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).
Pärt and colleagues (Pärt et al 1993) pioneered the development of a culture technique of rainbow trout gill cells in multiwell dishes. The techniques have also been developed for other species including freshwater tilapia, goldfish, puffer fish and Japanese eels, as well as the marine fish Sea bass (see Table 1 for references). It is not until collaboration with Wood in 1997 (Wood and Pärt, 1997) that the gill cells were cultured on permeable supports and shown to generate a polarised epithelium that can withstand the application of water to the apical surface (see Figure 1). At that time the seeding onto permeable supports was from a single fish, and cells were initially cultured in a flask, trypsinised and then seeded onto the insert, referred to single seeded inserts (SSI), this preparation contained only respiratory pavement cells (Wood and Pärt 1997; Fletcher et al 2000). Direct seeding cells onto inserts (SSDI) occasionally produced viable confluent inserts, but also lacked the mitochondrial rich cells (Wood et al 2002). In 2000 Fletcher and colleagues developed a novel double seeded technique (double seeded inserts, DSI), where cells from one fish are seeded directly onto inserts and after 24hrs the cells are washed and a cell preparation from a second fish are placed on top. The reason for this is that the first seeding appears to provide a scaffold on which mitochondrial rich cells are able to adhere and flourish (Figure 1). A tight epithelium is formed in 6 – 14 days and as a pavement cells:mitochondrial rich cell ratio (85:15) similar to that observed in vivo (Walker et al 2007).

The formation of tight junction between cells results in an increase in transepithelial resistance (TER). TER of >1 kOhms is reached with SSI from rainbow trout, goldfish and tilapia in symmetrical condition (L15 on both sides) after 6-14 days culture (Table 2). If the apical medium is replaced with sterile water the TER increases reaching >10 kOhms. The extent of TER formation is batch and season dependent. In general the TER for DSI preparations are a lot higher in symmetrical conditions reaching~30 kOhms if compared to values measured from SSI, and there is little change when water is added to the apical 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 fluxes (apical $\rightarrow$ basolateral vs. basolateral $\rightarrow$ 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 $\rightarrow$ basolateral) and an active efflux (basolateral $\rightarrow$ apical) of Na$^+$, (Wood and Pärт 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 Pärт 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
treatments have had very little effect on active influx rates in asymmetrical conditions either with SSI or DSI (Gilmour et al 1998; Kelly and Wood 2001a, 2002a,b) and suggests that another stimulus is required to activate active ion uptake processes in these cells.

Interestingly, T3 on SSI and DSI and prolactin on DSI stimulates the activity of Na⁺/K⁺-ATPase activity, an enzyme associated with the basolateral membrane and involved in the transfer of Na⁺ from the cell to the circulation in freshwater fishes (Kelly and Wood, 2001a, 2002a). Treatment of SSI or DSI with cortisol cause an increase in TER and a decrease in membrane permeability, that is reflected in lower unidirectional Na⁺ and Cl⁻ fluxes (Kelly and Wood, 2002b). Kelly and colleagues have extensively studied the effect of hormones on membrane permeability and tight junction formation in rainbow trout, goldfish and tilapia membrane cell cultures (Chaostis et al 2010, 2011a,b; Kelly and Chaostis, 2011). They have shown that hormones influence membrane permeability and integrity via the induction of the expression of claudins, ZO-1 and occludin proteins that are integral in tight junction formation (e.g. Günzel and Yu, 2013). The expression of these proteins decreases membrane permeability and is an explanation where these hormones may decrease ion efflux. The SSDI, SSI and DSI membranes from a number of species have been important in identifying factors that influence membrane permeability and passive ion and ammonia fluxes (Table 1). But, further work is required to identify the stimulus necessary to induce active Na⁺ and Cl⁻ uptake if this in vitro model is to be used to gain a better understanding of ionoregulation in freshwater fish.

Regulations – Environmental Risks

Cell lines vs. Primary cells

Both cell lines and primary cell cultures have their advantages and disadvantages. Cell lines are relatively easy to maintain, handle and can in theory be cultured indefinitely. But, re-culturing over time may results in the cells losing some of the morphological and 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 Selenastrum 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
can be further refined by building a species sensitivity distribution in which a statistical or empirical distribution function is used to fit the proportion of species affected (e.g. NOEC, LC50, EC50) as a function of stressor concentration or dose. From a species sensitivity distribution plot it can then be possible to determine what percentage of species are affected by any given concentration of the chemical. Depending on the quality of the data available, e.g. whether it is acute or chronic data, whether the SSD plots are representative of a large range of species and taxa, a further assessment (or safety) factor (e.g. 10 or 100 fold decrease) is applied. This approach to environmental risk assessment is a good way of ranking the hazard of chemicals, but a number of issues have been raised about the use of results from laboratory toxicity tests to derive meaningful environmental water quality standards and consequently, EQS is often under- or overprotective. Firstly, the tests are often performed on organisms that have been cultured in the laboratory for a number of generations and do not represent those in the wild. Secondly, the tests are performed in well-defined clean water that are not representative of natural water. Thirdly, tests are performed on single compounds, but seldom are organisms exposed to only one compound - though a number of studies are now evaluating contaminant mixtures. Fourthly, the tests are often performed on single species and do not take into account the complex interaction within an ecosystem. The use of cell-cultures instead of animals in toxicity tests may not necessarily solve this problem, but cell cultures have the potential to provide high-throughput screens to allow more in-depth investigation of chemicals that are more likely to be problematic, and they may also be used in biomonitoring (see below) which serves to detect if chemicals in natural waters have effects on biota.

All new chemical products have to undergo environmental risk assessment, which includes toxicity tests. For example, the OECD 203 acute fish test prescribed the use of between 42 and 60 fish per tests. There is a move towards reducing the use of animals in research and toxicity testing and there are initiatives worldwide to investigate alternative methods that 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-acetoxy methyl 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-dioxin (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^-9M 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
genres 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 $K_{\text{ow}}$ (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 $K_{\text{ow}}$ 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 dioxin-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
salts, galactose and pyruvate are added to the water to maintain the media isoosmotic to the cells. Dayeh et al (2002) utilised the later methods to assess the toxicity of 31 paper mill effluents to the RTgill-W1 cells and compared the toxicity to a rainbow trout acute toxicity test. Of the 31 sites only one was acutely toxic to rainbow trout and was also cytotoxic to the cell line. A similar study using industrial effluent identified increased vacuolisation and neutral red uptake in RTgill-W1 cells that was associated with elevated ammonia concentrations (Dayeh et al 2009). These results demonstrate the possibility of using cell lines for environmental monitoring.

Recent work in our laboratory has further developed this idea, but with the use of DSI of primary gill cells. The reason for using this approach is because the DSI are able to tolerate water on their apical surface and thus there is no need to amend the test media with additional salts. In the laboratory, we have demonstrated that the DSI can be used to predict metal toxicity (Walker et al 2007; 2008) and it was possible using microarray technology to identify genes expressed to specific metals, but after having tested many different potential biomarker genes MT still best reflects metal toxicity to fish (Walker et al 2007; 2008). Following on from our laboratory observations, we evaluate the viability of taking the primary gill cell culture to the field for environmental monitoring. For this purpose, we used the River Hayle in Cornwall as an example. This river has a history of mine inputs that has resulted in a polymetal pollution gradient (Cd, Cu, Zn, Ni) with little other contaminant input (Durrant et al 2011). The cultured gill cells were transported (over 1000kms) in temperature controlled Medi-boxes (over 30hrs) and exposed to the water directly taken from the river. The cells survived the transport and exposure to natural river water. The water induced expression of MT-A and B in the cells and we were able to demonstrate that this endpoint is a useful indicator of bioreactive metals in natural waters. The limited number of sampling points and a lack of dose-response data for individual metals made it difficult to determine the drivers of MT expression in the River Hayle. But, within the complex metal mixture a greater 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 nanoparticles 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.

Acknowledgement

This work was supported by a research grant, G1000081, from the National Centre for the
Replacement, Refinement & Reduction of Animal in Research (NC3R) to Prof Hogstrand and
Dr Bury.


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

<table>
<thead>
<tr>
<th>Author</th>
<th>Species and primary cell culture technique</th>
<th>Summary of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood and Pär (1997)</td>
<td>Rainbow trout - SSI</td>
<td>First SSI - Morphology, TER, TEP epithelium permeability, ion flux</td>
</tr>
<tr>
<td>Carlsson et al (1999)</td>
<td>Rainbow trout – wells</td>
<td>βNF; TCDD and B(k)F on EROD activity</td>
</tr>
<tr>
<td>Duranton et al (2000a)</td>
<td>Sea bass - wells</td>
<td>K⁺ channel activation by hypotonic shock</td>
</tr>
<tr>
<td>Duranton et al (2000b)</td>
<td>Sea bass – wells</td>
<td>Stretch activated K⁺ channels</td>
</tr>
<tr>
<td>Wood and Pär (2000)</td>
<td>Rainbow trout – wells and cover slips</td>
<td>Intradiluting media on membrane property</td>
</tr>
<tr>
<td>Visottiviseth and Chanwanan (2001)</td>
<td>Rainbow trout - SSI</td>
<td>Triphenyltin hydroxide toxicity</td>
</tr>
<tr>
<td>Carlsson and Pär (2001)</td>
<td>Rainbow trout - SSI</td>
<td>βNF; TCDD and B(k)F on EROD activity</td>
</tr>
<tr>
<td>Smith et al (2001)</td>
<td>Rainbow trout – SSI</td>
<td>Effect of copper on protein synthesis and O₂ consumption</td>
</tr>
<tr>
<td>Kelly and Wood (2001a)</td>
<td>Rainbow trout – SSI</td>
<td>3,5,3-Triiodo-L-thyronine (T₃) and cortisol on TER, permeability, ion transport and Na/K-ATPase activity</td>
</tr>
<tr>
<td>Kelly and Wood (2001b)</td>
<td>Rainbow trout – DSI</td>
<td>Ammonia fluxes</td>
</tr>
<tr>
<td>Kelly and Wood (2001c)</td>
<td>Rainbow trout - SSI</td>
<td>Cortisol and membrane permeability</td>
</tr>
<tr>
<td>Kelly and Wood (2002a)</td>
<td>Rainbow trout – SSI &amp; DSI</td>
<td>Prolactin on TER, membrane permeability, ion transport and Na/K-ATPase activity</td>
</tr>
<tr>
<td>Kelly and Wood (2002b)</td>
<td>Rainbow trout - DSI</td>
<td>Cortisol and tilapia serum on TER, membrane permeability, ion transport and Na/K-ATPase activity</td>
</tr>
<tr>
<td>Galvez et al (2008a)</td>
<td>Rainbow trout - DSI</td>
<td>Effect of organic matter on TEP</td>
</tr>
<tr>
<td>Galvez et al (2008b)</td>
<td>Rainbow trout</td>
<td>Cultures with enriched pavement or chloride cells</td>
</tr>
<tr>
<td>Sandbiclerhler et al (2011)</td>
<td>Rainbow trout – SSI</td>
<td>Claudin 28b expression; osmotic stress and cortisol</td>
</tr>
<tr>
<td>Chasiotis and Kelly (2011a)</td>
<td>R. Trout and Goldfish -SSI</td>
<td>Cortisol effect on permeability, claudin and ZO-1 expression</td>
</tr>
<tr>
<td>Chasiotis and Kelly (2011b)</td>
<td>Goldfish -- SSI</td>
<td>Oclunin expression and membrane permeability</td>
</tr>
<tr>
<td>Chow and Wong (2011)</td>
<td>Japanese Eel - wells</td>
<td>Hyperosmotic stress-induced signalling cascades</td>
</tr>
<tr>
<td>Kelly and Chasiotis (2011)</td>
<td>Rainbow trout - SSI</td>
<td>Effect of GCs and MGs agonists and antagonists on paracellular permeability</td>
</tr>
<tr>
<td>Chasiotis et al (2012)</td>
<td>Goldfish - SSI</td>
<td>Effect of ion poor water on epithelium permeability, and expression of tight junction proteins and use of siRNA</td>
</tr>
<tr>
<td>Kolosov and Kelly (2013)</td>
<td>Rainbow trout - SSI</td>
<td>Properties of the Tight junction protein triclinulin</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Culture technique</th>
<th>TER</th>
<th>TEP</th>
<th>Permeability cm s⁻¹ x10⁻⁷</th>
<th>Report cells present</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rainbow Trout</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wood and Pär't (1997)</td>
<td>SSI</td>
<td>Sym ~ 3.5</td>
<td>Sym ~ 0</td>
<td>Sym 1.7 - 5</td>
</tr>
<tr>
<td>Fletcher et al (2000)</td>
<td>SSI</td>
<td>Sym 1.2 - 21</td>
<td>Sym + 0.4</td>
<td>Sym 0.47</td>
</tr>
<tr>
<td>Carlsson and Pär't (2001)</td>
<td>SSI</td>
<td>Sym ~ 3.5</td>
<td>Sym +1.9</td>
<td>Sym 0.47</td>
</tr>
<tr>
<td>Smith et al (2001)</td>
<td>SSI</td>
<td>Sym + 0.4</td>
<td>Asym - 1.2</td>
<td>Asym 1.01</td>
</tr>
<tr>
<td>Kelly and Wood (2001a)</td>
<td>SSI</td>
<td>Asym ~ 5.8</td>
<td>Asym - 35</td>
<td>Asym ~ 5.8</td>
</tr>
<tr>
<td>Kelly and Wood (2001b)</td>
<td>DSI</td>
<td>Sym ~ 3.5</td>
<td>Sym ~ 0</td>
<td>Sym 6</td>
</tr>
<tr>
<td>Kelly and Wood (2001c)</td>
<td>SSI</td>
<td>Sym ~ 3.1</td>
<td>Sym + 0.95</td>
<td>Sym ~ 5.4</td>
</tr>
<tr>
<td>Kelly and Wood (2002a)</td>
<td>SSI</td>
<td>Sym 3.97</td>
<td>Sym 0</td>
<td>Sym 6</td>
</tr>
<tr>
<td>Zhou et al (2003)</td>
<td>DSI</td>
<td>Sym ~ 1.3</td>
<td>Sym + 0.95</td>
<td>Sym ~ 5.4</td>
</tr>
<tr>
<td>Zhou et al (2005)</td>
<td>DSI</td>
<td>Sym ~ 1.3</td>
<td>Sym + 0.95</td>
<td>Sym ~ 5.4</td>
</tr>
<tr>
<td>Shahsavaran et al (2006)</td>
<td>SSI</td>
<td>Sym ~ 1.3</td>
<td>Sym + 0.95</td>
<td>Sym ~ 5.4</td>
</tr>
<tr>
<td>Kelly and Wood (2008)</td>
<td>DSI</td>
<td>Sym 32.94</td>
<td>Sym + 0.4</td>
<td>Sym 6.24</td>
</tr>
<tr>
<td>Galvez et al (2008b)</td>
<td>DSI</td>
<td>Sym 32.94</td>
<td>Sym + 0.4</td>
<td>Sym 6.24</td>
</tr>
<tr>
<td>Chasiotis et al (2010)</td>
<td>SSI</td>
<td>Sym 2.9</td>
<td>Sym 50</td>
<td>Sym 50</td>
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<tr>
<td>Sandbichler et al (2011a)</td>
<td>SSI</td>
<td>Sym 6.8</td>
<td>Sym 15</td>
<td>Sym 15</td>
</tr>
<tr>
<td>Sandbichler et al (2011b)</td>
<td>SSI</td>
<td>Sym 10</td>
<td>Sym 15</td>
<td>Sym 15</td>
</tr>
<tr>
<td>Farkas et al (2011)</td>
<td>DSI</td>
<td>Sym 10</td>
<td>Sym 15</td>
<td>Sym 15</td>
</tr>
<tr>
<td>Kelly and Chasiotis (2011)</td>
<td>SSI</td>
<td>Sym 2 - 9</td>
<td>Sym 1.8 - 4</td>
<td>Sym 1.8 - 4</td>
</tr>
<tr>
<td>Kolosov and Kelly (2013)</td>
<td>SSI</td>
<td>Sym 4</td>
<td>Sym 4.5</td>
<td>Sym 4.5</td>
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<tr>
<td><strong>Goldfish</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chasiotis and Kelly (2011a)</td>
<td>SSI</td>
<td>Sym ~ 1.5</td>
<td>Sym 4.8</td>
<td>Sym 4.8</td>
</tr>
<tr>
<td>Chasiotis and Kelly (2011b)</td>
<td>SSI</td>
<td>Sym 0.8 - 1.8</td>
<td>Sym 7.3</td>
<td>Sym 7.3</td>
</tr>
<tr>
<td>Chasiotis et al (2012)</td>
<td>SSI</td>
<td>Sym ~ 1.2</td>
<td>Sym 5.3</td>
<td>Sym 5.3</td>
</tr>
<tr>
<td><strong>Tilapia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kelly and Wood (2002b)</td>
<td>SSI</td>
<td>Sym 1.8</td>
<td>Sym 0</td>
<td>Sym 5</td>
</tr>
<tr>
<td><strong>Sea Bass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rainbow trout gill cell line</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lee et al (2009)</td>
<td>RTgill-W1</td>
<td>Sym 5</td>
<td>Sym + 28</td>
<td>Sym + 28</td>
</tr>
</tbody>
</table>

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 = Transepithelial electrical resistance, TEP = transepithelial epithelial potential, PVC = pavement cells, MRC = mitochondrial rich cells.
Table 3. Examples of different endpoints and fish cell models used to evaluate toxicity of chemicals.

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

BF-2: Bluegill sunfish; PLHC-1: Topminnow liver; RTG-2: Rainbow trout gonad; RTL-W1: Rainbow trout liver; FHM: Fathead minnow; OLHNI2: Medaka fin; GFS: Goldfish scale; FiGCS: Fish Gill Cell System; NR: Neutral Red; AB: Alamar Blue; CFDA-AM: carboxyfluorescein diacetate acetoxymethylester; *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.
Figure 1

A

Insert

Gill cell epithelium

B

Symmetrical

Asymmetrical

C

i

ii

iii