University of Southampton

Faculty of Environment and Life Sciences

School of Ocean and Earth Sciences

The Effects of Fluid Shear Stress, Reduced Serum Supplementation, and a Selection of Chemicals on the Rainbow Trout Gill Cell Line RTgill-W1

by

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Thesis for the degree of Master of Philosophy

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Abstract

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Static, cell-based toxicity testing with the Rainbow trout gill cell-line, RTgill-W1 is a regulator-accepted proxy for fish acute toxicity testing. Yet, the static tests provide effect concentration data without considering fluid shear stress (FSS) present at the gill in vivo, or the adverse outcome pathway (AOP) of the test chemical. Additionally, pressure to cut use of foetal bovine serum in toxicity testing in line with reduction and replacement targets necessitates refinement of methodologies to include use of little or no animal derived serum.

Fluid shear stress mediates mechanosensitive pathways responsible for uptake and resistance to toxicants. The first of two experimental phases in this study utilised custom 3D printed chambers housing cell culture inserts for the application of FSS (0.2 dynes cm^2) over the cells in the presence and absence of (Cu). Expression of copper stress response genes (metallothionine a and b, *atp7a, mtf-1, atox-1, sod1, sod2)* and mechanosensitive cation channel, *peizo1* together with reactive oxygen species production were measured for comparison of static and FSS exposure.

The second study phase comprised measuring gene expression (*vitellogenin, chop, znt1, cyp1a1)* under benzo(a)pyrene, cadmium and clotrimazole exposures of 24 hours. In addition, RTgill-W1 cultures reduced to 1% FBS were tested against standard 5% cultures in 3,4-dichloroaniline (DCA) toxicity curves.

Fluid shear stress increased the metabolic sensitivity of RTgill-W1 to copper and altered the metal responsive gene expression pattern. FSS significantly increased *atp7a, peizo1 and sod* expression together with ROS production.

Gene expression analysis in RTgill-W1 alone was not predictive of adverse outcome pathways, particularly for PAHs owing to the lack of cyp1a1 activity in the cell line. However, responses of RTgill-W1 cultured in 1% FBS to DCA testing matched those of standard 5% FBS cultures.

Overall, the results show FSS affects RTgill-W1 responses both alone and in the presence of copper and should be considered in future fish toxicity test replacement methodologies. The preliminary results from 1% FBS RTgill-W1 cultures indicate possibility of fast, simple reduction of animal product use in RTgill-W1 based toxicity tests, but further work is required to characterise the FBS-reduced cultures. Moreover, the addition of more cell lines and assays is necessary for a successful AOP prediction suite.

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Research Thesis: Declaration of Authorship

Penelope C Fenton

The Effects of Fluid Shear Stress, Reduced Serum Supplementation, and a Selection of Chemicals on the Rainbow Trout Gill Cell Line RTgill-W1

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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Signature: Date: 08 February 2025

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This list is not complete without a special mention for another victim of my public speaking rehearsals, my constant four-legged companion, Tibbles.

Definitions and Abbreviations

General Abbreviations

AFT.....Acute fish test. An OECD protocol for testing acute toxicity in live fish.

AOP.....Adverse outcome pathway. The chain of events leading from chemical exposure to the physiological response or outcome in the exposed system.

BCF.....Bioconcentration factor. The measure of chemical accumulation in an exposed organism or tissue, relative to the concentration in the exposure medium/water.

FET.....Fish embryo test

FSS.....Fluid shear stress. Force exerted on a surface by fluid moving parallel to it. Measured in dynes/cm^2.

MIE.....Molecular initiating event. First molecular effect identified in a system after exposure. Usually triggers the adverse outcome pathway.

OECD.....Organisation for Economic Cooperation and Development

TEER.....Transepithelial electrical resistance. A non-invasive, label-free measure of cell layer barrier function and tight junction integrity through measurement of electrical resistance.

Genes

atp7a.....Encodes copper transporting ATPase. Also known as Menkes protein.

atox1.....Encodes a cytosolic copper chaperone.

Chop.....Encodes a component of the endoplasmic reticulum stress apoptotic pathway.

cyp1a1.....Encodes member of cytochrome P450 family. Key enzyme in metabolism of polyaromatic hydrocarbons.

efb1.....Encodes translation elongation factor 1 beta. Housekeeping gene.

mta.....Encodes metallothionein a. Low molecular weight metal binding protein.

mtb.....Encodes metallothionein a. Low molecular weight metal binding protein.

mtf1 ..... Encodes metal regulatory transcription factor 1. Induces transcription of metallothionein.

piezo1 ..... Encodes piezo type mechanosensitive ion channel component 1. Mechanosensory nonselective ion channel

sod1 ..... Encodes superoxide dismutase 1. Antioxidant enzyme and binder of copper and zinc

sod2 ..... Encodes superoxide dismutase 2. Antioxidant enzyme located in the mitochondria. Manganese dependent.

ubiquitin ..... Small regulatory protein. Conserved and ubiquitous housekeeping gene.

vitellogenin..... Egg yolk protein precursor

znt1..... Encodes zinc transporter 1. Responsible for cellular zinc export.

Chapter 1 Background Introduction

1.1 Heavy metal threats to freshwater resources and biodiversity

Pollution affects human health directly through parameters like water and air quality, and indirectly by resulting habitat and biodiversity loss (Landrigan et al., 2018). Complex ecological interplay requires that resource protection extends beyond qualities directly associated with human health (Gleeson et al., 2020), to include the biodiversity of ecosystems from which we abstract our water supply. Biodiversity provides an ecological safety buffer for food security and development of new medicines as well as being associated with reduction of zoonotic transmission. Freshwater biodiversity is disproportionately affected by the global biodiversity crisis compared with marine and terrestrial systems (Albert et al., 2020) and water stress is predicted to affect more than half the world population for at least one month each year by 2050 (Boretti and Rosa, 2019). Regulation of human activities through chemical testing and registration is vital and these tests must be powerful and ethical in the contexts of ecological protection and animal use.

Freshwater bodies, which constitute ~ 2 % of the Earth's surface, contain approximately one-third of vertebrate species on Earth (Strayer and Dudgeon, 2010; Albert et al., 2020). The distribution of these species is predominantly endemic (Abell et al., 2008) placing more importance on individual sites. In addition, the condition of these habitats can reflect the groundwater and soil quality in their drainage catchments, making them valuable indicators for the health of surrounding land (Silva et al., 2012). Agricultural practices contribute nonpoint source pollutants through runoff, while point source pollutants, such as pharmaceutical waste from water treatment plant outlets (Miller et al., 2021) and heavy metals from active mining (Freedman et al., 2012), directly enter rivers.

Mines cause point source metal pollution at levels toxic to fish (Durrant et al., 2011) and hazardous to humans (Zahra et al., 2017). Copper and cadmium are among the most common metal pollutants (Stankovic et al., 2014) and bioaccumulation can facilitate the presence of these substances at toxic levels in fish bound for human consumption, especially in the developing world (Ahmed et al., 2019). Copper is an essential micronutrient for all living things but is tightly regulated at a cellular level due to its toxic potential. Copper toxicity is driven by its potential bioactivity at every biological level of organisation (Kumar et al., 2013). For example, in the fish, toxicity manifests in dysregulation of ionic and osmotic balance through gill damage at the level of the organism, and oxidative stress at a cellular level (Exley et al., 1991). The river Hayle in Cornwall, UK is an example of a river with deadly levels of copper contamination. However, the brown trout in the Hayle survive the pollution from an abandoned mine that would be deadly to metal-naïve trout. Tolerance is not a solution to the

contamination; the reduced genetic diversity of the Hayle trout affects fish health and potentially the genetic diversity of the surrounding trout populations (Durrant et al., 2011). Heavy metals are only one group of pollutants affecting river health, however they pose particular risk due to the likelihood of deposit mobilisation by increased salination and flow-rate variation caused by extremes of weather (Freedman et al., 2012).

1.2 Fish in regulatory freshwater protection

Reliable toxicity testing protects human health and natural resources through influencing water quality standards. Programs such as the European Union Registration, Evaluation, Authorization and Restriction of Chemicals regulation 1907/2006 (REACH, 2021), the Environment Action Programme Decision No 591/2022 (EU Parliament, 2022) and the Organisation for Economic Co-operation and Development (OECD) high production volume chemical assessment initiative (OECD, 2018) all drive the development of reliable risk assessment for more than 30,000 existing chemicals and a “no data, no market” approach to new substances (Wlodkowic and Campana, 2021).

Fish are a relatable link to our freshwater habitats and a model vertebrate organism for freshwater ecotoxicity studies and therefore regulatory test protocols required by regulatory bodies for chemical registration. Fish are vital constituents of food webs (Lombardo et al., 2015) and figureheads for aquatic environmental protection. Moreover, they are excellent indicators for water health and chemical safety due to their constant exposure to water pollutants via the gills, where solutes are exchanged with more efficiency than in exchange systems of mammals, birds, and amphibians (Piiper and Scheid, 1975).

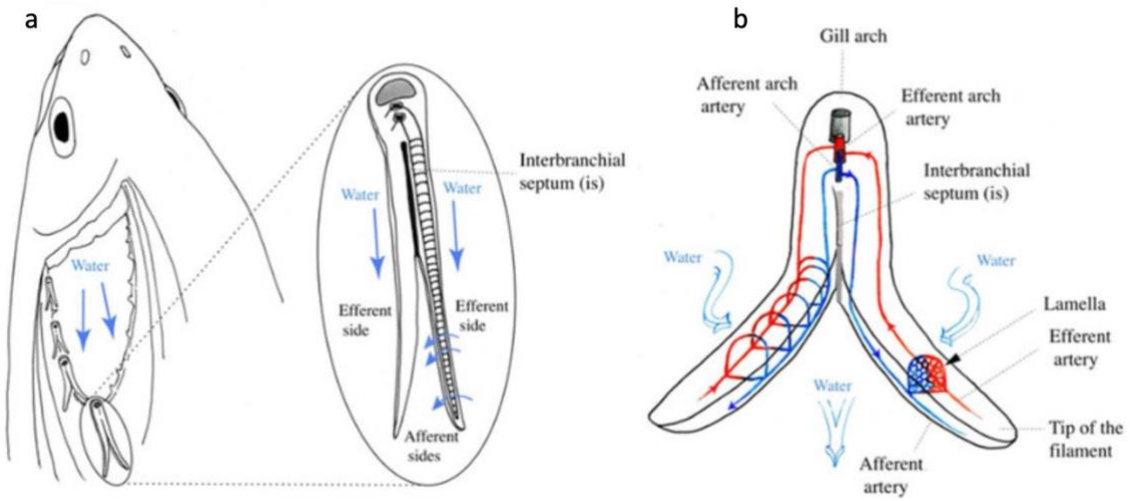


Figure 1. Teleost gill structure

Location of the gill arches (a) with two rows of gill filaments. Each filament is further divided into the afferent and efferent sides which each possess highly vascularised secondary lamella (b) (Image, Rességuier et al. 2020).

In the teleost, bilateral opercular cavities are separated by a single buccal cavity. The gills comprise 4 branchial arches supporting equally spaced primary lamellae from which filaments known as secondary lamellae protrude (Fig. 1). Membranes just one cell thick separate the internal filament from the external environment. Each level of organisation increases efficiency further by adding surface area which itself is correlated to fish size and level of activity (Hughes, 1966). The secondary lamellae are well vascularised and covered in epithelium comprising pavement, mitochondria rich cells (or ionocytes) and goblet cells (Wilson and Laurent, 2002). The gill epithelium is subject to continuous steady or pulsatile water flow, dependent on physical activity status, that elicits fluid shear stress on the apical surface, (Strother, 2013). Wall shear stress from blood vessels is constant on the basolateral surface and stretch from the contraction of pillar cells (Stensløkken et al., 2006) contributes to the typical mechanical stimuli acting on epithelium of the secondary lamellae in vivo. These fundamentals of cell physiology modulate vital barrier function (White et al., 2011), ion transport (Satlin et al., 2001) and adenosine triphosphate (ATP) synthesis (Yamamoto et al., 2018).

Damage to the gills is often the cause of death in instances of acute toxicity in the fish (Exley et al., 1991) due to disruption of vital functions such as acid-base, ionic and osmotic regulation, and the excretion of nitrogenous waste (Evans et al., 2005). This lethality is measured in acute fish tests (AFTs), live fish toxicity tests established for testing all chemicals synthesised or imported by the European Union in quantities of >10 tons/year (Schirmer et al., 2008). The OECD test number 203 is the accepted AFT guideline but requires the exposure of 7 fish per concentration to acute levels of toxicity for up to 96 hours (OECD, 2019) and leads to the generation of many litres of chemical waste (Tanneberger et al., 2013). Fifty percent lethal concentrations (LC50) drawn from fish dose response curves (OECD, 2019) are a crude endpoint, overlooking biological and physiological events leading up to death such as adverse outcome pathways and the molecular initiating events that trigger them. Chronic toxicity testing addresses some of these gaps and is carried out when risk is indicated by acute testing data, when longer-term exposure is expected (Schirmer et al., 2008) or the chemical in question is not expected to reach steady state within in 96 hours. Nevertheless, tests that generate a minimum of data from the death and unavoidable discomfort of millions of fish, contravene modern ethics. Growing interest in the emotional cognition of, and pain felt by fish can be seen in the establishment of organisations advocating specifically for fish welfare (Message and Greenhough, 2019). Such principles manifest in a societal desire for regulatory conservation in parallel with reduced animal testing. These pressures have catalysed the development of several alternatives to the AFT (Norberg et al., 2018; Lillicrap et al., 2016; Scott and Minghetti, 2020) and the initiation of new regulatory strategies to meet ethical expectations. With some alternatives, accessibility to testing may be extended to facilities without the available funding or space to carry out AFTs.

1.3 Alternative testing methods

The National Centre for the Replacement Refinement and Reduction of Animals in Research (NC 3Rs) was established in the UK in response to the principles of reduction, refinement, and replacement (3 Rs) which were established more than 50 years ago. Additionally, CEFIC-LRI Eco8/CEllSens (Fischer et al 2019) and the CREAM (Chemical Risk Effects Assessment Models project) (Grimm et al, 2009) were assembled and together, posited that a rainbow trout gill cell line, RTgill-W1 (Bols et al., 1994) and zebrafish embryos (Nagel, 2002) may serve as models to predict acute fish toxicity (Natsch 2018). Fish embryos and larvae are not protected by the Animals (Scientific Procedures) Act 1986 (Balls, 1986). In fact, the larvae only become a protected animal when they can feed independently. Therefore, the individual operator, study and establishment licenses required to test on protected animals are not necessary for work with non-feeding stages. Similarly, established cell lines and primary cells (providing live fish are not kept on the premises) require no such license, increasing accessibility to chemical safety testing. The only alternative test requiring less animal material than cell lines is computational modelling based on existing data.

AI or in silico modelling would satisfy the high demand of a multi-variable risk assessment of new and repurposed chemicals, and the need for animal replacement (Miller et all., 2018). However, any model is only as accurate as the toxicological response data it is built upon. Closure of the read-across gap between in vivo tests and proposed in vitro replacements is required to generate data in a manner acceptable to modern ethical expectations. This step cannot be skipped or resulting in silico models could present more risk than protection.

1.3.1 Primary cell culture

Primary gill cell cultures have been well reviewed as indicators of toxicity (Bury et al., 2014). They outperform cell lines in their retention of native tissue characteristics. The characteristic of layer permeability can be considered a measure of epithelial barrier function and is directly related to the number of tight junctions between cells (Claude and Goodenough, 1973). Apicolateral tight junctions comprise an interaction of transmembrane protein homo- and hetero-dimers between neighbouring cells and adhesion molecules such as ZO-1 (Angelow et al., 2008). Transmembrane proteins called claudins determine electrical permeability status due to the electrophysical distribution of amino acids in the extracellular domain (Hou et al., 2013). This permeability is measured non-destructively, through reading transepithelial electrical resistance (TEER) of a cell layer. It is quantitatively expressed in ohms-cm^2 (Ω-cm^2). Primary cultures of rainbow trout gill epithelia generate high TEER indicative of a tight epithelium (>5 KΩ cm^2) with realistic permeability (Fletcher et al., 2000). Double seeded cell culture inserts which are seeded twice, 24 hours apart generate a TEER of ~ 20 KΩ cm^2.

Moreover, cultures contain pavement cells, ioncocytes and mucus producing cells in realistic proportions (Fletcher et al., 2000, Laurent and Perry, 1993). Critically, ionocytes of the gill have shown increased metallothionein expression in response to metal contaminants (Burkhardt-Holm et al., 1999). This goes to the importance of a physiologically realistic heterogeneity in a cell culture model for branchial metal exposure. Primary gill cells, double seeded onto cell culture inserts generated metallothionein (MT) in response to metal contaminated waters at levels comparable with whole organisms (Walker et al, 2008; Minghetti et al., 2014).

Native characteristics lend primary gill cell cultures to relatively accurate branchial modelling for the toxicity of metals. Encouragingly, two fish can yield up to 72 cell culture inserts in experienced hands (Minghetti et al., 2014; Stott et al., 2015) which is attractive from an ethical standpoint. Primary cultures may present as the most physiologically accurate answer to AFTs. Their ability to form an epithelial layer characteristic of gill epithelium allows modelling of xenobiotic uptake which could replace live fish in the generation of bioconcentration factors (BCFs) providing information on levels of bioaccumulation relative to water chemical concentrations.

Fish euthanised for the purpose of tissue donation are not considered as being used in a procedure under UK animal welfare legislation (Balls, 1986). The use of some fish may produce data that is ultimately more valuable than that from cell lines or computational modelling. Some fish now, could mean protection for many more in the future. However, the isolation of primary cultures is laborious, usually requires an aquarium, and the resulting cultures are short-lived and difficult to reproduce (Lee et al., 2009). Additionally, undetected physiological variations in donor fish can lead to fluctuations in response data (Castaño et al., 2003), although this is also true of live fish tests as unseen physiological characteristics can interfere with results.

1.3.2 Fish Embryo Test

The OECD adopted fish embryo testing (OECD TG 236 [OECD, 2013]) in 2013. Data correlated well with acute fish testing (Wheeler and Brändli, 2009) and is now used in conjunction with other reliable, independently generated data to assess toxicity of chemicals with a range of modes of action and physiochemical properties (Braunbeck et al., 2015). Thusly, the German Standardization Organization (DIN) adopted this method to replace acute fish testing for routine whole effluent toxicity.

Acute fish test data is based on mortality and unusual observations in appearance and behaviour. Conversely, FET data is drawn from 4 indicators: embryo coagulation, the tail failing to detach from the yolk, impaired somite development and lack of a heartbeat (OECD, 2013). Klüver et al., (2015) demonstrated an insensitivity to neurotoxic substances when they compared acute fish test data and

FET data. The authors implemented behavioural analysis with a video tracking system accessing locomotor response. This approach increased FET sensitivity to outliers, but EC50s remained up to 51-fold higher than AFT LC50s.

Twenty embryos are used per concentration for every FET (OECD, 2013). Although this non-feeding stage is not protected, these procedures seem to move against the momentum drawing away from the use of animals. Such values are evident in the decision by the EPA to cease all mammal toxicity testing by 2035 (United States Environmental Protection Agency, 2019), although these measures do not yet extend to cover fish. Moreover, accessibility issues presented by the FET are like those of live fish testing. The need for an aquarium for the spawning fish rule this test out for some facilities. However, although FETs come with caveats, they constitute a valuable step toward animal replacement and the refinement of existing tests like AFT.

1.3.3 RTgill-W1

The rainbow trout gill cell line RTgill-W1 (Bols et al., 1994) has been approved by the OECD as a replacement for fish in acute toxicity testing (OECD, 2021). The new test aligns with societal and economical requirements for animal free chemical risk assessment by reducing both the number of animals used and the costs associated with using live animals (Hartung, 2017). The assay uses cell viability measurements to derive EC50s (concentration leading to a 50% loss in cell viability) following static exposures to a range of concentrations of test chemicals.

RTgill-W1 confers some accessibility to toxicity testing. For example, the cells can be cultured at 18 – 22°C, and as demonstrated by Dayeh et al., (2002) and tolerate protein free exposure medium preventing interference with substance bioavailability (Yue et al., 2016). Maintenance of osmolality and cell function requires the addition of salts, galactose and pyruvate which can in themselves alter the chemistry of, and the response to, test chemicals. However, a study demonstrated that RTgill-W1 monolayers can be stored at 6 °C for 78 weeks in ambient CO_2 for toxicity testing in the field (Brennan et al., 2012) which increases accessibility and versatility. Moreover, the authors reported agreement with bovine lung micro vessel endothelial cell responses which is encouraging for read-across. Both cell lines remained viable on microfluidic chips, yet the mammalian cell line required media changes and maintenance at 37°C. Cells were seeded onto Electric cell-substrate impedance-based sensing (ECIS) chips which, along with readers, are commercially available lending reproducibility to the procedure.

RTgill-W1 use comes with limitations. The morphology is distinctly non-epithelial and appears fibroblastic at all levels of confluence. Bols et al., (1994) identified two morphologically distinct epithelial cell types while deriving RTgill-W1 from primary rainbow trout gill filaments. Polygonal,

epithelial-like cells were lost over passages and the irregular, spindly cells predominated and became RTgill-W1. The cell line is however heterogenous, with reported presence of goblet cells and ionocytes (Lee et al., 2009). The authors of this study stated that the cell line forms a tight epithelium. However, a confluent RTgill-W1 monolayer reads from 30 Ω-cm^2 to 32 Ω-cm^2 (Mandal et al., 2020) while a tight epithelium is expected to produce a TEER of > 5 KΩ-cm^2 (Fletcher et al., 2000). Interestingly, RTgill-W1 expresses the adhesion molecules claudin -10e and 30 (Trubitt et al., 2015), claudin 8d (Mandal et al., 2020; Kelly and Chasiotis, 2011), 3a, -7, -12, -28b and -31 (Kelly and Chasiotis, 2011), along with ZO-1 (Trubitt et al., 2015). With these expression profiles it is unclear why RTgill- W1 lacks the cell-cell adhesion required for epithelial tightness and remains unsuitable for uptake-based study. Nonetheless, RTgill-W1 toxicity assay showed agreement with whole animal studies assuming non-specific modes of action (Fischer et al., 2019).

The broad aim of this project was to improve RTgill-W1 as a near-animal-free model for chemical risk assessment. The shortcomings of the cell line and its current regulatory usage were addressed in three main experiments. First, to address poor cell-cell adherence and exclusively static test conditions, flow was applied to cells to try to increase TEER in response to fluid sheer stress and to interrogate response to copper when compared to static exposure conditions. Second, to address use of animal products in RTgill-W1 culture, foetal bovine serum supplementation was reduced both with and without addition of a synthetic replacement and the resulting culture compared to standard cultures for response to a standard toxicant. Third, gene expression analysis was used to attempt to predict adverse outcome pathways and modes of action to fill knowledge gaps left by the crude endpoint of cell death. RTgill-W1 was exposed to chemicals with known adverse outcome pathways and gene expression compared to those pathways.

Chapter 2

Fluid Shear Stress Affects the Metabolic Impact of Copper Exposure in RTgill-W1

Abstract

Static, cell-based toxicity testing with the Rainbow trout gill cell-line, RTgill-W1 is a regulator-accepted proxy for fish acute toxicity testing. Yet, the static tests provide effect concentration data without considering fluid shear stress (FSS) present at the gill in vivo.

Fluid shear stress mediates many mechanosensitive pathways including some responsible for uptake and resistance to toxicants. This experiment utilised custom 3D printed chambers housing cell culture inserts for the application of FSS (0.2 dynes cm^2) over RTgill-W1 in the presence and absence of copper (Cu). Expression of Cu stress response genes (metallothionine A and B, *atp7a, mtf-1, atox-1, sod1, sod2)* and mechanosensitive cation channel, *peizo1* together with reactive oxygen species production were measured for comparison of static and FSS responses.

FSS increased expression of Cu transporter *atp7a* and superoxidase dismutase, and elevated reactive oxygen species production in the absence of Cu when compared to static controls. Under static conditions, Cu (0.163 µM to 2.6 µM) did not impact cell metabolism. However, FSS + Cu, concentrations exceeding 1.3 µM, caused a significant reduction in cell metabolism. Furthermore, metallothionein genes *mta* and *mtb* exhibited differential expression patterns between tested conditions. Static Cu exposure elevated *mta* expression, while FSS + Cu increased only *mtb* expression.

These findings report, for the first time, constitutive, FFS-induced and static copper-induced *piezo1* expression in RTgill-W1. RTgill-W1 response to FSS may be a consideration for development of testing methodologies and in further characterising the cell line.

Chapter 2 Fluid Shear stress affects copper toxicity in gill cell line RTgill-W1

2.1 Introduction

Physical stimuli at the gill in vivo are relevant at organism level, however, at ecosystem level, changes in river flowrate and surges in run-off lead to increased release of ions from heavy metal sinks such as soil and sediment (Freedman et al., 2012). Such changes are more frequent due to climate change and increase the ecological pressure of heavy metal pollution. Potential erosion-mobilised compounds must be assessed for potential ecotoxicity with consideration of the existing and climate change- driven physical variable of flow and resulting fluid shear stress on river fish and their gills.

The RTgill-W1 acute toxicity exposure regime in OECD 249 (Schirmer et al., 2021) and in most studies to date (Fischer et al., 2019; Scott et al., 2020; OECD, 2021), is static. Static conditions allow development of an unstirred layer apical to the cells of approximately 1.6 mm in standard 12 or 24 well culture plates (taken as an average form Hidalgo et al., 1991; Hilgers et al., 1990; Karlsson and Artursson, 1991; Shibayama et al., 2015). The layers become the rate-limiting factor on the permeation of compounds known to enter the cells (Stoker, 1973; Chang et al, 2021). This is not the case at the gill in vivo. where the epithelial cells are the interface between the water and circulation of the fish and are constantly exposed to fluid shear stress (FFS) generated by movement of water on the apical surface and capillary blood on the basolateral surface (Piiper and Scheid, 1984). FSS is a force typically measured in dynes. One dyne increases the velocity of one gram of matter by one centimetre per second in the direction it is acting. Physiological levels of FSS range from 0.1 dyne/cm^2 for interstitial fluid to > 60 dynes/cm^2 in small arterioles (Ballerman et al., 1998).

The force acts as a mechanosensory trigger for several known changes in cellular behaviour. Examples of such mechanosensory pathways are evident in endothelial and basal epithelial cell studies where increased expression of cell-cell adhesion molecules (Delon et al., 2019), cell polarisation (Jang et al., 2013), changed cytoskeletal arrangement (Flitney et al., 2009), increased membrane fluidity (Haidekker et al., 2000) and gene expression (Snouber et al., 2011: Conway et al., 2010) are altered by shear stress.

When cultured under flow, Madin-Darby canine kidney II (MDCKII) cells transport organic cations at increased rates that correlate with rising levels of shear stress. In addition, human organic cation transporter 2 (OCT2) RNA levels increase under shear stress (Jayagopal et al., 2019). Caco-2 cells exposed to fluid shear stress increased expression of adhesion molecules zonula occludens and tight

junctions (Delon et al., 2019). However, evidence of expression equating to increase in transepithelial resistance or cell-cell tightness is generally contradictory, dependant on very specific levels of FSS and comparison confounded by differing models and exposure conditions between studies (Drieschner et al., 2019: Kim et al., 2012: Tan et al., 2018). One study did report an increase in transepithelial tightness in the lung cell line NCI H441 when grown in a biomimetic microsystem that replicates the alveolar/capillary interface of the human lung. Moreover, the authors reported changes in morphology toward that of lung epithelium in vivo (Huh et al., 2010).

In an intestinal model, Kim et al (2012) mimicked the fluid mechanics of the intestine by growing Caco-2 cells on porous, flexible membranes in a flow-through chamber. This replication of peristaltic intestinal fluid force caused the cells to differentiate and develop intestinal villi and basal crypts. Additionally, fluid movement triggered differentiation of mucus-secretory, entero-endocrine and Paneth cells like those observed in vivo.

A study from Feng et al (2019) showed increased effectiveness of the cancer drug Vandetanib under FSS. The drug reduced cell viability in human umbilical vein endothelial cells under even low levels of shear stress (~ 0.1 dynes/cm^2) when compared to static controls. Additionally, in the specific context of metal toxicity, Conway et al (2010) reported elevated, expression of the metal binding protein, metallothionein in response to shear stress. All these results taken together although not in fish cells, lead to a hypothesis that RTgill-W1 toxicological responses could be altered by FSS and may be altered in the case of exposure to the common river pollutant, copper. In the following experiments, RTgill-W1 cells were exposed to copper under static conditions and with the addition of FSS. The effects of FSS on cell viability in the absence of copper was tested and the effect of each exposure condition on copper transport gene expression and reactive oxygen species production was measured.

2.2 Materials and methods

2.2.1 Flow chamber design and sterilisation

Flow chambers were designed on Tinkercad (Autodesk, California), to fit 12 or 24 - well permeable cell culture inserts (Falcon) and produced on a desktop 3D printer using acrylonitrile styrene acrylate (ASA) filament (Filamentive, Bradford, UK). The chambers were housed in pairs within autoclavable containers and connected to a peristaltic pump (Marlow- Watson, 12-channel). Manifold tubes were connected to medical grade silicone tubing of 1 mm internal diameter cut to desired length allowing for chamber set-up within a sterile tissue culture hood while the pump remained on the bench (Fig. 2).

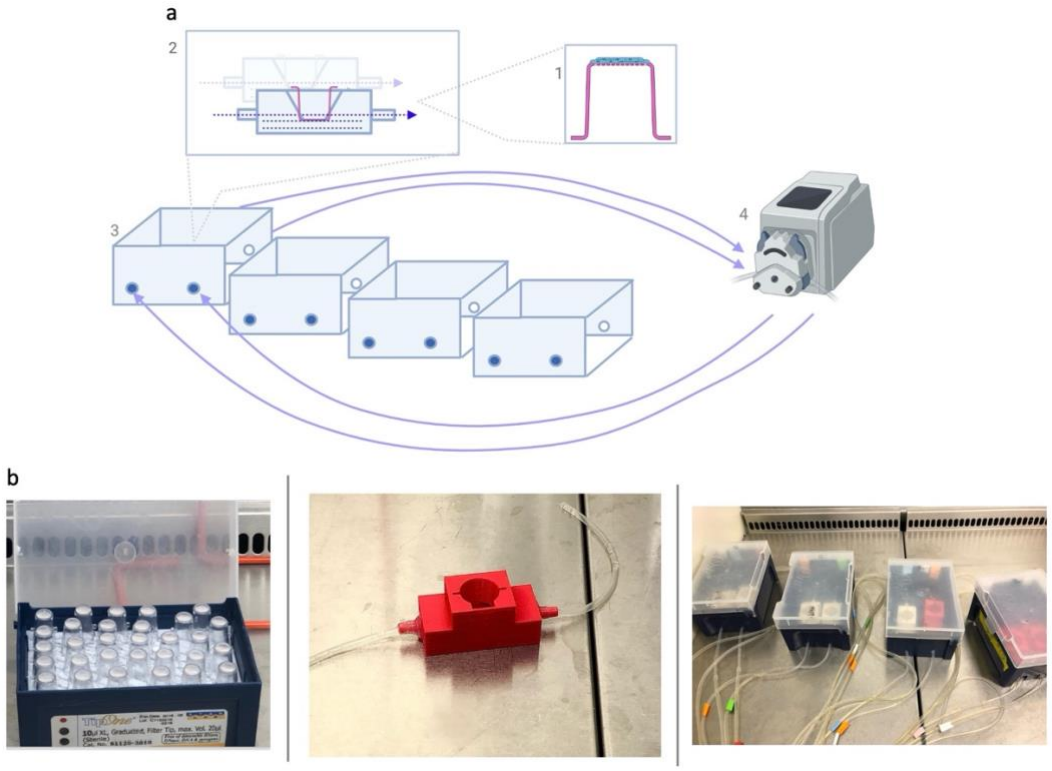


Figure 2. Flow-through system configuration

RTgill-W1 layers were cultured on the underside of cell culture insert (a1), the insert was placed in the chamber where the cell layer is subject to media flow (a2, dotted line, arrow shows flow direction). Two chambers were placed into each autoclavable housing (a3) and each chamber connected to a peristaltic pump (a4) by medical grade silicone tubing (solid blue line, arrow shows direction of flow). Photographs of inverted insert seeding chamber with 3D printed platform, and chambers in housing (b).

To sterilise the chamber and tubing, the assembled flow system was flushed with 70% industrial methylated spirit (IMS) for 10 minutes. The IMS was drained, and the system rinsed through with sterile L-15/ex [a simplified medium without FBS containing the following constituents (mg/L): CaCl2, 40.0; MgCl2, 93.7; Mg SO4, 97.7; KCl, 400.0; KH2PO4, 60.0; NaCl, 8000.0; Na2HPO4, 190.0; D+ Galactose, 900.0 and Na- Pyruvate, 550.0 (Schirmer et al., 1997)] and drained again. Finally, fresh L-15/ex was added and allowed to flush through for ten minutes before a final draining.

2.2.2 Cell culture and insert preparation

Cell line RTgill-W1 was purchased from ATTC (CRL-2523) and passages between 17 and 30 were used throughout these experiments. RTgill-W1 were cultured according to OECD TG249 in Leibovitz media (L15) without phenol red (Gibco, 21,083,027), containing 5% FBS (Sigma Aldrich, F7524) and 0.5% gentamycin (Fisher Scientific, 15,750,060), at 18 ± _1 ◦C. Media was changed every 5 days and confluent 75 cm2 flasks (Corning) were passaged every 12 days. The cells were seeded in L15 with FBS at a density of 400,000 cells/ mL on inverted 12 or 24-well inserts (Schnell et al., 2015). Briefly, inverted inserts were placed in a sterile humidity chamber (Fig. 2b) containing 10 mL of L-15/ex. The insert membrane (0.4 µm pore, 0.9 cm2 effective growth area, polyethylene terephthalate (PET) membrane (BD Falcon, cat. no. 353180) was primed with 100 µL L15 with FBS for 30 min. The L15 with FBS was aspirated, and each insert seeded with 150 µL of a RTgill-W1 suspension for 24-well and 300 µL for 12-well inserts. A dome of suspended cells was created upon each insert (Fig. 2b) and was left to incubate for 24 h after which, cells had attached and media on inserts was aspirated and replaced with 150 µL of fresh L15 with FBS. Seeded inserts were left to grow for a further 24 h.

2.2.3 Flow system set-up

Each box housing (Fig. 2a) received 2 mL of L-15/ex to maintain a humid atmosphere. Approximately 9 mL of L-15/ex was added to the chamber under flow of 2 mL / min generated by peristaltic pump (Fig. 2a) which was calculated according to (Drieschner et al., 2019) as 0.2 dyne / cm^2 ± 0.1 FSS using the following equation where, μ is the viscosity of the medium/liquid, Q is the flow rate, h is the channel height (cm) and w is the channel width (cm).

$$\tau = \frac{6\mu Q}{h^2 w}$$

The chamber and tubing were allowed to fill ensuring removal of trapped air. The seeded inserts were washed by placing in a 24-well companion plate (Falcon) containing sterile L-15/ex. Insert confluence was checked using an inverted microscope and any non-confluent inserts noted and discarded. One hundred and fifty µL of L15 with 5% FBS was added to the inside of each insert

(basolateral to the cell layer). Cells were exposed to L-15/ex or L-15/ex containing CuS04.5H2O at concentrations of 2.6 µM, 1.3 µM, 0.65 µM, 0.325 µM and 0.163 µM Cu. All concentrations reported refer to nominal Cu concentrations, which equates to nominal free Cu^{2+}concentrations of 2.2 µM, 1.1 µM, 0.55 µM, 0.28 µM and 0.14 µM, respectively calculated using ChemEQL (Eawag, Switzerland). Static controls were set up for the same treatments and all exposures lasted 24 h at 18 ± 2 ◦C in ambient CO_2.

2.2.4 Visualisation and TEER

To confirm cellular adherence after exposure to FSS cell layers were stained for actin with a nuclear counterstain. The cell-covered membranes from static and FSS exposures were removed from inserts. Each membrane was washed in PBS and cells fixed with a paraformaldehyde-based solution (10% TritonX-100, sigma and 32% paraformaldehyde in PBS) for 25 minutes. Cells were washed 3 times with 0.1% TritonX-100 in PBS and left in the wash solution for 5 minutes after the final wash step to permeabilise the fixed cells. The membranes were soaked in staining solution containing DAPI (Fisher Scientific) and phalloidin (Alexa Fluor™ _594 conjugate, Thermo Fisher) diluted 1:1000 in PBS for 20 min. After 3 washes with PBS, membranes were gently tapped dry and mounted on slides with round cover slips using DAKO permanent mounting media (Agilent Technologies). Cells were visualised at the centre of each membrane with a Zeiss axio-observer with excitation and emission wavelengths of 586 nm:603 nm and 359:457 nm for phalloidin conjugate and DAPI, respectively. Images were generated from merging DAPI and phalloidin conjugate channels at 200× magnification.

TEER was measured with the Endohm 12 chamber and Evom2 (World Precision Instruments, Hertfordshire, UK). Following either static or FSS 24 h exposures in L15 with FBS or L-15/ex, cell covered inserts were washed with PBS and placed in the Endohm chamber which was pre-filled with 3 mL L-15/ex to act as an electrolyte. The inserts were filled internally with 1 mL L-15/ex and electrode secured for the resistance reading. Three inserts were used for each condition and each measurement carried out in triplicate with a blank insert serving as negative control.

2.2.5 Measuring cell metabolism

Cell metabolism was assessed using the reduction of resazurin to resorufin by NADH and NADPH following the procedures described in OECD 249 TG. Briefly, after 24 h exposures the inserts from the flow chambers were removed into a companion well containing 500 µL PBS. For the static inserts, the exposure media was removed, and both the wells and inserts washed with 500 µL PBS. The PBS was removed, and the cells incubated with 500 µL L-15/ex containing 30 µL resazurin (resazurin sodium salt, Sigma Aldrich, 199,303) working solution (1.5 mg/mL resazurin sodium salt in PBS) and

incubated in the dark at 18 ± _1 ◦C for 30 mins on a rotator. Absorbance was measured using the Fluostar Omega (BMG Labtech, Ortenberg, Germany) plate reader set for absorbance of 570 nM (resorufin) and 600 nM (resazurin).

2.2.6 RNA extraction

All FSS and static insert exposures for gene expression analysis were conducted with 12- well tissue culture inserts to increase RNA yield. Cells were exposed to the two lowest concentrations of Cu (0.163 µM and 0.325 µM). Following exposure, the inserts containing the cells were placed in companion wells and washed with 500 µL PBS. PBS was removed and 350 µL of lysis buffer was added to remove cells from inserts and lyse membranes. mRNA was extracted using SurePrep™ _TrueTotal™ _(Thermo Fisher) plus a DNase (Invitrogen) digestion. RNA quality was checked via nanodrop (Thermo Fisher). cDNA was synthesised from extracted mRNA using iScript™ reverse transcriptase (Bio-rad).

2.2.7 QPCR

Primers were designed using primer blast (www.ncbi.nlm.nih.gov) and according to Rainbow trout gene sequences from GenBank (Table 1). Housekeeping genes selected for stability across treatments were ubiquitin and elongation factor beta (*efb*). Selection was confirmed in the reference gene selection utility of the CFX maestro software (Bio-rad) and normalised to both housekeeping genes. QPCR was carried out using iTaq Universal SYBR Green Supermix (Bio-rad). Reaction protocol followed 40 cycles of 95 ◦C for 5 s and 59 ◦C for 30 s using the CFX connect QPCR machine (Biorad). QPCR relative expression was calculated using 2–ΔΔCt in CFX maestro.

Table 1. List of Primers for Copper Handling and Mechanosensitive Genes

	Forward	Reverse
ubiquitin	GAAGCATTCCACCTGATC	GATGAAGGGTGGACTCTTT
efb	GCTACATCGAGGGGTGGGT	GGTTGTACCAGCGAAGAGCA
mta	ACACCCAGACAACTACTAC	GGTACAAAAGCTATGCTCAA
mtb	GCTCTAAAACTGGCTCTTGC	GTCTAGGCTCAAGATGGTAC
mtf1	CCTCTCAGTACGGTCACAGC	CCTGGGACTGGAACTGG
atp7a	CATGCCGGTGACTAAGAAGC	AATGAGGATCCAGGCGAACA
atox1	ATGTGAGGGATGCTCTGGTG	AGCCTCCTTTCCAGTCTTCT
piezo1	AAGAACCGCAATAGTCCGCA	GGTGCGGTCATTCCACTAGA
sod1	TGGTCCTGTGAAGCTGATTG	TTGTCAGCTCCTGCAGTCAC
sod2	TCCCTGACCTGACCTACGAC	GGCCTCCTCCATTAAACCTC

2.2.8 Reactive oxygen species measurement

Production of ROS under static and FSS conditions in the absence of Cu was compared. Confluent inserts were loaded with 5 µM cell permeant 2’ ,7’ -dichlorodihydrofluorescein diacetate (H2DCFDA, Thermo fisher) in L-15/ex for 90 min. The inserts were washed twice with PBS to remove excess dye and exposed to L-15/ex under static and FSS conditions for 2 h. These shorter exposure times were chosen due to the transient nature of ROS generation. After the exposures, the PET membranes were checked for consistent confluence then cut from the inserts using a new scalpel blade and wet mounted onto a microscope slide, face down. To ensure unbiased analysis of the ROS production a randomisation protocol was developed; Petri dishes where coded and matched to the static and FSS slide mounts which were placed inside. The codes were then hidden with tape, and dishes mixed up by hand. Each chosen Petri dish was coded with another number matched to the photographs taken of the inserts with an inverted microscope (EVOS FL, Invitrogen). Images were captured in triplicate from the centre of the inserts to avoid areas damaged by the removal process. Microscope settings were the same for each image and for quantification the image colour was inverted, the background removed, and the same threshold set for FSS and corresponding static images. The total area above threshold was assessed using ImageJ software.

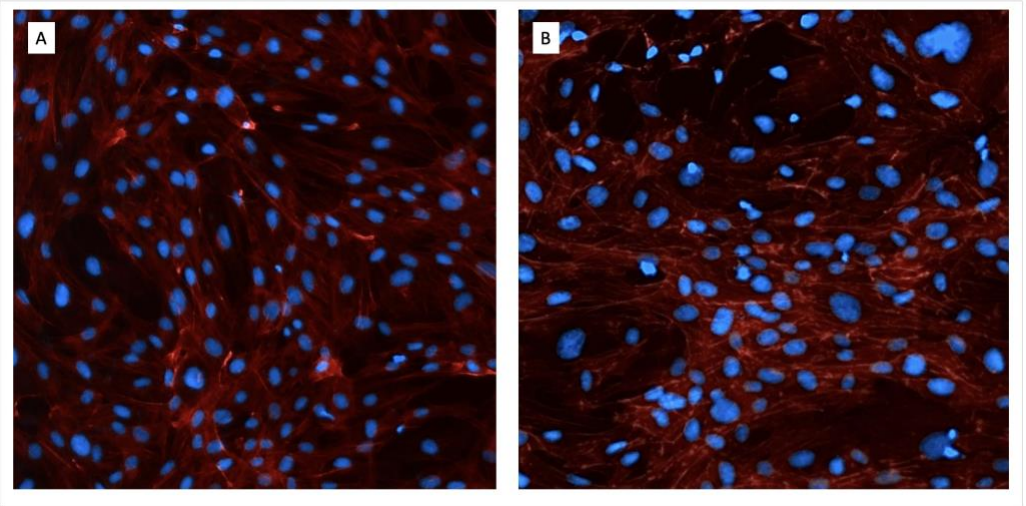
2.2.9 Statistics

All experiments were repeated at least 3 times with separate batches of cells. Statistical analysis was carried out using GraphPad Prism (version 8). Two-way Anova with Holm-Sidak post-hoc test was performed on log transformed data to assess differences between static and flow cell viability data at the different Cu concentrations. A One-way ANOVA with Tukey's post-hoc test was used to assess difference in gene expression under FSS and static Cu exposures. TEER under FSS and static L-15/ex exposures, static and FSS ROS readings were analysed for differences with Welch's *t*-test.

2.3 Results

2.3.1 Cell attachment and TEER

Cells remained attached to the inserts for the duration of FSS exposures (Fig. 3 a, b). There was no significant difference in TEER of cell layers between static and FSS exposed inserts (Fig. 3 c).



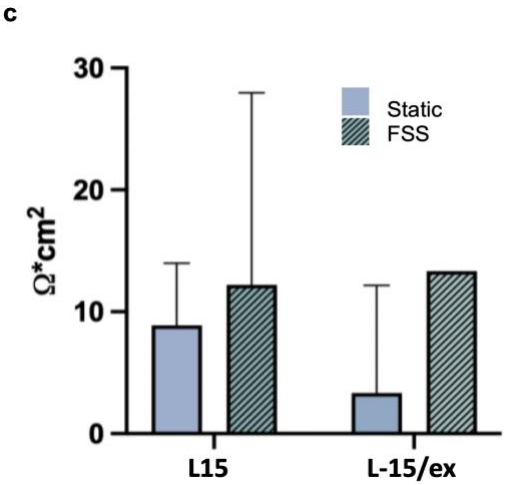


Figure 3. Visualisation of intact RTgill-W1 cell layer and transepithelial resistance

Cells were exposed to L-15/ex under static (a) or fluid shear stress (FSS) conditions (b) for 24 h. To demonstrate enduring cell attachment after FSS exposure, actin was stained with phalloidin (red) and nuclei counterstained with DAPI (blue). Visualisation was carried out with the Zeiss Axio observer at 200× magnification. (c) TEER measurements under static or FSS conditions, values represent an average of 3 measurements from 3 different batches of cells ± standard deviation. FSS L-15/ex showed no plottable error. No significant differences were found in TEER between static and FSS conditions (Welch's *t*-test).

2.3.2 Cell metabolism under static and FSS copper exposure

Cu up to a total concentration of 2.6 µM did not affect cell metabolism under static exposure conditions (Fig. 4). Cell metabolism decreased under FSS in a dose-dependent manner and was significantly reduced in cells exposed to > 0.163 µM Cu (Fig. 4).

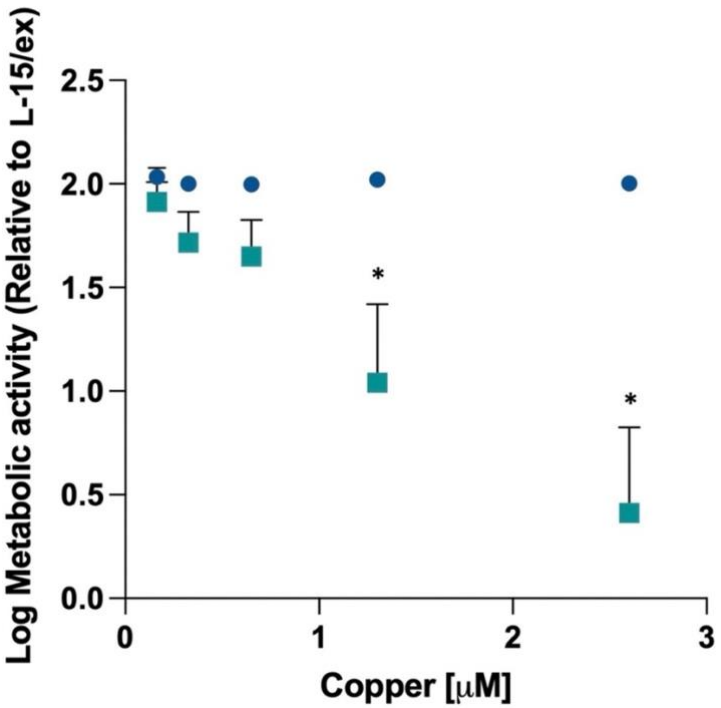


Figure 4. Metabolic activity of RTgill-W1 exposed to copper.

RT-gill W1 cells were exposed to various concentrations of copper (nominal concentrations, 0.163, 0.325, 0.65, 1.3, 2.6 µM) under static (circles) and FSS (squares) conditions. Metabolic activity, measured as the reduction of resazurin, was normalised to the respective measurements in static cells receiving no Cu. Values represent the average ± standard deviation of 3 inserts from different batches. Asterisks denote statistical significance between static and FSS ($p < 0.05$, ANOVA on log-transformed data with a Holm-Sidak post-hoc test). Error bars not shown where standard deviation is in the order of point size. ANOVA and Fishers multiple comparison outcomes presented in Table 2.

Table 2. Figure 4 ANOVA and Fishers Results.

ANOVA	Interaction	Cu Treatment Level	Column (FSS vs.Static)
DF	4	4	1
F	4.9	5.067	29.16
Variation	*	*	*

Multiple comparisons (Fishers)	Cu [µM]				
	0.163	0.325	0.65	1.3	2.6
Static vs. FSS	NS	NS	NS	*	*

2.3.3 Gene expression under fluid shear stress

The genes encoding the Cu transporter, *atp7a*, the mechanosensory ion channel *piezo1* and antioxidant enzymes *sod1* and *sod2* were significantly upregulated under FSS conditions (Fig. 5). *atp7a* showed a 12.7-fold increase and *piezo1* a 2.7-fold increase under FSS relative to the static L-15/ex control. Increases of 2.1- fold and 1.8-fold of *atox1* and *mtf1* respectively were not significant and *mta and mtb* were not affected by FSS alone (Fig. 5).

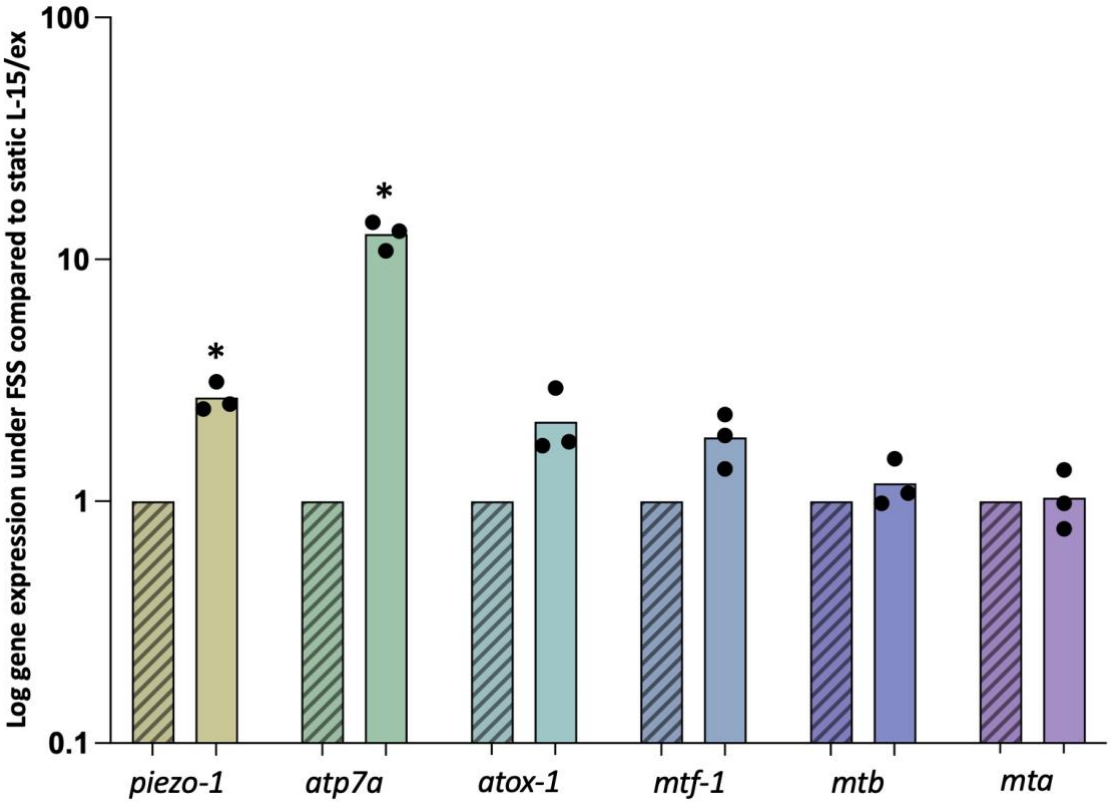


Figure 5. Effects of fluid shear stress on gene expression.

The expression of *atp7a*, *peizo-1*, *atox1*, *mtf-1*, *mta* and *mtb* following 24 h static (hatched column) or FSS L-15/ex exposure (clear column). Values represent individual inserts from different batches and are expressed relative to the corresponding static batch. The bars represent the average of the 3 values and the asterisks indicate significant difference to the static L-15/ex controls ($p < 0.0001$, Welch's t-tests performed on log transformed data).

2.3.4 Gene expression under FSS and copper exposure

Expression of *mta* was upregulated 31.3-fold by static Cu concentrations of 0.163 µM and 11.4-fold by 0.325 µM static Cu (Fig. 6 a). Conversely, there was a 36-fold reduction in *mta* expression under 0.163 µM Cu + FSS and > 50-fold reduction for 0.325 µM Cu under FSS. Differences between static concentrations were highly significant however, differences between extents of downregulation for each concentration under FSS, did not satisfy statistical significance.

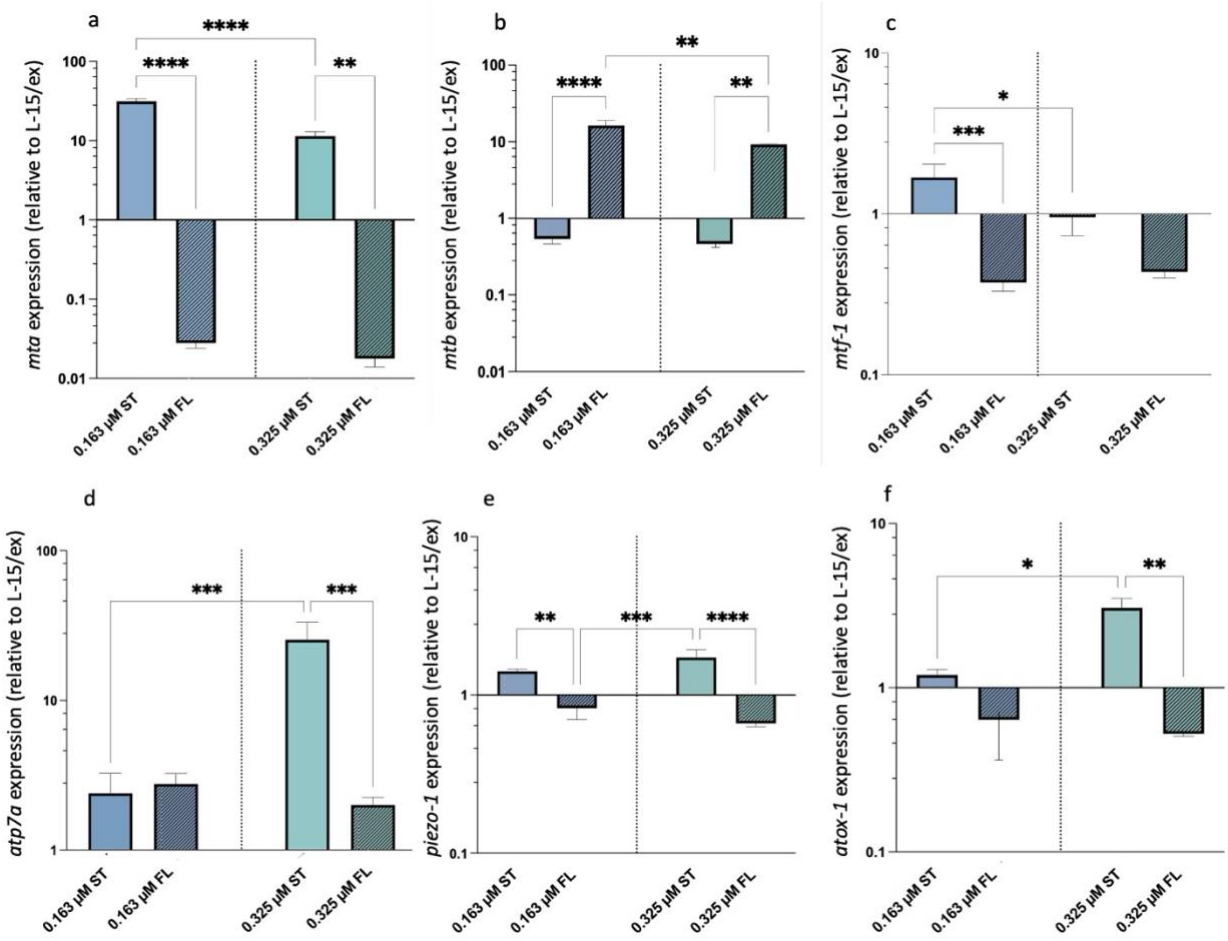


Figure 6. Effects of static and FSS copper exposures on gene expression

The expression of (a) *mta*; (b) *mtb*; (c) *mtf-1*; (d) *atp7a*; (e) *piezo-1* and (f) *atox1* on exposure to 0.163 and 0.325 µM Cu for 24 h under static (solid) or FSS (shaded) conditions. Values represent an average of three measurements of three separate exposures ± standard deviation. Values are normalised to the appropriate static or FSS L-15/ex without Cu controls. Asterisks indicate significant differences ($p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, one way ANOVA with Tukey's post-hoc test). ANOVA results and multiple comparisons (Fishers) are presented in Table 3.

Table 3. Figure 6 ANOVA and Fishers Results

ANOVA	MTA	MTB	MTF1	ATP7A	PIEZO	ATOX1
DF	3	3	3	3	3	3
F	91.79	59.27	25.44	25.25	46.86	13.77
Significance between means	*	*	*	*	*	*

Multiple Comparisons		Sig	DF
MTA	0.138 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM ST	*	8
	0.325 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM FL	*	8
	0.325 µM FL vs. 0.138 µM FL	NS	8
	0.325 µM FL vs. 0.275 µM ST	*	8

Multiple Comparisons		Sig	DF
ATOX1	0.138 µM FL vs. 0.138 µM ST	NS	8
	0.325 µM ST vs. 0.138 µM ST	*	8
	0.325 µM FL vs. 0.138 µM ST	NS	8
	0.325 µM ST vs. 0.138 µM FL	*	8
	0.325 µM FL vs. 0.138 µM FL	NS	8
	0.325 µM FL vs. 0.275 µM ST	*	8

Multiple Comparisons		Sig	DF
MTB	0.138 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM ST	NS	8
	0.325 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM FL	*	8
	0.325 µM FL vs. 0.138 µM FL	*	8
	0.325 µM FL vs. 0.275 µM ST	*	8

Multiple Comparisons		Sig	DF
ATP7A	0.138 µM FL vs. 0.138 µM ST	NS	8
	0.325 µM ST vs. 0.138 µM ST	*	8
	0.325 µM FL vs. 0.138 µM ST	NS	8
	0.325 µM ST vs. 0.138 µM FL	*	8
	0.325 µM FL vs. 0.138 µM FL	NS	8
	0.325 µM FL vs. 0.275 µM ST	*	8

Multiple Comparisons		Sig	DF
MTF1	0.138 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM ST	*	8
	0.325 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM FL	*	8
	0.325 µM FL vs. 0.138 µM FL	NS	8
	0.325 µM FL vs. 0.275 µM ST	NS	8

Multiple Comparisons		Sig	DF
PIEZO	0.138 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM ST	NS	8
	0.325 µM FL vs. 0.138 µM ST	*	8
	0.325 µM ST vs. 0.138 µM FL	*	8
	0.325 µM FL vs. 0.138 µM FL	NS	8
	0.325 µM FL vs. 0.275 µM ST	*	8

Expression *of mtb* was, in contrast to *mta*, upregulated by 0.163 µM and 0.325 µM Cu under FSS by 16.3 and 9.2-fold respectively (Fig. 6 b). Static Cu exposures caused little change in expression with approximately a 2-fold downregulation under both treatment concentrations. Upregulation of *mtf1* was only observed under 0.163 µM static Cu treatment (Fig. 6 c) but was downregulated under FSS by 0.163 µM and 0.325 µM respectively. Upregulation of *atp7a* was observed in all 4 treatments compared to the static L-15/ex control (Fig. 6 d). Static 0.163 µM, FSS 0.163 µM and FSS 0.325 µM caused 2.4-fold, 2.8-fold and 2-fold increases in *atp7a* expression, respectively. Differences between the three were not significant. However, there was a 25.4-fold increase *in atp7a* expression under static 0.325 µM Cu treatment. Static Cu treatments significantly increased *piezo-1* expression while FSS 0.325 µM Cu caused significant downregulation when compared to L-15/ex controls (Fig.6 e). No significant changes in *atox1* expression were observed under static or FSS treatments of 0.163 µM Cu. Static exposure of 0.325 µM increased *atox1* expression 3.1-fold and 0.325 µM Cu + FSS reduced expression by 2-fold (Fig. 6 f).

2.3.5 Reactive oxygen species generation under fluid shear stress

FSS exposed cells were above the threshold set in Image J for ROS detection over an average of 34% ± 2.8% of their area (Fig. 7 b), while images of static exposures were 8.4% ± 3.4% over threshold (Fig. 7 a). FSS induced increases in ROS were significant when compared to static exposures Fig 7 c). Superoxide dismutase, was upregulated under FSS by 22 % and 27 % for sod1 and sod 2 respectively. Fluorescent H2DCFDA images show ROS intensity (H2DCFDA green) (Fig. 7 a, b)

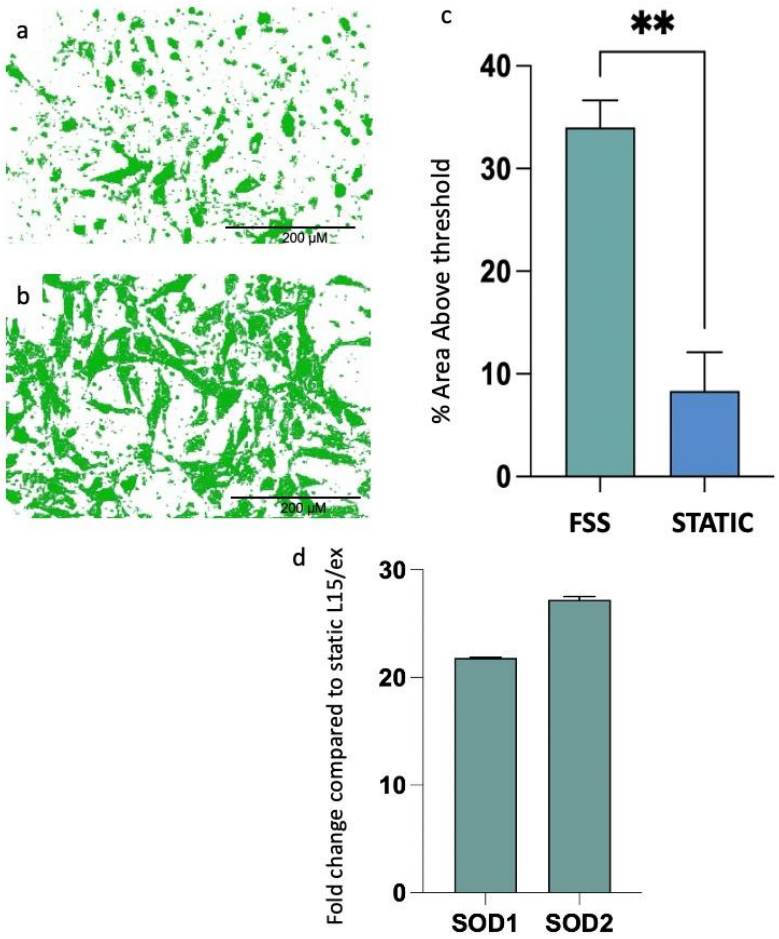


Figure 7. Reactive oxygen species and superoxide dismutase expression

Image J particle analysis for ROS in cells following 24 h exposure to L-15/ex under static (a) and FSS conditions (b). Quantification of ROS detection in static and FSS exposed cells presented as percent of area over set threshold determined by removal of background signal in ImageJ (c). values are average of images of three separate inserts from three separate exposures. The expression of sod1 and 2 in L-15/ex under FSS expressed relative to static L-15/ex (d). Values represent average ± standard deviation from 3 separate experiments. Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.001$, Welch's t-test).

2.4 Discussion

2.4.1 Adherence and TEER

Cell layers grown on solid surfaces and permeable supports often do not demonstrate the morphology or function of native cells in vivo. However, there are examples where culturing cell lines under FSS or membrane stretch has shown changes in cell morphology and functionality that better represent the intact tissue (Huh et al., 2010; Kim et al., 2012; Tan et al., 2018; Delon et al., 2019; Drieschner et al., 2019; Jayagopal et al., 2019). and even affect the potency of drugs and toxins (Feng et al., 2019). It was hypothesised that introducing FSS to RTgill-W1 exposures by applying flow, may alter cellular activities such as copper handling and TEER to better reflect that measured in intact tissue and primary gill cell cultures (Schnell et al., 2015). FSS did not affect the adhesion of the cells to the inserts (Fig. 3a, b) and there was no change in TEER over the 24 h FSS exposure when compared to static cultures (Fig. 3c). The level of FSS or exposure time may have been insufficient to increase TEER or additional stimulus could be required on the basolateral surface to replicate capillary blood flow present in vivo. It is also possible the cultures, while of heterogenous cellular composition (Lee et al. 2009), lack endocrine stimuli responsible for maintaining TEER.

2.4.2 The effects of fluid shear stress in the absence of Cu

Fluid shear stress without Cu treatment, significantly increased levels of *piezo1*, *atp7a*, (Fig. 5) and both *sod1* and *sod2* expression (Fig. 7) compared with static no-Cu treatment. The *piezo1* upregulation shown in this study agrees with previous work in which shear stress upregulated *piezo1* in murine osteoblastic cell line MC3T3-E1 (e.g. Song et al., 2020). The mechanism that translates shear stress to *piezo1* upregulation was not investigated in the present study, however, Lee et al. (2021) discovered that increased *piezo1* expression was facilitated through p38 MAP-kinase activity and transcription factor Atf2 binding directly to the *piezo1* promotor region. Studies in human and animal primary cells and cell lines have demonstrated elevated *piezo1* RNA correlates to an increase in Piezo1 protein synthesis (Caolo et al., 2020; Zhang et al., 2021). Thus, the increase in *piezo1* expression in the present study infers an increase *in piezo1* translation in the absence of protein quantification.

Ca^{2+} readily permeates gated Piezo1 channels (Ilkan et al., 2017; Gnanasambandam et al., 2015; Kuchel et al., 2021; Lee et al., 2021). For example, a *PIEZO1* knockout in HUAECs greatly inhibited shear stress-induced calcium influx (Wang et al., 2016) and the application of Yoda1, a *PIEZO1* agonist, caused a 170% increase in Ca^{2+} transient influx in human platelets. An elevated number of FSS activated Piezo1 channels (Coste et al., 2012) can increase intracellular Ca^{2+} (Liao et al., 2021).

This rise in cellular Ca^{2+} has been shown to induce ROS production (Santulli et al., 2015). It is possible that this was the case in RTgill-W1 under FSS where we observe elevated ROS production and expression of *sod1* and *sod2* in response to FSS (Fig. 7). Superoxide dismutase enzymes are known antioxidants whilst *Atp7a*, which was upregulated 30-fold by FSS (Fig. 5), has been shown to have an antioxidant role in mouse endothelium where calcium influx causes the transporter to activate extracellular SOD by presenting the Cu cofactors for enzyme activity (Sudhahar et al., 2013). In addition, a study of *Atp7a* knockout mouse embryonic fibroblasts showed increased sensitivity to oxidative stress demonstrating a significant role in this transporter in defence against ROS (Zhu et al., 2017) These findings taken with those in the present study suggest FSS-induced oxidative stress in RTgill-W1 is likely due to an increase in Ca^{2+} influx through Peizo1.

2.4.3 The metabolic effects of fluid shear stress and Cu

Metabolic activity was not reduced in cells exposed at 0.163 µM to 2.6 µM total Cu under static conditions but, was significantly reduced under FSS and exposure to Cu > 1.3 µM (Fig. 4). In static conditions, a study reported 5 µM total Cu reduced cell viability in RTgill-W1 (Bopp et al.,2008) with an EC50 of 29.2 µM Cu. Scott et al. (2020) showed a drop-in metabolic activity with an EC50 of 3.85 µM total Cu following 24 h exposure. These differences in response to copper concentrations when compared to one another and to our observations maybe due to the varied exposure times (2.5 h) and use of different exposure medium (a modified Earle's medium) with different copper binding properties. These studies also base their findings on measured Cu^{2+} concentrations, whilst in the current study we report nominal concentrations. Cu may bind to the plastic surfaces of the companion well and insert and Scott et al. (2020) reported a drop of Cu concentrations of 15% over 24 h in static conditions. It is unlikely that this accounts for discrepancies here as we observed increased toxicity, and such binding would artificially increase these values rather than reducing them. These points considered, a significant caveat to the observation that Cu toxicity is enhanced under FSS (Fig. 4) is the need to confirm the exposure concentration analytically. Again, the flow-through chamber is a closed system (Fig. 2) with a much larger surface area to that of a static chamber and a greater potential to adsorb Cu and reduce the exposure concentration. Thus, the effect concentration may be further reduced under FSS. A thinner unstirred layer apical to the cells under FSS than that observed in static exposures would enhance the permeation rate of Cu (Stoker, 1973) and increase toxicity so modelling the fluid dynamics at play could yield insight but was not in the scope of this study.

The cell maintains tight regulation of Cu and prevents a rise in intracellular free Cu^{2+} under homeostatic conditions, yet oxidative environments can reduce the Cu binding capacity of MT and allow release of Cu from the MT-Cu complex (Fabisiak et al., 1999). ROS induced by Cu + FSS

treatments could reduce cell viability through a combination of disruption to Cu control mechanisms together with FFS induced oxidative stress as demonstrated in the no Cu FSS test (Fig. 7).

2.4.4 The effects of fluid shear stress and Cu on gene expression

At low copper concentrations it is generally accepted that excess intracellular Cu is controlled via high affinity metal binding molecules such as MT (Gudekar et al., 2020). At higher concentrations of Cu, these metal binding proteins become saturated, and synthesis of new MT is unable to keep up with the influx. Here alternative detoxification processes are required. Such as cellular exocytosis where Cu is passed from ATOX1 to the trans Golgi network-associated ATP7A, forming Cu-rich vesicles for transfer to the cell membrane for excretion (Dameron and Harrison, 1998). The gene expression analysis from Cu exposure under static conditions concurs with these hypotheses. For example, at the metabolically inactive lower concentration of 0.163 µM static Cu, a value that also produces no effect under FSS (Fig. 4), there is a greater expression of *mtf1* and *mta*, and at the higher concentration of 0.325 µM static Cu, the lowest metabolically effective concentration under FSS conditions (Fig. 4), gene expression of *mtf1* and *atp7a* was greater than at 0.163 µM Cu (Fig. 5). In static conditions, *mtb* gene expression did not change with exposure to Cu, although *mtf1*, a metal transcription factor present in the promoter region of the *mtb* gene, expression increased (Fig. 5 c). This contradicts studies on primary fish gill cells where 0.6 µM Cu induced *mtb* expression (Walker et al., 2007). In contrast, a rainbow trout gut cell line showed no significant induction of *mtb* after 24-h exposures to either 0.3 µM or 0.6 µM Cu compared to L-15/ex controls (Ibrahim et al., 2020). Moreover, Cu uptake via the gills in live fish, was shown to peak and then subside, all within 10 h of Cu exposure (Kamunde et al., 2002). It may be that after 24-h of exposure to static, non-toxic concentrations, *mtb* expression in RTgill-W1 has saturated and subsequently waned.

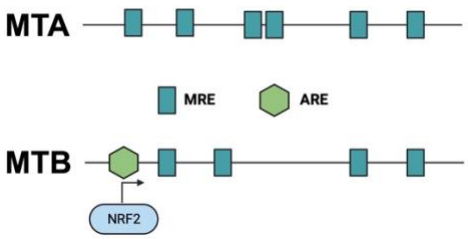


Figure 8. Metallothionein response elements and ARE-Nrf2 system

Metal response elements and antioxidant response elements illustrated on simplified *mta* and *mtb* genes. The mechanically activated transcription factor Nrf2 targets the antioxidant response element when activated by mechanical stimulus (Image modified from Bury et al., 2008).

At gene level, rainbow trout *mta* contains two more metal response elements (MRE) than *mtb* (Fig. 8), while *mtb* possesses an antioxidant response element (ARE) not seen in *mta* (Zafarullah et al., 1990; Olsson et al., 1995; Samson et al., 2001; Bury et al., 2008). Thus, *mtb* may be responding to oxidative stress via the ARE following Cu + FSS exposure rather than directly to Cu and is supported by the observation by Samson et al. (2001) of a functional oxidant responsive element in the rainbow trout *mtb* promoter. The existence of mechano-sensing transcription factors such as nuclear factor (erythroid-derived 2)-like (Nrf2), which target the ARE in response to mechanical cues (Takabe et al., 2011), also supports this hypothesis. Together, these factors may explain the absence of *mtb* response to static Cu exposure and the strong response under mechanical stimulus observed in this study. One could posit that crosstalk between the MRE-targeting transcription factor *mtf1* and the Nrf2-ARE system, may upregulate *mtb* gene expression (Fig. 5). There is evidence that piezo-1 can itself activate Nrf2 in response to FSS. Further explanation may lay in the differences in *mta* and *mtb* gene responsive elements where *mtb* possesses an ARE (Zafarullah et al., 1990; Olsson et al., 1995; Samson et al., 2001; Bury et al., 2008). In this scenario the induction of ARE and MRE transcription factors results in a greater stimulus of *mtb* expression, however a time-course study covering the early stages of exposure to both Cu and FSS would shed light on any choreographed genetic response between these two pathways.

The expression of *piezo-1* appears to have been downregulated by 24-hour Cu + FSS exposures (Fig. 6 e) while the gene was significantly upregulated by FSS alone (Fig. 5). It can be speculated that this was a protective mechanism to reduce stretch activated influx of Ca^{2+} and Cu or purely a consequence of ROS-mediated dysregulation of constitutive *piezo-1* expression by combined FSS and Cu driven ROS production. It would be interesting to measure expression from the addition of the Cu and at regular intervals up to 24 hours.

These results demonstrate toxicologically relevant differences in the response of RTgill-W1 to Cu exposures under FSS when compared to static assays. RTgill-W1 use in static toxicity tests, such as the OECD TG249, has been accepted as a surrogate for acute fish toxicity assessment due to a good corelation between in vitro and in vivo toxicity. However, while static RTgill-W1 tests correlate to fish lethality, nominal concentrations of 1.65 µM Cu have been shown to affect gill function and structure in vivo (Heerden et al., 2004). Therefore, alternative dynamic exposures may reflect sub-lethal effects on the gill in vivo and this can be explored in future work. Nonetheless, it is interesting that the addition of a stimulus found at the gills in vivo, results in a significantly lower effect concentration in RTgill-W1 when compared to static exposure.

Chapter 3

New approaches to predicting toxicity and reducing FBS use in routine cell culture

Abstract

The interaction between a chemical and its targets in the cell and throughout the levels of biological organisation can be summarised in a conceptual framework known as an adverse outcome pathway (AOP). Known AOPs could be used to predict chemical toxicity of new and untested legacy chemicals that share characteristics with reference compounds, reducing the need for live fish testing for chemical registration purposes. The adoption of a growing number of cell-based techniques creates pressure to reduce use of foetal bovine serum (FBS) in cell culture in alignment with ethical expectations and initiatives to reduce animal use in toxicity testing.

This work tested the ability of gene expression analysis by QPCR to identify cellular events in RTgill-W1 from known AOPs for cadmium, a heavy metal, benzo [a] pyrene, a polyaromatic hydrocarbon and clotrimazole, a pharmaceutical antiproliferative. In addition, RTgill-W1 cultures were reduced to 1% FBS supplementation and compared to standard 5% FBS cultures in 3,4-dichloroanaline toxicity curves.

RTgill-W1 gene expression alone could not predict adverse outcome pathways for Benzo [a] pyrene, cadmium or clotrimazole. However, RTgill-W1 cells cultured with 1% FBS exhibited the same responses as 5% FBS cultures in DCA curves. An 80% reduction in FBS use presents a speedy, accessible solution for reducing use of animal products in toxicity testing without special reagents or expensive proprietary FBS alternatives. QPCR of know AOP genes combined with additional assays carried out on a range of cell line models could be more predictive of AOPs.

Chapter 3 New approaches to predicting toxicity and reducing FBS use in routine cell culture.

3.1 Introduction

RTgill-W1 was utilised in a national toxicology program "Toxicology in the 21st Century" (Tox21) along with 13 mammalian cell lines to predict toxicity of chemicals to humans through multiple endpoints (Tice et al., 2013). These cell-based tests characterised chemical toxicity to humans more efficiently than traditional live animal tests such as those using rodents. This approach could be applied to predicting chemical toxicity to fish and therefore freshwater ecosystems while providing more information about the mediators of such toxicity than is acquired from acute fish testing and basic cell viability testing with RTgill-W1

The physiological impact of a substance begins with a key molecular initiating event at sub-cellular level. This trigger initiates a chain of causally linked events that fit within an adverse outcome pathway (AOP), a conceptual framework spanning the biological levels of organisation from molecular to organism and population level (Fig. 9). Identification of events in AOPs can help predict chemical toxicity in specific systems and with the aid of in-silico methods, the toxicity of untested compounds with similar structures to those with known AOPs. These frameworks could be applied to regulatory decision-making activities such as chemical registration and chemical research and development (Coady et al. 2019). Cellular AOP events can be identified through gene expression analysis and assays that measure enzyme activity and accumulation and flux of biomarkers like calcium, zinc and reactive oxygen species (Li et al. 2023; Nzengue et al. 2011).

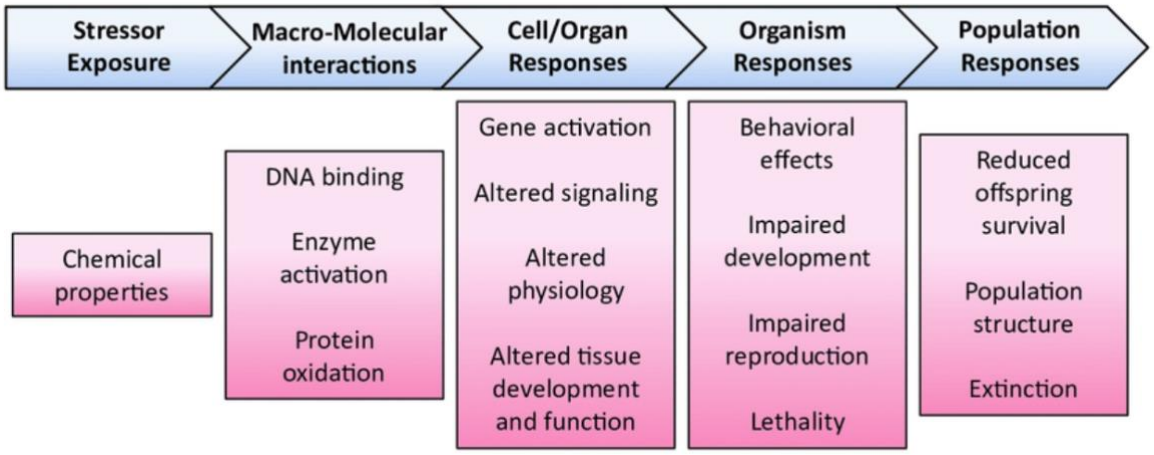


Figure 9. Representation of the adverse outcome pathway structure

Biological events (pink) that occur at each level of biological organisation (blue) throughout an adverse outcome pathway (figure, Connon et al. 2019).

3.1.1 Gene expression analysis to determine adverse outcome pathways

Gene activation and expression at the cell level of organisation in an AOP (Fig. 9) is more ecologically relevant than the more sensitive subcellular molecular interactions between a chemical and its first molecular target (Connon et al., 2019). In addition, the cellular level of organisation is the most easily replicated in vitro with minimal animal or animal product use.

Some examples of known AOP gene expression events in different chemical classes include the expression of *znt1,* a zinc transporter responsible for cadmium-driven zinc efflux from the cell (Chouchene et al. 2011) and an indicator of metal transcription factor *mtf1* activation (Langmade et al. 2000) in the cadmium AOP. In addition, benzo (a) pyrene (BAP) is a potent activator of cytochrome P450 (cyp) expression in most cellular systems (Franco, Sutherland and Lavado, 2018; Schirmer et al. 1998) where it is responsible for production of carcinogenic, genotoxic metabolites. Cyp1a1 is the most potent BAP bioactivator of this protease enzyme family (Schwarz, 2001). BAP has also been shown to reduce expression of oestrogenic biomarker vitellogenin, through endoplasmic reticulum antagonism (Liang et al. 2004; Petersen and Tollefsen, 2012; Nicolas 1999). The pharmaceutical fungicide clotrimazole induces apoptosis through an unfolded protein response and resulting endoplasmic reticulum stress involving increased expression of the chop gene (Isler et al. 2005). Additionally, Clotrimazole and other imidazoles have been shown to disrupt cyp activity (Bart and Scott, 2018).

3.1.2 Foetal bovine serum in testing

Relying heavily on cell cultures and predictive constructs to reduce animal testing naturally draws attention to the enduring reliance on foetal bovine serum (FBS) for culturing many cell lines, including RTgill-W1. Reduction in FBS use is driven by two main concepts; the close association of FBS production with aspects of the dairy industry, now broadly considered ethically unacceptable, and the ill-defined nature of the FBS itself.

Foetal bovine serum contains a cocktail of growth factors, hormones, and unidentified molecules. Many cell lines require such serum for attachment and cell spreading although the specific contributing factors in FBS that mediate these essential functions in different cell lines remain, in many cases, unknown. Factors affecting the performance of cell cultures in toxicity testing should be considered when developing new animal-free culture protocols and media formulations. For example, FBS reduced toxic effects of lead on human fibroblasts (DomíNguez et al. 2002) and cytotoxicity of zinc on murine fibroblast-derived cell line L929 was reduced as FBS concentration increased from 5 % to 10 % (Jablonská et al. 2021).

The albumin found in FBS at a concentration of ~ 4.5 mg/mL contains discreet binding sites with varied affinities for metal ions and other pharmaceutical and toxic compounds (Alhazmi et al. 2023). In a defined medium, free concentration of a test chemical can be modelled or calculated using speciation software. This is not possible when an undefined serum is present in the medium.

The ill-defined nature of FBS and batch to batch variability pose issues for reproducibility and cause difficulty in the formulation of animal- free alternatives. In addition, the differing requirements of individual cell lines rule out the solution being one single formulation. However, there has been some success culturing bovine muscle satellite cells (Stout et al. 2022) and human stem cells (Parker et al. 2007) without serum or with chemically defined serum replacements. Cultures used in regenerative medicine are often animal-free by necessity to rule out introduction of immunogenic molecules. Comparatively, there has been little success culturing RTgill-W1 under serum-free conditions. While the use of the serum-free exposure medium L-15/ex (Schirmer et al. 1997) allows for more accurate calculations of bioavailability of a test chemical in toxicity exposures, RTgill-W1 cells only remain viable in the simplified medium for 48 - 72 hours. This short window rules out FBS-free culturing and arguably does not allow for acclimatisation or for the cells to be considered free of the effects of the culturing serum at the point of test chemical treatment.

The aim of this experiment was to address these ethical and practical issues by gradually weaning RTgill-W1 cultures down from a standard 5% FBS, while the standard 5% cultures were used to test

the predictive power of QPCR to indicate known AOPs of three chemicals. For context, an aim for future work was to utilise gene analysis data acquired from established and characterised reduced FBS cultures.

3.2 Materials and methods

3.2.1 Cell culture

RTgill-W1 cultures were grown according to OECD TG249 at 18°C ±1 with media changes every 5 days and passaged every 12 days at 100% confluence. For standard culture conditions, Leibovitz (Gibco, 21,083,027) media without phenol red was supplemented with 5% foetal bovine serum and 0.5% gentamycin. Cells between passage 10 and 40 were used throughout this study.

3.2.2 Establishment of model chemical concentrations

Three toxins from different classes were chosen to test the predictive power of the QPCR analysis for AOPs: Cadmium, a heavy metal, BAP, an aromatic hydrocarbon, and clotrimazole, an antiproliferative fungicide.

Two test concentrations of each model toxin were chosen for further study. Toxicity curves were established for cadmium as per OECD TG249. Briefly, 6 concentrations were applied to wells seeded with RTgill-W1 at a density of 350,000 cells per well in triplicate. After 24-hours cell metabolism was measured against negative controls using resazurin to resorufin transformation. Fluorescence of resorufin was quantified by plate reader (BMG Labtech, fluostar). An EC50 and EC20 were calculated as 2 μM and 0.7 μM respectively (Fig.10).

This preliminary work was conducted in parallel with colleagues at the University of Gothenburg who were conducting similar tests with fish cell lines and provided some preliminary concentration guidance that was used in conjunction with available literature (Stadnicka-Michalak et al., 2018) to establish initial concentrations of clotrimazole and BAP. Clotrimazole concentrations of 4 μM and 0.4 μM and BAP concentrations of 10 μM and 1 μM were chosen for further study.

3.2.3 QPCR

Primers were designed using primer blast (www.ncbi.nlm.nih.gov) and according to Rainbow trout gene sequences from GenBank (Table 4). Housekeeping genes selected for stability across treatments were ubiquitin and elongation factor beta (*efb*). Selection was confirmed in the reference gene selection utility of the CFX maestro software (Bio-rad) and normalised to both housekeeping genes. QPCR was carried out using iTaq Universal SYBR Green Supermix (Bio-rad). Reaction protocol followed 40 cycles of 95 ◦C for 5 s and 59 ◦C for 30 s using the CFX connect QPCR machine (Biorad). QPCR relative expression was calculated using 2–ΔΔCt in CFX maestro. The following primers were used to analyse genes known as biomarkers of specific AOPs.

Table 4. List of Primers for AOP Marker Genes

	Forward	Reverse
vitellogenin	GAGCTAAGGTCCGCACAATTG	GGGAAACAGGGAAAGCTTCAA
cyp1a1	TGACCCGGAGCTGTGGAAGGAG	CAGCCTTTGGAGCAGGATGGCC
znt1	CGGACTCGTTCCATATGCTAT	ACCACGATGACAGAGCCCAG
chop	AACGCATACGCATTTGGAAG	GGTCTTGTCGCAGGTTTCTATC

3.2.4 Reduction of FBS in RTgill-W1 Cultures

Standard 5 % FBS cultures were initially weaned by a 0.5% reduction in FBS every 5 days when media was changed. Direct reductions were less time consuming and did not appear to affect cultures more than gradual reductions. FastGro (MP Biological, Worcester, MA) is a synthetic defined FBS replacement containing no animal derived compounds or growth factors derived from any source. FastGro was substituted for a percentage of FBS in concentrations from 2.5 % to 10 % in conjunction with FBS in concentrations from 5 % to 0.5 % (Table 5). Viable trials were cultured for at least 30 days with media changes every 5 days.

Table 5. List of FBS Reduction Trials

	FBS %	FastGro %
Standard Culture	5 %	None
Trial 1	2.5 %	2.5 %
Trial 2	2.5 %	5 %
Trial 3	1 %	10 %
Trial 4	0.5 %	10 %
Trial 5	1%	None

3.2.5 Comparison of reduced FBS and standard cell culture responses to 3,4- dichloroaniline

Reduced FBS cells (1% FBS) and standard culture RTgill-W1 cells (5% FBS) were seeded onto separate 24-well plates at a density of 350,000 cells/well and allowed to adhere and grow for 24 hours. Adhered cell layers were washed with PBS and treated in triplicate according to OECD test guideline 249 with 3,4-dichloroaniline at concentrations of 100 mg/L, 50 mg/L, 25 mg/L, 12.5 mg/L , 6.25 mg/L and 3.125 mg/L. After 24-hour exposures, treatments were aspirated, cell layers washed with PBS and 500 µL L-15/ex containing 30 µL resazurin (resazurin sodium salt, Sigma Aldrich, 199,303) working solution (1.5 mg/mL resazurin sodium salt in PBS) added to each well. Plates were placed onto a rotator in the dark and left for 30 minutes after which resazurin was aspirated and replaced with L-15/ex. Conversion of resazurin to resorufin in the mitochondria was quantified by measuring resorufin fluorescence with the Fluostar Omega (BMG Labtech, Ortenberg, Germany) plate reader set for excitation and emission wavelengths of 571 nm and 584 nm respectively.

3.3 Results

3.3.1 Cadmium toxicity curve

Cadmium effect concentrations in RTgill-W1 were established as 0.7 µM and 2 µM for EC20 and EC50 respectively (Fig. 10). Effect concentrations were calculated in GraphPad Prism (version 8).

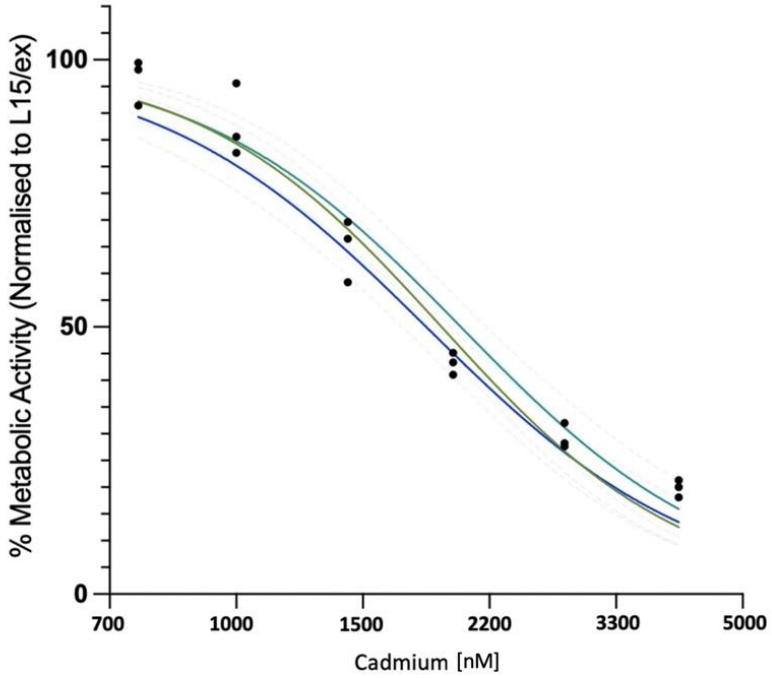


Figure 10. Cadmium toxicity curve

Each data point (circle) represents the mean of triplicate wells from one assay repeat (n=3). Lines show non-linear fit for metabolism inhibition vs normalised response with variable slope (Graphpad, Prism version 8) for each assay. R squared 0.976, 0.955, 0.956.

3.3.2 Gene expression

Cyp1a1 expression was 100 times greater than L15/ex controls in the presence of 1 µM and 10 µM BaP (Fig. 11 b). Downregulation of znt1 expression was observed at 1 µM BaP, while chop, piezo and vtg expression was not significantly affected (Fig. 11 b). Twenty-fold downregulation of znt1 by 1 µM and 10 µM cadmium was evident while chop, cyp1a1 and vtg were not affected when compared to controls (Fig. 11 a). Clotrimazole downregulated znt1 at 10 µM while 1 µM had no effect. In addition, chop, cyp1a1, piezo1 and vtg were not significantly affected (Fig. 11 c). Inter-run errors and some intra-run errors were unusually high for some of the genes analysed. While figures indicate genetic upregulation or downregulation (Fig. 11) and no asterisk, effects are not statistically significant and should be interpreted with discretion.

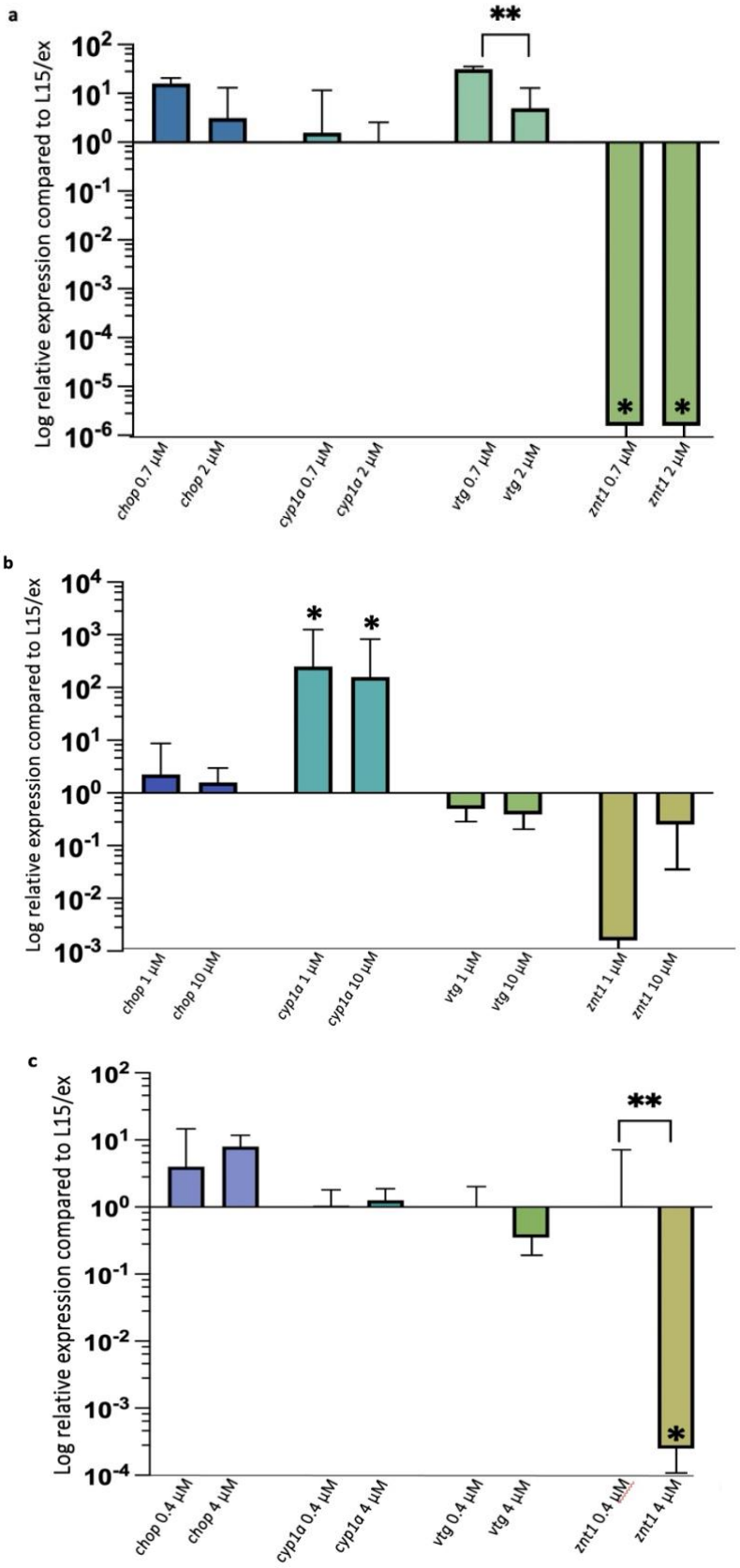


Figure 11. Gene expression under chemical exposures

Expression of genes associated with modes of action and adverse outcome pathways under 24-hour exposures to cadmium (a), benzo [a] pyrene (b), clotrimazole (c).

Values represent means of three exposures. Asterisks denote statistical significance (t-tests [treated vs. control], ANOVA with Fishers * p< 0.05, ** p< 0.01). ANOVA and Fishers results are presented in Table 6.

Table 6. Figure 11 ANOVA and Fishers Results

ANOVA	Cadmium	BAP	Clotrimazole
DF	3	3	3
F	11.25	0.34	1.9
Significance between gene means	*	NS	NS

Multiple Comparisons (Fishers)		0.7 µM	2 µM	0.7 vs 2 µM
Cadmium	Chop vs. Cyp	*	NS	Chop NS
	Chop vs. Vit	*	NS	Cyp NS
	Chop vs. Znt1	*	NS	Vit *
	Cyp vs. Vit	*	NS	Znt NS
	Cyp vs. Znt	NS	NS	
	Vit vs. Znt	*	NS	
		1 µM	10 µM	1 vs. 10 µM
BAP	Chop vs. Cyp	NS	NS	Chop NS
	Chop vs. Vit	NS	NS	Cyp NS
	Chop vs. Znt1	NS	NS	Vit NS
	Cyp vs. Vit	NS	NS	Znt NS
	Cyp vs. Znt	NS	NS	
	Vit vs. Znt	NS	NS	
		0.4 µM	4 µM	0.4 vs. 4 µM
Clot	Chop vs. Cyp	NS	NS	Chop NS
	Chop vs. Vit	NS	*	Cyp NS
	Chop vs. Znt1	NS	*	Vit NS
	Cyp vs. Vit	NS	*	Znt *
	Cyp vs. Znt	NS	NS	
	Vit vs. Znt	NS	NS	

3.3.3 Reduction of FBS in culture medium

RTgill-W1 cells cultured in 1% FBS demonstrated normal adherence although doubling time was increased by 50% (Fig. 12a). Cells cultured at 1% FBS showed no significant deviation in metabolic activity when compared to standard 5% FBS cells in DCA tests (Fig. 12b). The cells were frozen down and successfully thawed after a week stored at -80 °C.

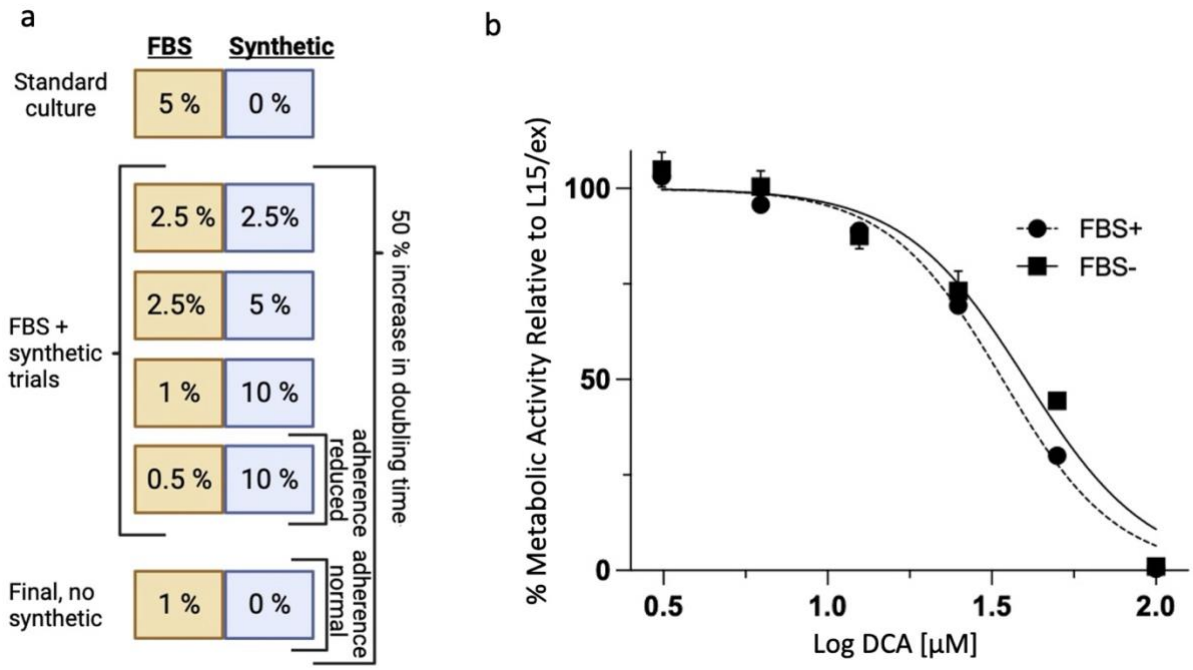


Figure 12. Reduction of FBS in RTgill-W1 Cultures

Cultures of RTgill-W1 were cultured with reduced FBS. (a) Visualisation of trials conducted with reduced FBS (brown) and increasing concentrations of synthetic serum (FastGro, MP Biomedical, [blue]). (b) Adherent cultures with 1 % FBS (FBS-) supplementation and no synthetic supplementation were compared to standard 5 % FBS (FBS+) cultures in a 24 hr DCA toxicity curve. The responses of the two culture methods were not statistically different for any tested concentration (Unpaired t-tests with Welch's correction). Points represent the mean of three individual tests, bars show SEM. Lines show non-linear fit for metabolism inhibition vs. normalised response with variable slope, (R^2 0.978 FBS+, 0.966 FBS - [Graphpad, Prism version 8]).

3.4 Discussion

3.4.1 Gene expression adherence to known AOPs

An 80% reduction of FBS in RTgill-W1 culture offers simple, rapid FBS reduction in toxicity testing with RTgill-W1. Moreover, the expression patterns suggested by this preliminary work present insight into the suitability of RTgill-W1 for predicting AOPs.

Some gene expression results in the present study agree with the accepted AOPs of the toxins tested, while some deviate. For example, while increased expression and activity of cyp1a1 in response to BaP is expected in whole fish (Williams and Hubberstey, 2014) and some hepatic cell lines (Franco, Sutherland and Lavado, 2018; Schirmer et al. 1998), BaP-induced cyp1a1 activity has not been detected in RTgill-W1. Therefore, the 9-fold increase in cyp1a1 expression (Fig. 11b) suggests AhR activation by the BaP ligand, but an absence of cyp1a1 translation. This is an indication of unsuitability of RTgill-W1 in predicting AOPs for potential cytochrome enzyme substrates including PAHs and an indication of requirement of additional cell line models.

Typically, znt1 expression in zebrafish gill increases in response to zinc supplementation and is reduced in instances of zinc depletion (Zheng et al. 2008). In a carp epithelial cell line zinc and cadmium (~ 0.6, 3, 15 μM total Cd) increased znt1 expression to similar levels (Muylle et al. 2006. Although, accounting for the binding properties of the Leibovitz medium used in exposure it can be inferred that znt1 expression is increased by <0.6 μM free Cd. In addition, cadmium exposure increases znt1 expression in rainbow trout gut cell line RTgillGC (Ibrahim et al. 2020) but reduces znt1 expression in zebrafish ovaries (Chouchene et al. 2011). Taken in the context of these findings, downregulation of znt1 in RTgill-W1 in response to 0.2 and 0.7 μM free cadmium (Fig. 8a) is unusual. However, given the varied tissues, and organ derivation of tested cell lines and use of exposure media with vastly different metal chelation potentials, concentration comparison between studies is unreliable.

Vitellogenin upregulation indicates oestrogen receptor activation by both 0.7 and 2 μM Cd (Fig 11a). The endoplasmic reticulum mediates effects of Cd activated oestrogen receptors by producing vitellogenin. The increased mRNA levels do not confirm vitellogenin production but confirm translocation of activated receptors to the nucleus and binding to the vitellogenin oestrogen response element. This response is expected but would likely be more sensitive in a liver or gonad cell line where greater response to oestrogen is typical (Nagler et al. 2007). Clotrimazole has been shown to reduce Cyp1a1 activity in rainbow trout gill filaments. This is another instance where RTgill-w1 is not a suitable model (Fig 11c). However, clotrimazole increases chop expression in melanoma cell line A375 (Rouaud et al. 2016) which agrees with upregulation by 4 μM clotrimazole (Fig. 11c).

Chapter 4 General discussion and conclusion

4.1 Impact on Existing Methods

This work suggests that the static well-plate based RTgill-W1 tests such as OECD TG 249 are not representative of the toxic effects of copper on fish gills in-vivo, and by extension may underestimate copper toxicity in the whole fish due to the link between gill damage and fish mortality. If this discrepancy is present for copper testing, it could be extrapolated that other heavy metals or chemicals sharing part or parts of the copper handling pathways, would be affected. Piezo 1 involvement supports the potential for FFS modulation of toxicity due to the growing body of evidence that the cation channel modulates many cellular pathways upon mechanosensitive activation (Borbiro and Rohacs, 2016).

The success in growing RTgill-W1 with 80% less FBS leaves room to ask why these simple, accessible approaches are not being utilised while instead focus remainins on expensive and complicated solutions for complete FBS elimination. This all-or-nothing approach is likely driven by pressure to produce completely animal free proposals for funding in the field of new approach methodologies. However, convenience, cost and stable reproducibility should be considered in balance with FBS removal. Culturing with reduced FBS will be more time-consuming compared to standard cultures. With doubling times increasing, it would be advisable to scale up culture protocols to ensure enough cells are available for the required throughput. The requirement for extra space could be a limiting factor for some laboratories but arguably one easier to overcome than the price associated with producing growth factors or purchasing proprietary serum replacements. Moreover, in this work, the reduced FBS cultures were only tested against standard cultures for one reference chemical. It Is possible the reduced FBS cells are not suitable for testing toxicity of other toxicants, especially those where FBS is known to be protective, such as zinc.

4.2 Future Work and Development

Identifying mechanisms leading to increased toxicity of copper under FSS could help persuade the scientific community and ultimately regulators that static exposures are lacking. Microarray technology could help elucidate the pathways modulating increased toxicity. It would be particularly useful to investigate the interaction between metallothioneins MTA and MTB and their transcription factors in response to FSS. If the NRF2/ARE system is implicated in increased copper toxicity, then all static metal exposures used to model gill or fish toxicity may be less powerful than currently thought.

Copper exposure concentrations should be measured to ensure these findings are robust enough to influence opinion or policy. Moreover, comparing static and FSS exposures of additional heavy metals and toxicants is required, together with variation in FSS levels to assess any flow-rate specific effects that could be applied to model toxicity with the consideration of activity level, watercourse flow-rates and gill shunting.

To validate the 1% FBS cell cultures, they must be compared to standard 5% cultures in exposures to a selection of heavy metals, pharmaceuticals and industrial toxicants. Providing evidence of the power of the reduced FBS model will ensure it stands up against any concerns related to extended growing time and space requirements. Moreover, identifying the minimum of specific factors in FBS required to culture RTgill-W1 would be complicated by the ill-defined nature of FBS. If individual factors were isolated through time consuming processes of elimination, the available growth factor products are expensive and producing them in useful quantities in yeast or bacteria is especially costly for laboratories not set up for such biotechnology protocols.

Gene expression of toxicity biomarkers alone were unsuccessful in predicting AOPs. However additional assays (see Table 7) and more cell line models would increase sensitivity and scope of application. For example, using a selection of cell lines to represent different tissues and their metabolic function could provide a more holistic data set while filling data gaps such as the lack of cyp activity in RTgill-W1. Candidate cell lines such as ZF4 from zebrafish embryo (Driever and Rangini 1993), PLHC-1 (Huuskonen et al. 1998) derived from guppy liver and RTL-W1, and RTgutGC from rainbow trout liver and gut respectively (Lee et al. 1993; Kawano et al. 2011) could address such shortfalls. Specifically, the addition of hepatic cell lines could extend suite sensitivity to endocrine disrupting compounds and increase sensitivity to compounds primarily metabolised in the liver. Furthermore, assays and biomarker probes (see Table 7) could build a predictive suite that measures actual enzyme activity and physiological change, building upon the foundations of gene expression data.

Table 7. Suggested Additional Assays for Prediction of AOPs

Test Compound	Adverse outcome pathway	Measurable marker	Assay/endpoint
Fungicides	Unfolded protein response	Endoplasmic reticulum stress	Chop expression
PAHs	AhR signalling	AhR activation	EROD activity
			CYP1A expression
PAHs Fungicides	ER signalling	ER activation/antagonism	Vitellogenin expression
Heavy metals	Metal stress	Mtf1 activation	ZnT1 expression
Fungicides heavy metals	Metabolic activity, Membrane and lysosomal integrity	Cell viability	Resazurin, CFDA-AM, neutral Red
Fungicides	Apoptosis	Programmed cell death	Caspase-3 activity
Heavy metals	Mitochondrial toxicity	Mitochondrial potential	Mito-MPS dye
PAHs	Reactive oxygen species production	Reactive oxygen species	Dihydrofluorescein dye
PAHs Heavy metals	Disrupted Ca^{2+} homeostasis	Ca^{2+} release	Fluo-4 dye
Heavy metals	Disrupted Zn^{2+} homeostasis	Zn^{2+} release	FluoZin-3AM

Apoptosis is a cellular event with such a measurable mediator enzyme activity, and assay kits and reagents are readily available. Apoptosis is a method of defence and maintenance comprising conserved molecular cell suicide machinery. Cysteine proteases called initiator caspases are converted from precursors to active proteases through apoptosis-triggered proteolytic mechanisms and are the initiators of the caspase cascades responsible for apoptosis (Asadi et al. 2021). An effector caspase is activated by initiator caspases and will go on to cleave substrates known as death substrates. These cleavages result in dysregulation of cellular machinery, DNA repair mechanisms and failure of cell structure. Caspase-3 is an effector caspase, and its activity can be used to identify the specific apoptotic pathway which is present in cadmium toxicity (Gao et al. 2013). Kits such as EnzChek™ (Invitrogen) can indicate caspase-3 activity when caspase- 3 cleaves a quencher from a specific fluorescent substrate**.** Some AOP biomarkers are shared with other classes of chemicals but could be differentiated by either an increase or decrease of that marker. For example, cellular calcium can be increased by PAHs (Mayati et al. 2012) and reduced by cadmium (Tvermoes et al. 2011). Moreover, disruption of zinc homeostasis caused by heavy metal exposure could be measured by probing cellular zinc (Zhao et al. 2009) and loss of mitochondrial potential assayed to indicate antifungal exposures (Sakamuru et al. 2012).

4.3 Conclusions

The broad aim of this project was to improve RTgill-W1 as a near-animal-free model for chemical risk assessment. This work addressed poor cell-cell adherence, exclusively static test conditions, the use

of animal products in RTgill-W1 culture and trialled gene expression analysis to predict adverse outcome pathways. This work shows previously un-reported evidence of physiological responses from RTgill-W1 cells to FSS including altered cellular responses to Cu and piezo 1 activity. In addition, with the above considerations, RTgill-W1 could be further improved as a near-animal-free model for chemical risk assessment. With development of more specialised exposure chambers or plates, one assay could potentially run FBS- FSS exposures with enough cell material left over for gene analysis, thus in part satisfying the main aims of this project.

Although RTgill-W1 did not respond to FSS with any increase in cell-cell adhesion, other levels of FSS may be tested. It may be that the cell line will never be suitable for uptake studies. However, this work has contributed to the characterisation of this important cell line and highlighted potentially harmful shortcomings of current static testing protocols such as OECD TG249. An 80% reduction in FBS is a promising starting point for accessible and morally responsible RTgill-W1 culture and discovering the need for a much more extensive suite of tests for AOP prediction is a small advance toward deriving more powerful data from RTgill-W1 tests. Most importantly, any characterisation or improvement of the important RTgill-W1 model reduces the risk of compromising the protection of freshwater ecosystems with incomplete information.

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