reported in Tanneberger et al 2013). Thus there is a societal pressure to reduce the numbers of animals used for these tests and identify alternative strategies. A number of studies have used the rainbow trout gill cell line RTgill-W1 to test with water samples. These cells are unable to tolerate water on their apical surface and thus either the water has to go through an extraction process and subsequent resuspension of the extract in a solvent that can be administered to the cells, or the water is used to dilute double strength L15 media, or

466 salts, galactose and pyruvate are added to the water to maintain the media isoosmotic to the
467 cells. Dayeh et al (2002) utilised the later methods to assess the toxicity of 31 paper mill
468 effluents to the RTgill-W1 cells and compared the toxicity to a rainbow trout acute toxicity
469 test. Of the 31 sites only one was acutely toxic to rainbow trout and was also cytotoxic to the
470 cell line. A similar study using industrial effluent identified increased vacuolisation and
471 neutral red uptake in RTgill-W1 cells that was associated with elevated ammonia
472 concentrations (Dayeh et al 2009). These results demonstrate the possibility of using cell
473 lines for environmental monitoring.
474
475 Recent work in our laboratory has further developed this idea, but with the use of DSI of
476 primary gill cells. The reason for using this approach is because the DSI are able to tolerate
477 water on their apical surface and thus there is no need to amend the test media with
478 additional salts. In the laboratory, we have demonstrated that the DSI can be used to predict
479 metal toxicity (Walker et al 2007; 2008) and it was possible using microarray technology to
480 identify genes expressed to specific metals, but after having tested many different potential
481 biomarker genes MT still best reflects metal toxicity to fish (Walker et al 2007; 2008).
482 Following on from our laboratory observations, we evaluate the viability of taking the primary
483 gill cell culture to the field for environmental monitoring. For this purpose, we used the River
484 Hayle in Cornwall as an example. This river has a history of mine inputs that has resulted in
485 a polymetal pollution gradient (Cd, Cu, Zn, Ni) with little other contaminant input (Durrant et
486 al 2011). The cultured gill cells were transported (over 1000kms) in temperature controlled
487 Medi-boxes (over 30hrs) and exposed to the water directly taken from the river. The cells
488 survived the transport and exposure to natural river water. The water induced expression of
489 MT-A and B in the cells and we were able to demonstrate that this endpoint is a useful
490 indicator of bioreactive metals in natural waters. The limited number of sampling points and
491 a lack of dose-response data for individual metals made it difficult to determine the drivers of
492 MT expression in the River Hayle. But, within the complex metal mixture a greater
493 correlation between Zn, Cd and Ni concentration and MT-A expression and Cu and Ni

concentration and MT-B expression, suggesting isoform specific responses to metals (Minghetti et al 2013 unpublished data).

Other Applications and way forward

Multi – organ systems (Fish-on-a-chip)

Recent advancements in microengineering and microfluidic dynamics has seen the development of a number of human tissues-on-a-chip (e.g. lung (Huh et al 2010); kidney proximal tubule (Jang et al 2013); kidney renal tubular epithelium (Jang et al 2011); gut (Imura et al (2009); and liver (Lee et al 2007)). With respect to fish cells, Glawdel et al (2009) developed a microfluidic system integrated with an electroosmotic pump for the culture of RTgill-W1 cell line and showed using a cell viability assay that this system could be used for toxicity testing. These advancements utilise microengineered devises to form a structure that enables cells to grow in 3D to better mimic the organ structure and function. Static 2D cultures do not mimic the gradients in oxygen and shear generated by the circulatory systems that tissues within the body experience. As an example, Huh et al (2010) developed a human lung-on a-chip devise that is made of two silicone rubber poly(dimethyl-siloxane) (PDMS) channels separated by a 10µm extracellular matrix gel (ECM) covered membrane with engineered pores at the right size to mimic the alveolar –capillary interface (Huh et al 2011). PDMS is a compound that has revolutionised the developed of microfabricated devices for cell culture, because it is relatively cheap and pliable, importantly it allows for gas exchange and is optically transparent, and ECM gels provide the scaffold on which cells adhere and grow into 3D structures. Two lung cell types, alveolar and capillary endothelial cells are co-cultured on either side of the membrane. Once they are confluent the alveolar cell chamber is exposed to an air-liquid interface and lung capillary endothelial cells microvascular chamber to microfluid dynamics mimics that of blood flow in the capillaries. The ingenuity of this devise is that it also mimicked the rhythmic breathing by applying

suction to an adjacent chamber. This enhanced the production of surfactants by the alveolar cells and maintained the integrity cultured cell membrane. From a toxicological perspective, this device has been used to assess the effects of nanoparticels on the lung. Exposure of the alveolar chamber to 12nm silica particles demonstrated cross-talk between the two cell types and the induction of an endothelial inflammatory response in the microvascular chamber. The exposure also induced the capture of neutrophiles by the endothelial cells that facilitated the uptake of the particles. Reactive oxygen species (ROS) generation on exposure to silica or Cs/Se quantum dots was only induced in cells when breathing was mimicked and this action also enhanced the acute inflammatory response.

The technology has advanced further with the prospect of developing a human-on-a-chip (Huh et al 2011), where different cell types are cultured on one microdevice that are linked by a microfluidic circulatory system. In a two chamber system linking cultures of liver and lung cells, the exposure of the liver cells to naphthalene, which is converted to the toxic metabolite 1,2-naphthalenediol and 1,2-naphthoquinone, induced glutathione depletion in the lung 'tissue' (Viravaidya et al 2004). The advancements in such devices in human cell culture arena came about because of the recognition that animal models where often unsuitable for research into human drug development and toxicity studies. The research has demonstrated that mimicking the organ 3D structure and circulatory system greatly enhances the performance of these cultures. The challenge to construct an equivalent Fish-on-a-chip device will require further research to develop a biomimetic microsystem that includes many of cell cultures that represent the organs within a fish.

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References

Airaksinen, S., Råbergh, C.M.I., Sisrone, L. And Nikinmaa, M. (1998). Effects of heat shock and hypoxia on protein synthesis in trainbow trotu (*Oncorhynchus mykiss*) cell. *J. exp. Biol*. **201**, 2543 -2551.

Avella, M., Pärt, P and Ehrenfeld, J. (1999). Regulation of Cl^- secretion in seawater fish (*Dicentrarchus labrax*) gill respiratory cells in primary culture. *J. Physiol*, **516.2**, 353—363.

Babich, H. and Borenfreund E. (1987). Polycyclic aromatic hydrocarbons in vitro cytotoxicity to bluegill BF-2 cells: Mediation by S-9 microsomal fraction and temperature. *Toxicol. Lett*. **36**, 107-116.

Babich, H., Puerner, J.A. and Borenfreund, E. (1986). In vitro cytotoxicity of metals to Bluegill (BF-2) cells. *Arch. Environ. Contam. Toxicol*. **15**, 31-37.

Babín, M.M. and Tarazona, J.V. (2005). In vitro toxicity of selected pesticides on RTG-2 and RTL-W1 fish cell lines. *Environ. Pollut*. **135**, 267-274.

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

Bols, N.C., Dayeh, V.R., Lee L.E.J. and Schirmer K (2005). Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. Biochemistry and Molecular Biology of Fishes, vol. 6T. P. Mommsen and T. W. Moon (Editors)

Bondesson, I., Ekwall, B., Hellberg, S., Romert, L., Stenberg, K. and Walum, E. (1989) MEIC- a new international multicenter project to evaluate the relevance to human toxicity of in vitro cytotoxicity tests. *Cell. Biol. Toxicol*. **5,** 331-347.

Brack, W. and Schirmer, K. (2003). Effect-directed identification of oxygen and sulphur heterocycles as major polycyclic aromatic cytochrome P4501A-inducers in a contaminated sediment. *Environ. Sci. Technol*. **37**, 3063-3070.

Brandão, J.C., Bohets, H.H.L., van de Vyver, I.E. and Dierickx, P.J. (1992). Correlation between the in vitro cytotoxicity to cultured fathead minnow fish cells and fish lethality data for 50 chemicals. *Chemosphere* **25**, 553-562.

Bui, P., Bagherie-Lachidan, M. and Kelly, S.P. (2010) Cortisol differentially alters claudin isoforms in cultured puffer fish gill epithelia*. Mol. Cell. Endocrinol*. **317**, 120–126.

Bury, N.R., Chung, M. Walker, P. Sturm, A. and Hogstrand, C. (2008)**.** Cortisol stimulates the zinc signalling pathway and expression of metallothioneins and ZnT1 in epithelial cells. *Am. J. Physiol*. **294**, R623-R629.

Butler, R. and Nowak, B.F. (2004) A dual enzyme method for the establishment of long and medium-term primary cultures of epithelial and fibroblastic cells from Atlantic salmon gills. *J. Fish Biol*. **65**, 1108–1125.

Caminada, D., Escher, C. and Fent, K. (2006). Cytotoxicity of pharmaceuticals found in aquatic systems: Comparison of PLHC-1 and RTG-2 fish cell lines. *Aquat. Toxicol*. **79**, 114-123.

Caminada, D., Zaja, D., Smital, T. and Fent, K. (2008). Human pharmaceuticals modulate Pgp1 (ABCB1) transport activity in the fish cell line PLHC-1. *Aquat. Toxicol*. **90**, 214-22.

Carlsson, C. and Pärt, P. (2001) 7-Ethoxyresorufin *O*-deethylase induction in rainbow trout gill epithelium cultured on permeable supports: asymmetrical distribution of substrate metabolites. *Aquat. Toxicol*. **54**, 29–38.

Carlsson, C., Pärt, P. and Brunström (1999) 7-Ethoxyresorufin *O*-deethylase induction in cultured gill epithelial cells from rainbow trout. *Aquat. Toxicol.* **47,** 117–128.

Castaño, A., Cantarino, M.J., Castillo, P. and Tarazona, J.V. (1996). Correlations between the RTG-2 cytotoxicity test EC50 and in vivo LC50 rainbow trout bioassay. *Chemosphere* **32**, 2141-57.

Castaño, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee, L.E.J., Mothersill, C., Pärt, P., Repetto, G., Sintes, J.R., Rufli, H. Smith, R., Wood, C., and Segner, H (2003). The use of fish cells in ecotoxicology - The report and recommendations of ECVAM workshop 47. *ATLA* **31**, 317-351.

Chasiotis, H., Wood, C.M. and Kelly, S.P. (2010) Cortisol reduces paracellular permeability and increases occludin abundance in cultured trout gill epithelia. *Mol. Cell. Endocrinol.* **323**, 232–238.

Chasiotis, H. and Kelly, S.P. (2011a).Permeability properties and occludin expression in a primary cultured model gill epithelium from the stenohaline freshwater goldfish. *J Comp. Physiol. B* **181**, 487–500.

Chasiotis, H. and Kelly, S.P. (2011b).Effect of cortisol on permeability and tight junction protein transcript abundance in primary cultured gill epithelia from stenohaline goldfish and euryhaline trout. *Gen. Comp. Endocrinol*. **172**, 494–504.

Chasiotis, H., Kolosov, D. and Kelly, S.P. (2012) Permeability properties of the teleost gill epithelium under ion-poor conditions. *Am. J. Physiol.* **302**, R727–R739.

Chapman, P.M. (2007). Determining when contamination is pollution-Weight of evidence determinations for sediments and effluents. *Environmental International* **33**, 492-501.

Chow, S.C. and Wong, C.K.C (2011). Regulatory function of hyperosmotic stress-induced signalling cascades in the expression of transcription factors and osmolyte transporters in freshwater Japanese eel primary gill cell culture. *J. exp. Biol*. **214**, 1264-1270

Colvin, R.A., Holmes, W.R., Fontaine, C.P. and Maret, W. (2010). Cytosolic zinc buffering and muffling: Their role in intracellular zinc homeostasis. *Metallomics **2***, 306-317.

Cravedi, J.P., Boudry, G., Baradat, M., Rao, D., and Debrouwer, L. (2001) Metabolic fate of 2,4-dicghloraniline, prochloraz and nonylphenol diethoxylate in rainbow trout: a comparative in vitro/in vivo approach. *Aquat. Toxicol*. **53**, 159-172.

Davoren, M., Ni Shuilleabhain, S., Hartl, M.G.J., Sheehan, D., O'Brien, N.M., O'Halloran, J., Van Belt, F.N.A.M., and Mothersill, C. (2005). Assessing the potential of fish cell lines as tools for the cytotoxicity testing of estuarine sediment aqueous elutriates. *Toxicol. In Vitro* **19**, 421-431.

Dayeh, V.R., Schirmer, K. and Bols, N.C. (2002) Applying whole-water samples directly to fish cell cultures in order to evaluate the toxicity of industrial effluent. *Wat. Res*. **36**, 3727-3738.

Dayeh, V.R., Schirmer, K. and Bols, N.C. (2009) Ammonia-containing Industrial Effluents, Lethal to Rainbow Trout, Induce Vacuolisation and Neutral Red Uptake in the Rainbow Trout Gill Cell Line, RTgill-W1. *ATLA* **37**, 77-87

de Wolf, W., Comber, M., Douben, P. Gimeno, S., Holt, M., Leonard, M., Lillicrap, A., Sim, D., van egmond, R., Weisbrod, A and Whale, G. (2007). Animal use replacement, reduction and refinement: Development of an integrated testing strategy for bioconcentration of chemicals in fish. *Integr. Environ. Assess. Manage*. **3**, 3-17. 5807-5813.

Dobson, P.D. and Kell, D.B. (2008). Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nature Reviews Drug Discovery* **7**, 205-220.

Doney, S.C. (2010). The growing human footprint on coastal and open ocean biochemistry. *Science* **328**, 1512-1516.

Duranton, C., Mikulovic, E., Tauc, M., Avella, M. and Poujeol, P. (2000). Potassium channels in primary cultures of seawater fish gill cells. I. Stretch-activated K1 channels *Am. J. Physiol.* **279**: R1647–R1658.

Duranton, C., Mikulovic, E., Tauc, M., Avella, M. and Poujeol, P. (2000). Potassium channels in primary cultures of seawater fish gill cells. II. Channel activation by hypotonic shock. *Am. J. Physiol.* **279**: R1659–R1670.

Durrant, C., Stevens, J.R., Hogstrand, C. and Bury, N.R. (2011). The effect of metal pollution on the population genetic structure of brown trout (*Salmo trutta* L.) residing in the River Hayle, Cornwall, UK. *Environ. Poll.* **159**, 3595-3603.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005) The Multifunctional Fish Gill : Dominant Site of Gas Exchange , Osmoregulation , Acid-Base Regulation , and Excretion of Nitrogenous Waste*. Physiol. Rev.* **85**, 97–177

Farkas, J., Christian, P., Gallego-Urread, J.A., Roose, N., Hassellövd, M., Tollefsen, N.E. and Thomas, K.V. (2011) Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. *Aquat. Toxicol.* **101**, 117–125.

Fischer, S., Loncar, J., Zaja, R., Schnell, S., Schirmer, K., Smital, T. and Luckenbach, T. (2011). Constitutive mRNA expression and protein activity levels of nine ABC efflux transporters in seven permanent cell lines derived from tissues of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **101**, 438-446

Fletcher, M., Kelly, S.P., Pärt, P., O'Donnell, M.J., and Wood C.M. (2000) Transport proterties of cultured branchial epithelia from freshwater rainbow trout: A novel preparation with mitochondria-rich cells. *J. Exp. Biol* **203,** 1523-1537.

Flouriot, G., Vaillant, C., Salber, G., Pelissero, C., Guriaud, J.M. and Valotaire, Y. (1993) Monolayer and aggregate cultures of rainbow trout hepatocytes: Long-term and stable liver-specific expression in aggregates *J. Cell Sci***. 105**, 407-416.

Galvez, F., Reid, S.D., Hawking, G. and Goss, G.G. (2002). Isolation and characterization of mitochondrion-rich cell types from the gills of rainbow trout. *Am. J. Physiol.* **282,** R658-R668.

Galvez, F., Donini, A., Playle, R.C., Smith, D.S. O'Donnell, M.J. and Wood. C.M. (2008a). A matter of potential concern: Natural organic matter alters the electrical potential of fish gills**.** *Environ. Sci. Technol***. 42,** 9385-9390.

Galvez, F., Tsui, T., and Wood C.M. (2008b)**.** Cultured trout gill epithelia enriched in pavement cells or in mitochondria-rich cells provides insights into Na^+ and Ca^{2+} transport. *In vitro Cell Dev An* **44**, 415-425.

Gilmour, K.M., Pärt, P., Prunet, P., Pisam, M., McDonald, D.G. and Wood, C.M. (1998). Permeability and morphology of a cultured branchial epithelium from rainbow trotu during prolonged exposure to freshwater. *J. Exp. Zool*. **281**, 531-545.

Glawdel, T., Elbuhen, C., Lee, L.E.J. and Ren, C.C. (2009). Microfluidic system with integrated electroosmotic pumps, concnetration gradient generator and fish cell line (Rtgill-W1) – towards water toxicity tests. *Lab Chip* **9**, 3243-3250.

Gubbels-van Hal, I., & Pelkmans, J. (2009). Is REACH going well. Centre for European Policy Studies Policy Brief No 198/November 2009, available from http://www.ceps.eu.

Günzel D. and Yu, A.S. (2013). Claudins and the modulation of tight junction permeability. *Physiol Rev*. **93**, 525-569.

Hansen, H.J.M., Kelly, S.P., Grosell, M., and Wood, C.M (2002) Studies on lipid metabolism in trout (*Oncorhynchus mykiss*) branchial cultures. *J Exp Zool* **293**, 683-692.

Hinton, D.E., Segner, H. and T. Braunbeck (2001). Toxic Responses of the liver. In *Target Organ Toxicity in Marine and Freshwater Teleosts* (Eds D. Schlenk & W.H. Benson), pp 224-268 Taylor & Francis, London, UK.

Huh, D., Mathtews, B.D., Mammoto, A., Montoya-Zavala, M., Hsin, H.Y. and Ingber, D.E. (2010). Reconstituting organ-level lung function on a chip. *Science* **328**, 1662-1668.

Huh, D., Hamilton, G.A. and Ingber, D.E. (20110). From 3D cell culture to organs-on-chips. *Trends Cell Biol*. **21**, 745-754.

Jang, K-J. and Suh, K.Y. (2011). A multi-layer microfluidic device for efficient culture and analysis of renal tubular cells. *Lap Chip* **10**, 36-42.

Jang, K-J., Mehr, A.P., Hamilton, G.A., McPartlin, L.A., Chung, S., Suh, K-Y. and Ingber, D.E. (2013). Human kidney proximal tubulte-on-a-chip for drug transport and nephrotoxicity assessment. *Integr. Biol* DOI: 10.1039/c3ib40049b.

Jönsson, M.E., Carlsson, C., Smith, R.W. and Pärt, P (2006). Effects of copper on CYP1A activity and epithelial barrier properties in the rainbow trout gill. *Aquat. Toxicol*. **79**, 78–86.

Imura, Y., Asano, Y., Sato, K. and Yoshimura, E.A. (2009) microfluidic system to evaluate intestinal absorption. *Anal Sci.* **25**, 1403-1407.

Kelly, S.P., Fletcher, M., Pärt, P. and Wood, C.M. (2000). Procedures for the preparation and culture of 'reconstructed'rainbow trout branchial epithelia. *Methods in Cell Science* **22**, 153–163.

Kelly, S.P. and Wood, C.M. (2001a). The physiological effects of 3,5*,3*-Triiodo-L-thyronine alone or combined with cortisol on cultured pavement cell epithelia from freshwater rainbow trout gills. *Gen. Comp. Endocrinol*. **123,** 280–294.

Kelly, S.P. and Wood, C.M. (2001b). The cultured branchial epithelium of the rainbow trout as a model for diffusive fluxes of ammonia across the fish gill. *J. Exp. Biol*. **204**, 4115–4124

Kelly, S.P. and Wood, C.M. (2001c). Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills. *Am. J. Physiol*. **281**, R811-R820.

Kelly, S.P. and Wood, C.M. (2002a). Prolactin effects on cultured pavement cell epithelia and pavement cell plus mitochondria-rich cell epithelia from freshwater rainbow trout gills. *Gen. Comp. Endocrinol*. **128,** 44-56.

Kelly, S.P. and Wood, C.M. (2002b). Cultured gill epithelia from freshwater tilapia (*Orechormis niloticus*): Effect of cortisol and homologous serum supplements from stressed and unstressed fish. *J. Membrane Biol*. **190,** 29-42.

Kelly, S.P. and Wood, C.M. (2003). Dilute culture media as an environmental or physiological simulant in cultured gill epithelia from freshwater rainbow trout. *In vitro Cell Dev B* **39**, 21-28

Kelly, S.P. and Wood, C.M. (2008). Cortisol stimulates calcium transport across cultured gill epithelia from freshwater rainbow trout. I*n vitro Cell Dev An* **44**, 96-104.

Kelly, S.P. and Chasiotis, H. (2011) Glucocorticoid and mineralocorticoid receptors regulate paracellular permeability in a primary cultured gill epithelium. *J. Exp. Biol*. **214**, 2308-2318

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J.-M., and Ait-Aissa, S. (2010). Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. *Environ. Poll. 158*, 74-83.

Knauer, K., Lampert, C. and Gonzales-Valero, J. (2007). Comparison of in vitro and in vivo acute fish toxicity in relation to toxicant mode of action. *Chemosphere* **68**, 1435-1441.

Kolosov, D. And Kelly, S.P. (2013). A role for tricellulin in the regulation of gill epithelium permeability. *Am J Physiol* **204**, R1139-R1148.

Krøvel, A.V., Softland, L., Tortensen, B. and Olsvik, P.A. (2008). Transcriptional effects of PFOS in isolated hepatocytes from Atlantic salmon Salmo salar L. *Comp. Biochem. Phys. C.* **148**, 14-22.

Larsen, B.K. and Perkins, E.J. (2001). Target organ toxicity in the kidney. In *Target Organ Toxicity in Marine and Freshwater Teleosts* (Eds D. Schlenk & W.H. Benson), pp 90 -150 Taylor & Francis, London, UK.

Laville, N., Ät-Ässa, S., Gomez, E., Casellas, C. and Porcher, J.M. (2004). Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. *Toxicology* **196**, 41-55.

Lee, L.E.J., Dayeh, V.R., Schirmer, K. and Bols, N.C. (2009). Applications and potential uses of fish gill cell lines: examples with RTgill-W1. *In Vitro Cell.Dev.Biol*. **45**, 127–134

Lee, P.J., Hung, P.J. and Lee L.P. (2007) An artificial liver sinusoid with a microfluidic endothelial-like barrier for primary hepatocyte culture. *Biotechnol Bioeng*. **97**, 1340-1346.

Leguen, I., Carlsson, C., Perdu-Durand, E., Prunet, P., Pärt, P. And Cravedi, J.P. (2000). Xenobiotic and steroid biotransformation activities in rainbow trout gill epithelial cells in culture. *Aquat. Toxicol*. **48**, 165–176

Leguen, I., Peron, S. and Prunet, P. (2011). Effects of iron on rainbow trout gill cells in primary culture. *Cell Biol Toxicol* **27**:311–319

Lilius, H., Sandbacka, M. and Isomaa, B. (1995). The use of freshly isolated gill epithelial cells in toxicity testing. *Toxciol. In Vitro* **9**, 299-305.

Liu, J., Kuznetsova, L.A., Edwards, G.O., Xu, J., Ma, M., Purcell, W.M., Jackson, S.K., Coakley, W.T. (2007). Functional three-dimensional HepG2 aggregate cultures generated from an ultrasound trap: comparison with HepG2 spheroids. *J. Cell Biochem*. **102**, 1180-1189.

Lončar, J., Popović, M., Zaja, R. and Smital, T. (2010). Gene expression analysis of teh ABC efflux transporters in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.* **151C**, 209-215.

Meier-Abt, F., Mokrab, Y. and Mizuguchi, K. (2005) Organic anion transporting polypeptides of teh OATP/SLCO superfamily: Identification of new members in nonmammalian species, comparative modelling and a potential transport mode. *J. Membr. Biol.* **208,** 213-227.

Nishomoto, M., Yanagida, G.K., Stein, J.E., Baird, W.M. and Varanasi, U. (1992). The metabolism of benzo[*a*]pyrene by English sole (*Parophyrs vetulus*): Comparison between isolated heapocytes in vitro and liver in vivo. *Xenobiotica* **22**, 949-961.

OECD Guidelines for testing chemicals. Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure (2012). www.oecd-library.org

O'Donnell, M.J.O., Kelly, S.P., Nurse, C.A. and Wood, C.M. (2001). A maxi Cl- channel in cultured pavement cells from the gills of the freshwater rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol* **204**, 1783–1794

Olsson, P.E.; Kling, P.; Erkell, L.J.; and Kille, P. (1995) Structural and functional analysis of the rainbow trout (*Oncorhyncus mykiss*) metallothionein-A gene. *Eur. J. Biochem*. **230**, 34924-34928.

Pichardo, S., Jos, A., Zurita, J.L., Salguero, M., Cameán, A.M. and Repetto, G. (2007). Acute and subacute toxic effects produced by microcystin-YR on the fish cell lines RTG-2 and PLHC-1. *Toxicol. In Vitro* **21**, 1460-1467.

Pärt, P., Norrgren, L., Bergström, and Sjöberg, P. (1993). Primary culture of epithelial cells from rainbow trout gills. *J. exp. Biol.* **175**, 219–232 .

Romano, A., Kottra, G., Barca, A., Tiso, N., Maffia, M., Argenton, F., Daniel, H., Storelli, C. and Verri, T. (2006) High-affinity peptide transporter PEPT2 (slc15a2) of the zebrafish *Danio rerio*: functional properties, genomic organization and expression analysis. *Physiol. Genom.* **24**, 207-217.

Romøren, K. Fjeld, X.T.L., Pole´, A.B.S., Smistad, G., Thu, B.J. and Evensen Ø. (2005). Transfection efficiency and cytotoxicity of cationic liposomes in primary cultures of rainbow trout (*Oncorhynchus mykiss*) gill cells. *Biochim Biophys Acta* **1717**, 50 – 57.

Saito, H., Koyasu, T., Yoshida, K., Shigeoka, T. and Koike, S. (1993). Cytotoxicity of 109 chemicals to goldfish GSF cells and relationships with 1-octanol/water partition coefficients. *Chemosphere* **26**, 1015-1028.

Samson, S.L. and Gedamu, L. (1995) Metal-responsive elements of the rainbow trout metallothionein-B gene function for basal and metal-induced activity. *J. Biol. Chem*. **270**, 6864 – 6871.

Sánchez-Fortún, S., Llorente, M.T. and Castaño, A. (2005). Genotoxic effects of selected biocides on RTG-2 fish cells by means of a modified Fast Micromethod Assay. *Aquat. Toxicol*. **73**, 55-64.

Sandbacka, M., Pärt, P. and Isomaa, B. (1999). Gill epithelial cells as tools for toxicity screening—comparison between primary cultures, cells in suspension and epithelia on filters. *Aquat. Toxicol*. **46**, 23–32.

Sandbichler, A.M., Farkas, J. Salvenmoser, W. and Pelster, B. (2011a). Cortisol affects tight junction morphology between pavement cells of rainbow trout gills in single-seeded insert culture. *J. Comp. Physiol. B* **181**, 1023–1034.

Sandbichler, A.M., Egg, M., Schwerte, T., and Pelster, B. (2011b). Claudin 28b and F-actin are involved in rainbow trout gill pavement cell tight junction remodeling under osmotic stress. *J exp Biol* **214**, 1473-1487.

Schnell , S., Bols, N.C., Barata, C. and Porte, C. (2009). Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. *Aquat. Toxicol*. **93,** 244-252**.**

Schnell, S, Olivares, A., Piña, B., Echarvarri-Erasun, B., Lacorte, S. and Porte, C. (2013). The combined use of the PLHC-1 cell line and the recombinant yeats assay to assess the environmental quality of estuarine and coastal sediments. **Mar. Pollut. Bull.** ahead of print http://dx.doi.org/10.1016/j.marpolbul.2013.09.047

Schirmer, K. (2006). Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicology*, **224**, 163-183.

Segner, H. (1998). Isolation and primary culture of telesotean hepatocytes. *Comp. Biochem. Physiol*. **120A**, 71-81.

Segner, H. (2004). Cytotoxicity assays with fish cells as an alternative to the acute lethality test with fish. *ATLA* **32**, 375-382.

Segner, H., and Cravedi, J.P. (2001) Metabolic activity in primary cultures of fish hepatocytes. *Altern. Lab. Anim*. **29**: 251-257.

Shahsavarani, A., McNeill, B., Galvez, F., Wood, C.M., Goss, G.G., Hwang, P.P. and Perry, S.F. (2006). Characterization of a branchial epithelial calcium channel (ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*). *J exp Biol* **209**, 1928-1943.

Smith, R.W., Jönsson, M., Houlihan, D.F. and Pärt, P. (2001). Minimising aerobic respiratory demands could form the basis to sub-lethal copper tolerance by rainbow trout gill epithelial cells *in vitro. Fish Physiol. Biochem*. **24**, 157–169

Strmac, M. and Braunbeck, T. (2000). Isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*) as a tool to discriminate between differently contaminated small river systems. *Toxicol. In Vitro* **14**, 361-377.

Sugano, K., Kansy, M., Artursson, Avdeef, A., Bedels, S., Di, L., Ecker, G.F., Faller, B., Fischer, H., Gerebtzoff, Lennernaes, H. and Senner, F. (2010). Coexistence of passive and carrier-mediated processes in drug transport. *Nature Reviews Drug Discovery* **9**, 597-614.

Tamura, I., Kanbara, Y., Saito, M., Horimoto, K., Satoh, M., Yamamoto, H. and Oyama Y. (2012). Triclosan, an antibacterial agent, increases intracellular Zn(2+) concentration in rat thymocytes: its relation to oxidative stress. *Chemosphere* **86:**70-75.

Tanneberger, K., Knöbel, M., Busser, F.J., Sinnige, T.L., Hermens, J.L. and Schirmer K. (2013). Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environ Sci Technol*. **47**, 1110-1119.

Tse, W.K.F., Chow S.C. and Wong C.K.C. (2008). The cloning of eel osmotic stress transcription factor and the regulation of its expression in primary gill cell culture. *J. Exp. Biol.* **211**, 1964-1968.

Uno, T., Ishizuka, M., and Itakura, T. (2012). Cytochrome P450 (CYP) in fish. *Environ. Toxicol. Pharmacol.* **34**, 1-13.

Verri, T., Terova, G., Romano, A., Barca, A., Pisani, P., Storelli, C. and Saroglia, M. (2012). The SoLute Carrier (SLC) family series in teleost fish: In: *Functional Genomics in Aquacutlure*. (Eds. Sargolia, M & A. Liu) pp X-X. John Wiley & Sons.

Viravaidya K., Sin A. and Shuler, M.L. (2004). Development of a microscale cell culture analog to probe naphthalene toxicity. *Biotechnol Prog*. **20**, 316-323.

Visoottiviseth, P. and Chanwanna, N. (2001). A newly established cell culture from hybrid catfish (*Clarias gariepinus* x *Claria macrocephalus*) for screening toxicity of triphenyltin hydroxide. *Appl. Organometallic Chem*. **15**, 463 – 472.

Walker, P.A., Bury, N.R. and Hogstrand, C. (2007). Influence of culture conditions on metal-induced responses in a cultured rainbow trout gill epithelium. *Environ. Sci. and Technol.* **41**, 6505-6513

Walker P.A. , P. Kille, A. Scott, N.R. Bury. and C. Hogstrand. (2008). An in vitro method to assess toxicity of waterborne metals to fish. *Toxicol. Appl. Pharmacol.* **30**, 67-77.

Weisbrod, A.V., Sahi, J., Segner, H., James, M.O., Nichols, J., Schultz, I., Erhardt, S., Cowan-Ellsberry, C., Bonnell, M., and Hoeger, B. (2009). The state of in vitro science for use in bioaccumaulation assessments for fish. *Environ. Toxicol. Chem* **28**, 86-96.

Wise Sr, J.P., Goodale, B.C., Wise, S.S., Craig, G.A., Pongan, A.F., Walter, R.B., Douglas Thompson, W., Ah-Kau, N., AbouEl-Makarim, A., Mitani, H., Spalding, M.J. and Mason, M.D. (2010). Silver nanospheres are cytotoxic and genotoxic to fish cells. **Aquat. Toxicol**. 97, 34-41.

Wood, C.M. and Pärt, P. (1997). Cultured branchial epithelia from freshwater fish gills. *J. Exp. Biol*. **200,** 1047–1059.

Wood, C.M. and Pärt, P (2000). Intracellular pH regulation and buffer capacity in CO_2/HCO_3^- buffered media in cultured epithelial cells from rainbow trout gills. *J Comp Physiol B* **170**, 175 -184.

Wood, C.M. (2001). Toxic Responses of the gill. In *Target Organ Toxicity in Marine and Freshwater Teleosts* (Eds D. Schlenk & W.H. Benson), pp1-89 Taylor & Francis, London, UK.

Wood, C.M., Eletti, B. and Pärt, P. (2002). New methods for the primary culture of gill epithelia from freshwater rainbow trout. *Fish Physiol. Biochem.* **26,** 329–344.

Wood, C.M. Kelly, S.P., Zhou, B., Fletcher, M., O'Donnell, M., Eletti, B. And Pärt, P. (2002) Cultured gill epithelia as models for the freshwater fish gill. *Biochim. Biophys. Acta* **1566**, 72– 83.

Xu, C., Li, C.Y. and Kong, A.N. (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharmacol. Res.* **28**, 249–268.

Zalasiewicz, J., Willimas, M., Steffen, W. and Crutzen, P. (2010). The new world of the anthropocene. *Environ. Sci. Technol.* **44,** 2228-2231.

Zhou, B.S., Kelly, S.P., Ianowski, J.P., and Wood, C.M. (2003) Effects of cortisol and prolactin on Na^+ and Cl^- transport in cultured branchial epithelia from FW rainbow trout*. Am J Physiol* **285**, R1305-R1316.

Zhou, B., Nichols, J., Playle, R.C. and Wood, C.M. (2005). An in vitro biotic ligand model (BLM) for silver binding to cultured gill epithelia of freshwater rainbow trout (*Oncorhynchus mykiss*). *Toxicol. Appl. Pharmacol.* **202**, 25– 37

Zhou, B., Liu, C., Wang, J., Lamb P.K.S. and Wu, R.S.S. (2006). Primary cultured cells as sensitive *in vitro* model for assessment of toxicants-comparison to hepatocytes and gill epithelia. *Aquat. Toxicol*. **80**, 109–118

910 Table 1. Summary of published primary gill cell culture research

Author	Species and primary cell culture technique	Summary of study
Pärt et al (1993)	Rainbow trout - flasks	First trout gill cell culture methodology
Wood and Pärt (1997)	Rainbow trout – SSI	First SSI - Morphology, TER, TEP epithelium permeability, ion flux
Gilmour et al (1998)	Rainbow trout – SSI	Apical application of water, morphology and permeability
Airaksinen et al (1998)	Rainbow trout - flasks	Effect of heat and hypoxia on protein synthesis
Avella et al (1999)	Sea bass– SSI	Vasotocin and isoproterenol effect on Cl^- secretion
Carlsson et al (1999)	Rainbow trout – wells	βNF; TCDD and B[k]F on EROD activity
Sandbacka et al (1999)	Rainbow trout – cell suspension, wells, SSI	Toxicity test screening
Fletcher et al (2000)	Rainbow trout -DSI	First methods for DSI. TER, membrane permeability, ion transport and Na/K-ATPase activity
Duranton et al (2000a)	Sea bass - wells	K+- channel activation by hypotonic shock
Duranton et al (2000b)	Sea bass – wells	Stretch activated K+ channels
Leguen et al (2000)	Rainbow trout – flasks	βNF and TCDD on EROD activity
Wood and Pärt (2000)	Rainbow trout – wells and cover slips	Intracellular pH regulation
Visottiviseth and Chanwanaa (2001)	Hybrid catfish - wells	Triphenyltin hydroxide toxicity
Carlsson and Pärt (2001)	Rainbow trout - SSI	βNF; TCDD and B[k]F on EROD activity
Smith et al (2001)	Rainbow trout – SSI	Effect of copper on protein synthesis and O_2 consumption
Kelly and Wood (2001a)	Rainbow trout – SSI	3,5,3-Triiodo-L-thyronine (T3) and cortisol on TER, permeability, ion transport and Na/K-ATPase activity
Kelly and Wood (2001b)	Rainbow trout – DSI	Ammonia fluxes
O'Donnell et al (2001)	Rainbow trout – wells	Patch clamp of maxi Cl^- channel
Kelly and Wood (2001c)	Rainbow trout - SSI	Cortisol and membrane permeability
Kelly and Wood (2002a)	Rainbow trout – SSI & DSI	Prolactin on TER, membrane permeability, ion transport and Na/K-ATPase activity.
Kelly and Wood (2002b)	Tilapia – SSI	Cortisol and tilapia serum on TER, membrane permeability, ion transport and Na/K-ATPase activity
Hansen et al (2002)	Rainbow trout - DSI	Lipid metabolism
Wood et al (2002)	Rainbow trout - DSI	Double seeding techniques development
Kelly and Wood (2003)	Rainbow trout -SSI	Effects of diluting media on membrane property
Zhou et al (2003)	Rainbow trout - DSI	Cortisol and prolactin on TER, membrane permeability, ion transport and Na/K-ATPase activity
Butler and Nowak (2004)	Atlantic Salmon – wells	Establishment of salmon gill cell line (RGE2)
Zhou et al (2005)	Rainbow trout – DSI	Ag binding and uptake rates
Romøren et al (2005)	Rainbow trout – wells	Cationic liposome transfection
Shahsavarani et al (2006)	Rainbow trout – SSI & DSI	Calcium uptake
Jönsson et al (2006)	Rainbow trout – wells & SSI	Effect of Cu on βNF induced EROD activity
Zhou et al (2006)	Tilapia DSI	TCDD, B[a]P; PCB and PBDE toxicity test
Walker et al (2007)	Rainbow trout - DSI	Metal induction of metallothionein and Ca^{2+} fluxes
Walker et al (2008)	Rainbow trout- DSI	Metal induced gene expression
Galvez et al (2008a)	Rainbow trout- DSI	Effect of organic matter on TEP
Kelly and Wood (2008)	Rainbow trout -DSI	Effects of cortisol on calcium transport
Bury et al (2008)	Rainbow trout DSI	Zinc/cortisol interactions and MT expression
Galvez et al (2008b)	Rainbow trout	Cultures with enriched pavement or chloride cells
Tse et al (2008)	Japanese Eel -wells	Regulation of the osmotic stress transcriptional factor
Bui et al (2010)	Puffer fish– wells	Effect of cortisol on claudin expression
Chasiotis et al (2010)	Rainbow trout – SSI	Effect of cortisol on occludin expression and permeability
Sandbichler et al (2011)	Rainbow trout – SSI	Morphology and effect of cortisol and membrane permeability
Sandbichler et al (2011)	Rainbow trout – SSI	Claudin 28b expression: osmotic stress and cortisol
Farkas et al (2011)	Rainbow trout - DSI	Silver nanoparticle uptake and cytotoxicity
Leguen et al (2011)	Rainbow trout – wells	Iron toxicity
Chasiotis and Kelly (2011a)	R. Trout and Goldfish -SSI	Cortisol effect on permeability, claudin and ZO-1 expression
Chasiotis and Kelly (2011b)	Goldfish –SSI	Occludin expression and membrane permeability
Chow and Wong (2011)	Japanese Eel – wells	hyperosmotic stress-induced signalling cascades
Kelly and Chasiotis (2011)	Rainbow trout -SSI	Effect of GCs and MCs agonists and antagonists on paracellular permeability
Chasiotis et al (2012)	Goldfish - SSI	Effect of ion poor water on epithelium permeability, and expression of tight junction proteins and use of siRNA
Kolosov and Kelly (2013)	Rainbow trout -SSI	Properties of the Tight junction protein tricellulin

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912 SSI – single seeded inserts; DSI – double seeded inserts; βNF - β-Napththoflavone; TCDD - 2,3,7,8-Tetrachlorodibenzo-*p*-
913 dioxin; B(k)F - benzo[k]fluoranthene; EROD - 7-Ethoxyresorufin *O*-deethylase; B[*a*]P - . benzo(a)pyrene; PCB- polychlorinated
914 biphenyl;PBDE - polybrominated diphenyl ether (PBDE); MT – metallothionein; GC – glucocorticoids; MC – mineralocorticoid.
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Table 2. Summary of properties of primary gill cell cultures on inserts and the gill cell line

	Culture technique	TER	TEP	Permeability cm s-1 x10-7	Report cells present
Rainbow Trout					
Wood and Pärt (1997)	SSI	Sym ~ 3.5 Asym ~ 15	Sym ~ 0 Asym ~-35	Sym 1.7 - 5	PVC
Fletcher et al (2000)	SSI	Sym 1.2 - 21	Sym – + 0.4		PVC
	DSI	Sym 1.3 -34	Sym +1.9 Asym - -10.3	Sym 0.47 Asym 1.01	PVC, MRC
Carlsson and Pärt (2001)	SSI	Asy - ~8 - 17			
Smith et al (2001)	SSI	Asym - ~6-17			
Kelly and Wood (2001a)	SSI	Sym ~ 18.2 Asym ~31	Sym +2.77 Asym - -1.2	Sym ~ 1.1 Asym ~ 2	
Kelly and Wood (2001b)	DSI	Sym 3.5 Asym ~ 4.2	Sym - +2.4		
Kelly and Wood (2001c)	SSI	Sym- 1.3 Asym – 5.8	Sym - +.0.95 Asym - -12.69	Sym ~ 4.5 Asym ~ 9.2	
Kelly and Wood (2002a)	SSI & DSI	Sym 3.97 Sym 15.28	Sym 0 Asym -9.25	Sym 6	
Zhou et al (2003)	DSI	Sym – 34 Asym – 23	Sym - +32-45 Asym -7.54		
Zhou et al (2005)	DSI	Asym ~25	Asym ~ -5		
Shahsavarani et al (2006)	SSI				PVC
	DSI				PVC, MRC
Jönsson et al (2006)	SSI	Asym 7.5 - 9		3.8 – 5.8	
Walker et al (2007)	DSI	Sym 34 Asym 30	Asym -14.2		
Walker et al (2008)	DSI				
Kelly and Wood (2008)	DSI	Sym 32.84 Asym 19.96	Sym - +20.47 Asym – 6.24		
Galvez et al (2008b)	DSI	Asym ~30	Sym ~ 5 Asym ~ -11		PVC, MRC
Chasiotis et al (2010)	SSI	Sym 2.9 Asym 10		Sym 22 Asym 50	
Sandbichler et al (2011a)	SSI	Sym 6.8 Asym ~ 15			PVC
Sandbichler et al (2011b)	SSI	Sym 10			
Farkas et al (2011)	DSI	Sym >30			
Kelly and Chasiotis (2011)	SSI	Sym 2 - 9		Sym 1.8 – 4	
Kolosov and Kelly (2013)	SSI	Sym~ 4		Sym 4.5	
Goldfish					
Chasiotis and Kelly (2011a)	SSI	Sym ~ 1.15 Asym 4.2		Sym 4.8 Asym 7.3	PVC, MRC
Chasiotis and Kelly (2011b)	SSI	Sym 0.8 – 1.8		Sym 3.5 – 5.2	
Chasiotis et al (2012)	SSI	Sym ~ 1.2		Sym ~ 5.3	
Tilapia					
Kelly and Wood (2002b)	SSI	Sym 1.8 Asym 18.6	Sym 0 Asym -13.42	Sym 5 Asym 3.2	
Zhou et al (2006)	DSI	Sym 5-8 Asym 12-15			
Sea Bass					
Avella et al (1999)	SSI	Sym 5	Sym + 28		
Rainbow trout gill cell line					
Lee et al (2009)	RTgill-W1 Monolayer				PVC, MRC, goblet-like cells

Note – values are from control treatments in each paper; Sym = symmetrical condition (media:media), Asym = asymmetrical conditions (water:media), SSI = single seeded inserts, DSI double seeded inserts. TER = Transepitehlial electrical resistance, TEP = transepithelial epithelial potential, PVC = pavement cells, MRC = mitochondrial rich cells

923 Table 3. Examples of different endpoints and fish cell models used to evaluate toxicity of
924 chemicals.

Toxicity endpoint	Chemical	Cell Model	In vitro/In vivo comparison	Reference
Cell viability (NR)	18 Metal salts	BF-2	Strong correlation for cationic metals r=0.83; cell line up to 2 orders of magnitude less sensitive for cationic metals; cell line more sensitive towards chromate	Babich et al., 1986
Cell viability (NR)	18 organic pollutants	BF-2	Strong correlation: r=0.98 (ex. 2,4 dinitrophenol); cell line up to 3 orders of magnitude less sensitive	Babich & Borenfreund, 1987
Cell viability (MTT); Biotransformation (EROD Inhibition)	9 Human pharmaceuticals	PLHC-1, primary rainbow trout hepatocytes (PRTH),		Laville et al., 2004
Cell viability (NR)	18 Plant protection Products	PLHC-1	Lower sensitivity of the in vitro assay	Knauer et al., 2007
Efflux inhibition (P-glycoprotein activity)	33 Human pharmaceuticals	PLHC-1		Caminada et al., 2008
Cell viability (MTT)	21 Human pharmaceuticals	PLHC-1, RTG-2	No correlation found (PLHC-1 vs. fish) r=0.48	Caminada et al., 2006
Cell viability (NR, Protein content)	Microcystin-YR	PLHC-1, RTG-2		Pichardo et al., 2007
Cell viability (MTT, NR)	6 Pesticides	RTG-2, RTL-W1		Babín & Tarazona, 2005
Genotoxicity	2 Biocides	RTG-2		Sánchez-Fortún et al., 2005
Cell viability (NR, ATP content, Cell detachment,)	16 chemicals of different classes	RTG-2	Strong correlation ($r \geq 0.97$); cell line 1 to 2 orders of magnitude less sensitive	Castaño et al., 1996
Cell viability (NR)	50 chemicals	FHM	Strong correlation: r=0.89 (ex. outlier); cell line up to 3 orders of magnitude less sensitive	Brandão et al., 1992
Cell viability (AB, CFDA-AM)	11 Human pharmaceuticals	RTL-W1		Schnell et al., 2009
Genotoxicity	Silver nanospheres	OLHNI2		Wise Sr. et al., 2010
Cell viability	109 chemicals of different classes	GFS	Strong correlation (log/log: r=0.96); cell line 1 order of magnitude less sensitive	Saito et al., 1993
Transcriptomics (apoptosis, P450 and Phase II enzymes, lipid metabolism and ion-regulation)	Perfluoroctane sulfonate (PFOS)	primary Atlantic salmon hepatocytes		Krovel et al., 2008
Cell viability (Calcein-AM)	30 MEIC chemicals [a]	Hepatocytes and gill epithelia cells in suspension (both from rainbow trout)	Primary hepatocytes: weak correlation r=0.72; Gill cells in suspension: strong correlation r= 0.85; cells in suspension 1 to 2 orders of magnitude less sensitive	Lilius et al., 1995
Transcriptomics (MT)		Primary rainbow trout gill cells grown as a DSI epithelium (FiGCS)	Expression of metallothionein predicted zinc and silver toxicity in a number of water compositions; Sensitivity similar to that *in vivo.*	Walker et al., 2008

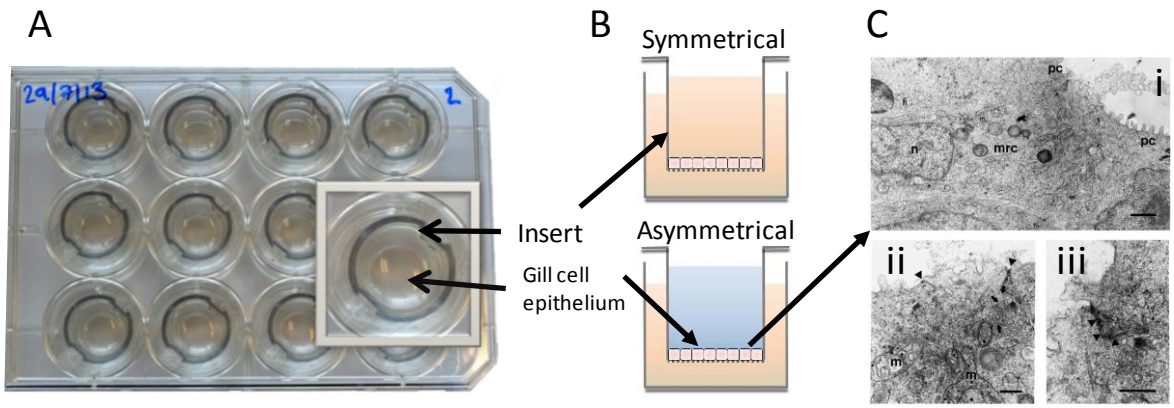
925 BF-2: Bluegill sunfish; PLHC-1: Topminnow liver; RTG-2: Rainbow trout gonad; RTL-W1: Rainbow trout liver; FHM: Fathead
926 minnow; OLHNI2: Medaka fin; GFS: Goldfish scale; FiGCS: Fish Gill Cell System; NR: Neutral Red; AB: Alamar Blue; CFDA-
927 AM: carboxyfluorescein diacetate acetoxymethylester; [a] MEIC=Multicenter Evaluation of *in vitro* cytotoxicity.

Figure Legends

Figure 1. (A) Multiwell cell culture plate with inserts containing semipermeable supports used to culture gill cell epithelia. The picture shows double seeded inserts (DSI) of rainbow trout gill cells that have developed high transepithelial resistance and are ready for experimentation. Arrows show the location of the insert and the epithelium in the magnified detail. (B) Representation of a cross view of a DSI in symmetrical (media:media) and asymmetrical (water:basolateral conditions. (C) Transmission electron micrographs of (i) a mitochondria-rich cell in a cultured double-seeded insert (DSI) epithelium; (ii) a magnified portion of the apical area of the mitochondria-rich cell (apical exposure delineated by arrowheads, and branching tubular system indicated by curved arrows); and (iii) a tight junction (indicated by arrowheads) between the mitochondria-rich cell and an adjacent pavement cell (m, mitochondrion; mrc, mitochondria-rich cell; n, nucleus; pc, pavement cell). Scale bars: (A) 1 mm; (B,C) 400 nm. Adapted with kind permission from Journal of experimental Biology (Fletcher et al., 2000)

Figure 2. Overview of xenobiotic biotransformation in in a generic cell, including Phase I and Phase II enzymatic processes and transporters in Phase 0 and Phase III. Abbreviations: X = xenobiotic; CYPs = Cytochrome P450 family of proteins; X-OH = hydroxylated xenobiotic; X-O-R = Xenobiotic conjugated with hydrophilic species, such as glutathione, sulfate, glycine, acetyl-group or glucuronic acid; ROS = Reactive Oxygen Species; Cat = Catalase; SOD = Superoxide Dismutase; T = Transporter. Phase 0 Transporters include SLCO1, SLCO2, SLC15, SLC22 and SLC47; Phase III transporters include multidrug resistance-associated proteins and other abc transporters.

954 Figure 1



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958 Figure 2

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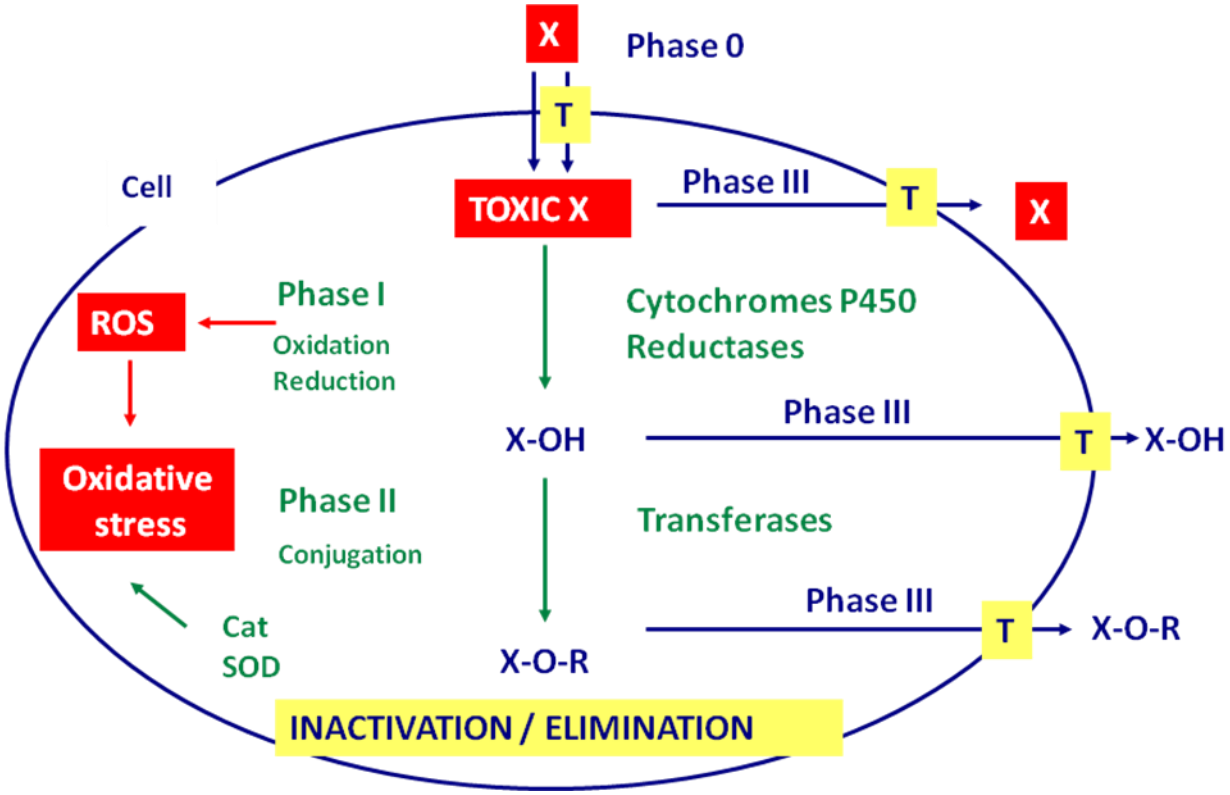

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