© 2014. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2014) 217, 639-650 doi:10.1242/jeb.095430

REVIEW

Gill cell culture systems as models for aquatic environmental monitoring

Nic R. Bury, Sabine Schnell and Christer Hogstrand*

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 focuses in particular on a system of cultured fish gill cells grown into an epithelium that permits direct treatment with water samples.

KEY WORDS: FIGCS, Biomonitoring, Environmental risk assessment, Fish, *In vitro*, Toxicology

Introduction

The Industrial Revolution caused a rapid rise in the 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 as a result of 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 have been great advances in agricultural 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 geologists have termed the current epoch the anthropocene (Zalasiewicz et al., 2010).

The increase in agricultural and industrial production and consumption of raw materials has resulted in the creation of 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 whether these standards are being adhered to, many jurisdictions 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 in order to set standards several species are tested. In the USA ~3 million fish are used in WET procedures (see 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 better define environmental regulations, and for use in biomonitoring. A number of excellent reviews are available assessing the use of cell lines for toxicity testing (Bols et al., 2005; Castaño 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 with cell line alternatives.

The primary gill cell culture

The gill epithelium is a complex tissue comprising a number of different cell types such as pavement, mitochondria-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 et al., 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 for 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 was not until a 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 Fig. 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 as single-

King's College London, Division of Diabetes and Nutritional Science, Franklin–Wilkins Building, 150 Stamford Street, London SE1 9NH, UK.

*Author for correspondence (christer.hogstrand@kcl.ac.uk)

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.095430

List of abbreviations

3Rs	replace, reduce or refine
ABC	ATP-binding cassette
AChE	acetylcholinesterase
AhR	aryl hydrocarbon receptor
AM	acetoxymethyl ester
AOP	adverse outcome pathway
BaP	benzo[a]pyrene
BCF	fish bioconcentration factor
CETA	concentration equilibrium transport assay
DSI	double-seeded inserts
EC	effect concentration
ECHA	European Chemicals Agency
ECM	extracellular matrix gel
ED	effect dose
EQS	environmental quality standards
EROD	7-ethoxyresorufin-*O*-deethylase
K_{ow}	octanol/water partition coefficient
LC	lethal concentration
LD	lethal dose
LOEC	lowest observed effect concentration
MRE	metal response element
MT	metallothionein
MTF-1	metal-regulatory transcription factor 1
NOEC	no observed effect concentration
PAMPA	parallel artificial membrane permeability assay
PBDE	polybrominated diphenyl ether
PBT	persistence, bioaccumulation and toxicity
PCB	polychlorinated biphenyl
PCP	pentachlorophenol
PDMS	poly(dimethyl-siloxane)
QSAR	quantitative structure–activity relationship
REACH	Registration, Evaluation, Authorisation and restriction of new CHemicals
ROS	reactive oxygen species
SSD	species sensitivity distribution
SSDI	direct seeding of cells onto inserts
SSI	single-seeded inserts
TCDD	1,3,7,8-tetrachlorodibenzo-*p*-diozin
TEP	transepithelial potential
TER	transepithelial resistance
WET	water effluent testing

seeded inserts (SSI); this preparation contained only respiratory pavement cells (Wood and Pärt, 1997; Fletcher et al., 2000). Direct seeding of cells onto inserts (SSDI) occasionally produced viable confluent inserts, but also lacked the mitochondria-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 24 h the cells are washed and a cell preparation from a second fish is placed on top. The reason for this is that the first seeding appears to provide a scaffold on which mitochondria-rich cells are able to adhere and flourish (Fig. 1). A tight epithelium is formed in 6–14 days, with a pavement cell:mitochondria-rich cell ratio (85:15) similar to that observed *in vivo* (Walker et al., 2007).

The formation of a tight junction between cells results in an increase in transepithelial resistance (TER). TER of >1 kΩ is reached with SSI from rainbow trout, goldfish and tilapia in symmetrical conditions (L15 medium on both sides) after 6–14 days culture (Table 2). If the apical medium is replaced with sterile water, the TER increases to >10 kΩ. The extent of TER formation is batch and season dependent. In general the TER for DSI preparations is a lot higher in symmetrical conditions, reaching ~30 kΩ, if compared with 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 the extremely low permeability to ^{3}H-PEG-4000 (Table 2). In symmetrical conditions the transepithelial potential (TEP) 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 a higher passive permeability to Na^+ and Cl^-. The tightness of the epithelium, changes in electrical potential across the membrane under different culture conditions and evidence that the cell inserts respond differently whether water is added to the apical or basolateral compartment (a far greater increase in TER when water is added apically) indicates that a polarised epithelium has 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 elsewhere (Wood et al., 2002). The criterion for active ion uptake is a disagreement between the measured fluxes (apical → basolateral versus basolateral → apical) and that predicted based on the Ussing flux ratio equation (Wood et al., 2002). Under symmetrical conditions (L15 in both apical and basolateral compartments), rainbow trout or tilapia SSI, which lack the mitochondria-rich cells, show similar unidirectional influx and efflux rates with slight deviations from the Ussing flux ratio, indicating non-diffusive uptake (Wood and Pärt, 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 contain mitochondria-rich cells, there is an active influx of Ca^{2+} from water in asymmetrical conditions, 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 with either SSI or DSI (Gilmour et al., 1998; Kelly and Wood, 2001a; Kelly and Wood, 2002a; Kelly and Wood, 2002b), suggesting 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; Kelly and Wood, 2002a). Treatment of SSI or DSI with cortisol causes an increase in TER and a decrease in membrane permeability, which 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 (Chasiotis et al. 2010; Chasiotis and Wood, 2011a; Chasiotis and Wood, 2011b; Kelly and Chasiotis, 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 for how these hormones may decrease ion efflux. The SSDI, SSI and DSI

Table 1. Summary of published primary gill cell culture research

Author	Species and primary cell culture technique	Abstract 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
Visoottiviseth 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 and 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, 2005	Rainbow trout – wells	Cationic liposome transfection
Shahsavarani et al., 2006	Rainbow trout – SSI and DSI	Calcium uptake
Jönsson et al., 2006	Rainbow trout – wells and 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., 2011a	Rainbow trout – SSI	Morphology and effect of cortisol and membrane permeability
Sandbichler et al., 2011b	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	Rainbow 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

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; MT, metallothionein; GC, glucocorticoids; MC, mineralocorticoid.

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.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.095430

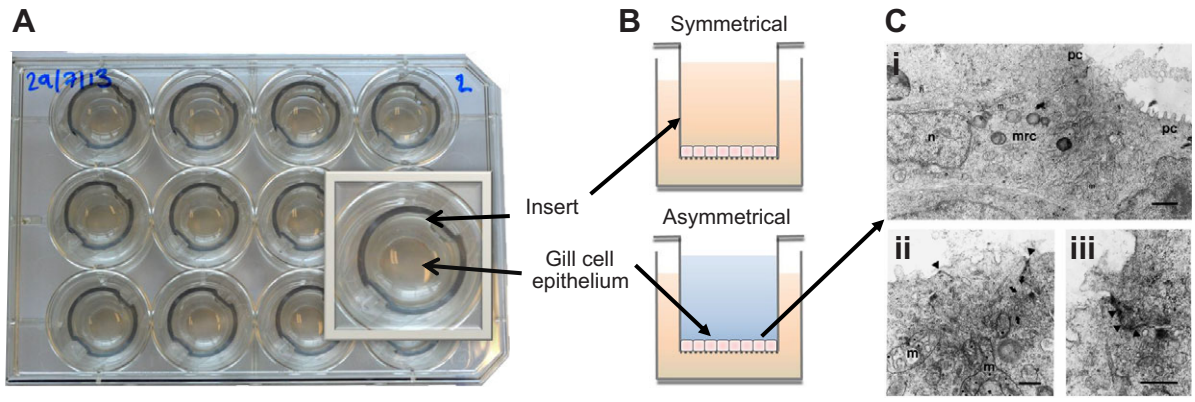

Fig. 1. The primary gill cell culture insert system. (A) Multiwell cell culture plate with inserts containing semipermeable supports used to culture gill cell epithelia. The picture shows double-seeded inserts (DSI) of rainbow trout gill cells that have developed high transepithelial resistance and are 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 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: (Ci) 1 mm; (Cii,Ciii) 400 nm. Adapted with kind permission from *The Journal of Experimental Biology* (Fletcher et al., 2000).

Regulations – environmental risks

Cell lines versus primary cells

Both cell lines and primary cell cultures have their advantages and disadvantages. Cell lines are relatively easy to maintain and handle, and can in theory be cultured indefinitely. But, re-culturing over time may result in the cells losing some of the morphological and physiological characteristics of the tissue from which they were first isolated. However, Lee et al. (Lee et al., 2009) have reported the presence of pavement cells, mitochondria-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 these cells can only be kept for a limited period. However, 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 (OECD, 1992)] identify the concentration of contaminants that cause mortality, and

Table 2. Summary of properties of primary gill cell cultures on inserts and the gill cell line

		Range of TER ($k\Omega\ cm^2$)	Range of TEP (mV)	Range of permeability ($cm\ s^{-1}\times10^{-7}$)	Reported cells present	References
Rainbow trout						
SSI	Sym	1.2 to 21	0 to +2.8	1.7 to 5	PVC	Wood and Pärt, 1997; Fletcher et al., 2000; Carlsson and Pärt, 2001; Smith et al., 2001; Kelly and Wood, 2001a; Kelly and Wood, 2001c; Kelly and Wood, 2002a; Shahsavarani et al., 2006; Jönsson et al., 2006; Chasiotis et al., 2010; Sandbichler et al., 2011a; Sandbichler et al., 2011b; Kelly and Chasiotis, 2011; Kolosov and Kelly, 2013
	Asym	5.8 to 31	−1.2 to −35			
DSI	Sym	1.3 to 34	+1.9 to +45	0.47	PVC, MRC	Fletcher et al., 2000; Kelly and Wood, 2001b; Kelly and Wood, 2002a; Wood et al., 2002; Zhou et al., 2003; Zhou et al., 2005; Walker et al., 2007; Walker et al., 2008; Kelly and Wood, 2008; Galvez et al., 2008b; Farkas et al., 2011
	Asym	4.2 to 30	−5 to −14.2	1.01		
Goldfish						
SSI	Sym	0.8 to 1.8		3.5 to 5.2	PVC, MRC	Chasiotis and Kelly, 2011a; Chasiotis and Kelly, 2011b; Chasiotis et al., 2012
	Asym	4.2		7.3		
Tilapia						
SSI	Sym	1.8	0	5		Kelly and Wood, 2002b; Zhou et al., 2006
	Asym	18.6	−13.4	3.2		
DSI	Sym	5 to 8				
	Asym	12 to 15				
Sea bass						
SSI	Sym	5	+ 28			Avella et al., 1999
Rainbow trout gill cell line					PVC, MRC, goblet-like cells	Lee et al., 2009

Note, values are from control treatments in each paper.
Sym, symmetrical conditions (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, mitochondria-rich cells

the potency of a toxicant to cause mortality is often referred to as the lethal concentration (LC) or dose (LD) that kills 10, 20, 50% of the population (LC or $LD_{10/20/50}$). Similarly, the 'no observed effect concentration' (NOEC) is the highest concentration tested without an effect and the 'lowest observed effect concentration' (LOEC) is the lowest concentration tested where mortality was observed. Chronic toxicity tests [e.g. OECD 229 (OECD, 2009)] can include mortality during a longer time of exposure, but also evaluate growth and/or reproduction as an end point from which effect concentration or dose (EC or ED) can be calculated. To assess the safety of a chemical in terms of the environment, toxicity tests are conducted on a limited number of organisms, typically a prokaryote, a plant, an invertebrate and a vertebrate (standard organisms in a battery of tests may include zebrafish to represent fish, *Daphnia magna* to represent aquatic invertebrates and *Raphidocelis subcapitata* 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 varies 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 LC_{50} 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, LC_{50}, EC_{50}) as a function of stressor concentration or dose. From a species sensitivity distribution plot it is then 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 the data are acute or chronic and whether the species sensitivity distribution (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 underprotective 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 is 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 whether 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 (OECD, 1992) acute fish test prescribed the use of between 42 and 60 fish per test. 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 analysed the *in vitro* cytotoxicity of compounds to fish cell lines and compared this with *in vivo* acute toxicity data (reviewed in Segner, 2004; Schirmer, 2006). If acute toxicity is due to cytotoxicity, usually via 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 (Segner, 2004)] and appear to be useful in ranking hazardous compounds, but more often than not, fish cell culture systems underestimate the toxicity by up to 3 orders of magnitude (Table 3) (see reviews by Segner, 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 use 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. (Tanneberger et al., 2013) used the RTgill-W1 cell line, derived from rainbow trout gill cells, to screen 35 compounds of differing modes of action. They integrated three measures of cell cytotoxicity: Alamar Blue as a measure of metabolic activity; 5-carboxyfluorescein 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 EC_{50} and LC_{50} values for the compounds tested. Of those, the acetylcholinesterase (AChE) inhibitors showed an even better correlation between EC_{50} and LC_{50} of 1.

Two major projects have evaluated the use of the primary gill cell culture system as an alternative for toxicity screening. Sandbacka et al. (Sandbacka et al., 1999) and Lilius et al. (Lilius et al., 1995) compared the responses of gill cells in primary culture or 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 were incubated with calcein-acetoxymethyl ester (AM); the accumulated intracellular calcein-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 ATP-binding cassette transporters (ABC transporters) that are present in culture gill cells (Fischer et al., 2011) and will, if active, export the compound from the cell. TER was used as a measure of membrane integrity in cell culture on inserts. The cell culture and suspension showed similar EC_{50} values for the compounds compared, and the 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 end point for cell viability. Seldom have we observed a significant reduction in TER for DSI exposed to metals resuspended in water in the laboratory (Walker et al., 2008) or to natural waters contaminated with metals (M. Minghetti, S.S., M. A. Chadwick, C.H. and N.R.B., unpublished results) for 24 h. Likewise, Zhou et al. (Zhou et al., 2006) observed no change in TER on exposure to aryl hydrocarbon receptor (AhR) agonist toxicants; thus, other end points maybe more appropriate as a measure of a toxic response.

Table 3. Examples of different endpoints and fish cell models used to evaluate toxicity of 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 and 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 and 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≥0.97); cell line 1–2 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 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		Krøvel et al., 2008
Cell viability (calcein-AM)	30 MEIC chemicals	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 1–2 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

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.

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 provide 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. (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 to PBDEs, generating 24 h EC_{50} values in the 10^{-6} to 10^{-9} mol l^{-1} range. These results are as predicted because TCDD, BaP and several congeners in Aroclor 1254 are strong AhR 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 3 h of exposure. Thus, EROD activity is probably not a good marker for PBDE toxicity, but it may be possible to devise surrogate toxicity end points 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 substances (S.S., N.R.B. and C.H., unpublished data).

In the laboratory, DSI respond 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 MT-A and MT-B, 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 (MREs) in the promoter of metal-responsive genes. For example, the promoter region of rainbow trout MT-A possesses six MREs, four of which are arranged in tandem (Olsson et al., 1995), and MT-B possesses four 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 substances that are produced in excess of 1000 kg. A regulatory requirement is to provide data on the persistence, bioaccumulative and toxic (PBT) properties of compounds. The OECD 305 (OECD, 2012) 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 OECD 305 guidelines have recently been revised and the number of fish used per test has been reduced to 80. It was initially estimated that 30,000 chemicals may require re-evaluation, but this may be a vast underestimate and the number of substances registered with the European Chemicals Agency (ECHA) by the 1 December 2008 deadline was 146,000 (Gubbels-van Hal and Pelkmans, 2009). Bioaccumulation tests for each of these compounds would use a worryingly high number of animals and money, with the current EU environmental testing market valued in excess of £500 million. To reduce animal numbers and cost, under REACH legislation BCF testing is only required for substances whose production exceeds 100,000 kg, or if the physiochemical properties suggest a lipophilic compound (log$P$>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 require re-evaluation, the 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 the 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 2012, 500,800 fish were used for scientific procedures, of which 34,700 were for toxicological procedures. However, it must also be remembered that REACH is only applicable to the EU and other regions such as the USA 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 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 chemical, BCF calculations can be inaccurate. Experimental data on accumulation can potentially be generated with cell cultures, but identifying suitable cell culture end points is difficult because the current OECD 305 BCF test requires a period of accumulation to steady-state followed by a lengthy depuration period. With respect to uptake, the cultured gill cells grown on semipermeable supports offer an alternative to whole-animal studies. The cultured gill epithelium expresses a number of tight junction proteins, such as the claudins and ZO-1, that enable the cells to forms a tight epithelium with a transepithelial electrical resistance in excess of 20 kΩ (e.g. Chasiotis et al., 2010; Chasiotis and Kelly, 2011a; Kolosov 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 probably transcellular. Usually, the uptake is directly proportional the compound's logK_{ow} (octanol/water partition coefficient), a measure of lipophilicity. A number of studies have used parallel artificial membrane permeability assays (PAMPA) to determine the relationship between logK_{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 a summation of that via passive diffusion or carrier-mediated processes. Recently, 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 (L. C. Stott, S.S., C.H., N.R.B., unpublished results). Interestingly, in concentration equilibrium transport assays (CETA) the 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 homologues of these transporters have been sequenced from fish species or identified in fish genomes (Meier-Abt et al., 2006; 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 the import of xenobiotics are collectively referred to as phase 0 proteins (Fig. 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 enzymes that catalyse the mono-oxygenation reaction of a wide range of natural and synthetic compounds (Uno et al., 2012). Phase II reactions involve enzymes that catalyse sulfonation (sulfontransferases, SULT), acetylation (*N*-acetyltransferases), methylation (methyltransferases), glutathione conjugation (glutathione-*S*-transferase) and glucuronidation (glucuronosyl transferase) of the polar moieties of products from phase I reactions. The xenobiotic conjugates from phase II are exported from the cell

The Journal of Experimental Biology (2014) doi:10.1242/jeb.095430

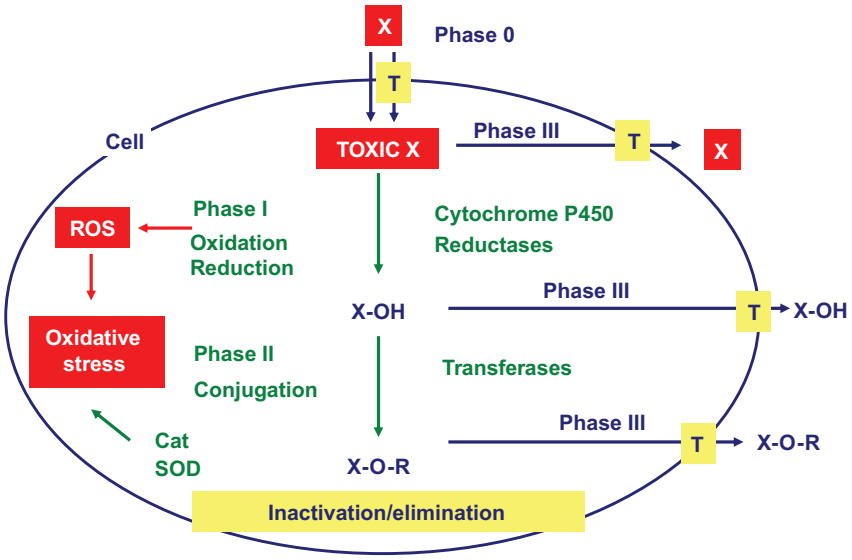

Fig. 2. Overview of xenobiotic biotransformation in a generic cell. The scheme includes phase I and phase II enzymatic processes and transporters in phase 0 and phase III. X, xenobiotic; X-OH, hydroxylated xenobiotic; X-O-R, xenobiotic conjugated with hydrophilic species, such as glutathione, sulfate, 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 ATP binding cassette (ABC) transporters.

by a family of proteins called the ABC transporters and there are eight 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 possesses the CYP450A1 enzymes necessary for xenobiotic biotransformation (Carlsson et al., 1999; 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, abcc2, abcc3 and abcc5 (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 ABCs (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 epithelium 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 extent 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 (Weisbrod 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 spheroid cultures 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 and 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 a useful tool to assess water-borne 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). Conversely, *in vitro* bioassays using cultured cells have been shown to be very useful tools for characterizing 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 that allows *in vitro* exposure (e.g. reconstitution of powdered culture media with sediment elutriates, concentrated organic sediment extracts, etc.) (Davoren et al., 2005). Because of this processing, difficulties arise when trying to associate observed effects with the 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 et al. (Davoren et al., 2005) assessed three different fish cell lines and their potential to serve as tools for cytotoxicity testing of estuarine sediment aqueous elutriates. 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. (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. (Schnell et al., 2013) where controls sites could be clearly teased apart from contaminated sites. Strmac and Braunbeck (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 the two rivers as well as between free water phase and sediment were detected by applying different end points focusing on cytological and biochemical changes. These examples illustrate how useful cell culture system permanent cell lines and primary cells can be for characterizing the environmental quality of sediments.

Water samples

In the US, over 3 million fish are used for WET testing to assess whether end-of-pipe effluence 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 number 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 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. (Dayeh et al., 2002) utilised these last methods to assess the toxicity of 31 paper mill effluents to the RTgill-W1 cells and compared the toxicity with 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 that 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; Walker et al., 2008) and it was possible using microarray technology to identify genes expressed in response to specific metals, but after having tested many different potential biomarker genes, MT still best reflects metal toxicity to fish (Walker et al., 2007; Walker et al., 2008). Following on from our laboratory observations, we evaluated 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 1000 km) in temperature-controlled Medi-boxes (over 30 h) 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 end point is a useful indicator of bioreactive metals in natural waters. The limited number of sampling points and the 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 between Cu and Ni concentration and MT-B expression was found, suggesting isoform-specific responses to metals (M. Minghetti, S.S., M. A. Chadwick, C.H. and N.R.B., unpublished results).

Other applications and the way forward

Multi-organ systems (fish-on-a-chip)

Recent advancements in microengineering and microfluidic dynamics have led to 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., 2010); gut (Imura et al., 2009); and liver (Lee et al., 2007)]. With respect to fish cells, Glawdel et al. (Glawdel et al., 2009) developed a microfluidic system integrated with an electroosmotic pump for the culture of a RTgill-W1 cell line and showed using a cell viability assay that this system could be used for toxicity testing. These advancements utilise microengineered devices to form a structure that enables cells to grow in 3D to better mimic 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. (Huh et al., 2010) developed a human lung-on a-chip device that is made of two silicone rubber poly(dimethyl-siloxane) (PDMS) channels separated by a 10 μm 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 development of microfabricated devices for cell culture because it is relatively cheap and pliable; importantly, it allows 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 cell microvascular chamber to microfluid dynamics mimics that of blood flow in the capillaries. The ingenuity of this device is that it can also mimick rhythmic breathing by applying suction to an adjacent chamber. This enhances the production of surfactants by the alveolar cells and maintains the integrity of the 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 12 nm 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 neutrophils 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 metabolites 1,2-naphthalenediol and 1,2-naphthoquinone, induced glutathione depletion in the lung ‘tissue’ (Viravaidya et al., 2004). The advancements in such devices in the human cell culture arena came about because of the recognition that animal models were 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 the cell cultures that represent the organs within a fish.

Competing interests
The authors declare no competing financial interests.

Author contributions
N.R.B., S.S. and C.H. all contributed to the writing of this review article.

Funding
This work was supported by research grants from the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) [ref. no. 26675] awarded to C.H. and N.R.B., and Natural Environment Research Council [ref. no. NE/I001204/1] awarded to N.R.B. and C.H.

References

Airaksinen, S., Råbergh, C. M. I., Sistonen, L. and Nikinmaa, M. (1998). Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *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**, 353-363.

Babich, H. and Borenfreund, E. (1987). Polycyclic aromatic hydrocarbon 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. In *Biochemistry and Molecular Biology of Fishes*, Vol. 6 (ed. P. Mommsen and T. W. Moon), pp. 43-84. Amsterdam: Elsevier.

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 sulfur heterocycles as major polycyclic aromatic cytochrome P4501A-inducers in a contaminated sediment. *Environ. Sci. Technol.* **37**, 3062-3070.

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, R., Smital, T. and Fent, K. (2008). Human pharmaceuticals modulate P-gp1 (ABCB1) transport activity in the fish cell line PLHC-1. *Aquat. Toxicol.* **90**, 214-222.

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

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.

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**, 2141-2157.

Castaño, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee, L. E. J., Mothersill, C., Pärt, P. et al.; ECVAM Workshop 47 (2003). The use of fish cells in ecotoxicology. The report and recommendations of ECVAM Workshop 47. *ATLA* **31**, 317-351.

Chapman, P. M. (2007). Determining when contamination is pollution – weight of evidence determinations for sediments and effluents. *Environ. Int.* **33**, 492-501.

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

Chow, S. C. and Wong, C. K. C. (2011). Regulatory function of hyperosmotic stress-induced signaling 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 Debrauwer, L. (2001). Metabolic fate of 2,4-dichloroaniline, prochloraz and nonylphenol diethoxylate in rainbow trout: a comparative *in vivo*/*in vitro* approach. *Aquat. Toxicol.* **53**, 159-172.

Davoren, M., Ní Shúilleabháin, S., Hartl, M. G. J., Sheehan, D., O'Brien, N. M., O'Halloran, J., Van Pelt, F. N. 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. *Water 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., Sijm, 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. Manag.* **3**, 3-17.

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

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

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

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

Durrant, C. J., 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. Pollut.* **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-Urrea, J. A., Roos, N., Hassellöv, M., Tollefsen, K. 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 different 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 properties 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., Salbert, G., Pelissero, C., Guiraud, 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 properties 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. Biol. Anim.* **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., Elbuken, C., Lee, L. E. J. and Ren, C. L. (2009). Microfluidic system with integrated electroosmotic pumps, concentration gradient generator and fish cell line (RTgill-W1) – towards water toxicity testing. *Lab Chip* **9**, 3243-3250.

Gubbels-van Hal, I. and Pelkmans, J. (2009). Is REACH going well. Centre for European Policy Studies Policy Brief Number 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 Braunbeck, T. (2001). Toxic responses of the liver. In *Target Organ Toxicity in Marine and Freshwater Teleosts* (ed. D. Schlenk and W. H. Benson), pp 224-268. London: Taylor and Francis.
Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Hsin, H. Y. and Ingber, D. E. (2010). Reconstituting organ-level lung functions on a chip. *Science* **328**, 1662-1668.
Huh, D., Hamilton, G. A. and Ingber, D. E. (2011). From 3D cell culture to organs-on-chips. *Trends Cell Biol.* **21**, 745-754.
Imura, Y., Asano, Y., Sato, K. and Yoshimura, E. (2009). A microfluidic system to evaluate intestinal absorption. *Anal. Sci.* **25**, 1403-1407.
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.* **5**, 1119-1129.
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.
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.
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 (*Oreochromis niloticus*): effect of cortisol and homologous serum supplements from stressed and unstressed fish. *J. Membr. 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. Biol. Anim.* **39**, 21-28.
Kelly, S. P. and Wood, C. M. (2008). Cortisol stimulates calcium transport across cultured gill epithelia from freshwater rainbow trout. *In Vitro Cell Dev. Biol. Anim.* **44**, 96-104.
Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J.-M. and Aït-Aïssa, S. (2010). Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. *Environ. Pollut.* **158**, 74-83.
Knauer, K., Lampert, C. and Gonzalez-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.* **304**, R1139-R1148.
Krøvel, A. V., Søfteland, L., Torstensen, B. and Olsvik, P. A. (2008). Transcriptional effects of PFOS in isolated hepatocytes from Atlantic salmon Salmo salar L. *Comp. Biochem. Phys.* **148C**, 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* (ed. D. Schlenk and W. H. Benson), pp. 90-150. London Taylor and Francis.
Laville, N., Aït-Aïssa, 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, 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.
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. Anim.* **45**, 127-134.
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. *Toxcicol. In Vitro* **9**, 299-305.
Liu, J., Kuznetsova, L. A., Edwards, G. O., Xu, J., Ma, M., Purcell, W. M., Jackson, S. K. and 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 the ABC efflux transporters in rainbow trout (Oncorhynchus mykiss). *Comp. Biochem. Physiol.* **151C**, 209-215.
Meier-Abt, F., Mokrab, Y. and Mizuguchi, K. (2006). Organic anion transporting polypeptides of the OATP/SLCO superfamily: identification of new members in nonmammalian species, comparative modeling and a potential transport mode. *J. Membr. Biol.* **208**, 213-227.
Nishimoto, M., Yanagida, G. K., Stein, J. E., Baird, W. M. and Varanasi, U. (1992). The metabolism of benzo(a)pyrene by English sole (*Parophrys vetulus*): comparison between isolated hepatocytes in vitro and liver in vivo. *Xenobiotica* **22**, 949-961.
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.
OECD (1992).*Test No. 203: Fish, Acute Toxicity Test*. OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing. doi: 10.1787/9789264069961-en
OECD (2009). *Test No. 229: Fish Short Term Reproduction Assay*. OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing. doi: 10.1787/9789264076211-en
OECD (2012).*Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure*. OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing. doi: 10.1787/9789264185296-en
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**, 344-349.
Pärt, P., Norrgren, L., Bergström, B. and Sjöberg, P. (1993). Primary culture of epithelial cells from rainbow trout gills. *J. Exp. Biol.* **175**, 219-232.
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.
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. Genomics* **24**, 207-217.
Romøren, K., Fjeld, X. T. L., Poléo, A. B., 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.
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.
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.* **77**, 282-289.
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, P., Avdeef, A., Bendels, S., Di, L., Ecker, G. F., Faller, B., Fischer, H., Gerebtzoff, G. et al. (2010). Coexistence of passive and carrier-mediated processes in drug transport. *Nat. Rev. Drug Discov.* **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* (ed. M. Sargolia, and A. Liu), pp 219-230. Oxford: 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* × *Claria macrocephalus*) for screening toxicity of triphenyltin hydroxide. *Appl. Organomet. 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. Technol.* **41**, 6505-6513.
Walker, P. A., Kille, P., Hurley, A., Bury, N. R. and Hogstrand, C. (2008). An in vitro method to assess toxicity of waterborne metals to fish. *Toxicol. Appl. Pharmacol.* **230**, 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 bioaccumulation assessments for fish. *Environ. Toxicol. Chem.* **28**, 86-96.
Wise, J. P., Sr, Goodale, B. C., Wise, S. S., Craig, G. A., Pongan, A. F., Walter, R. B., Thompson, W. D., Ng, A. K., Aboueissa, A. M., Mitani, H. et al. (2010). Silver nanospheres are cytotoxic and genotoxic to fish cells. *Aquat. Toxicol.* **97**, 34-41.
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.
Wood, C. M. (2001). Toxic responses of the gill. In *Target Organ Toxicity in Marine and Freshwater Teleosts* (ed. D. Schlenk and W. H. Benson), pp. 1-89. London: Taylor and Francis.
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., 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.
Xu, C., Li, C. Y. and Kong, A. N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* **28**, 249-268.
Zalasiewicz, J., Williams, M., Steffen, W. and Crutzen, P. (2010). The new world of the Anthropocene. *Environ. Sci. Technol.* **44**, 2228-2231.
Zhou, B., 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., Lam, P. K. 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.

The Journal of Experimental Biology