ADVANCING IN VITRO FISH ALTERNATIVES FOR REGULATORY ECOTOXICITY TESTING

By

Justin Scott

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Dissertation Approved:

Dr. Matteo Minghetti - Dissertation Adviser

Dr. Jason Belden

Dr. Mark Krzmarzick

Dr. Scott McMurry

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ABSTRACT

While fish toxicity testing methods have been vital in predicting toxicity of pollutants dissolved in water, advancements with *in vitro* ecotoxicological techniques have continued to gained momentum. *In vitro* alternatives allow identification of a toxicant's mode of action, high throughput approaches, reduced test duration and the number of animals used. This dissertation investigated novel animal alternative methods (i.e., fish embryo and cytotoxicity assays) and their ability to predict acute toxicity of chemicals dissolved in water or of actual effluents in fish *in vivo*. Fathead minnow (*Pimephales promelas*) embryos and the rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1, were successful in predicting toxicity of a broad list of legacy pollutants, chemicals of emerging concern (CECs) and wastewater samples similarly to fish. Moreover, the 24 hours cytotoxicity assay using RTgill-W1 cells was able to predict toxicity in all samples deemed toxic to fish using the 96- hour larvae assays. Additionally, to facilitate direct water sample exposures in fish gill cell monolayers, RTgill-W1 cells were cultured on transwell inserts. When cells are cultured on transwell inserts for 14-day in symmetrical culture media a monolayer is formed. In this culturing condition, RTgill-W1 cells were able to tolerate freshwater but not seawater exposure on the apical side. Furthermore, mixing between apical and basolateral compartments and lack of the formation of tight junction proteins reinforced the fact that RTgill-W1 cells do not form a truly tight epithelium. Finally, incorporation of additional rainbow trout cell lines from the liver, RTL-W1, and the intestine, RTgutGC, along with RTgill-W1 cells were used for cytotoxicity assays and identification of biomarkers to determine tissue specific mechanisms of toxic action and sensitivity to different classes of pollutants. RTgill-W1 cells were found to be the most sensitive to metal toxicity through cytotoxicity and gene expression of metallothionein. Our findings have highlighted embryo and cell lines applicability and reliability as advanced alternatives to traditional acute fish assays, and a more sensitive and specific toxicity forensic tool.

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CHAPTER I

INTRODUCTION

This chapter was adapted from the publication: Scott J, Minghetti M. 2020. An Introduction to Interdisciplinary Toxicology: Chapter 34 – Toxicity testing: in vitro models in ecotoxicology. Pope CN, Liu J, eds, An Introduction to Interdisciplinary Toxicology: From Molecules to Man. Elsevier, London, UK, pp 477–486. Academic Press 2020. https://doi.org/10.1016/B978-0-12-813602-7.00034-X.

Effluent testing and chemical registration

With the inception of the Clean Water Act of 1972 emphasis has been placed on strategies for reducing anthropogenic effects of chemicals and wastewater effluents to aquatic environments (USEPA 2003). This includes both legacy and priority pollutants of concern, as well as chemicals of emerging concern (CECs). Legacy chemicals consist of persistent, bioaccumulative and toxic (PBT) chemicals (i.e., metals, polycyclic hydrocarbons, and polychlorinated biphenyls) and CECs of chemicals or materials that have been detected in the environment, but lack information on toxicological impacts and regulatory mandates (i.e., quaternary ammonium compounds, poly and perfluorinated alkyl substances, and nanomaterials) (Hutchinson et al. 2013). Moreover, with an increased production and usage of CECs, emphasis on understanding their fate and toxicological impacts is becoming increasingly important.

Pollutant sources such as wastewaters and effluents have direct connections to fishable, swimmable, and navigable surface waters through routine point source discharges. Discharge facilities which are permitted by regulatory agencies are required to treat and monitor these effluents to reduce the amount of pollutants that enter aquatic environments (USEPA 2010). Treated and untreated discharge wastewaters, or effluents, are routinely monitored by government and private testing laboratories to ensure adverse effects to aquatic environments are prevented. One aspect to this is Whole Effluent Toxicity (WET) testing, which uses both biological endpoints and confirmatory chemical analysis to holistically ensure toxicity is properly identified. Effluent contaminants must maintain stringent regulatory limits due to their relevance and frequency to the discharge operations which is incorporated into their discharge permit. Overall, the rationale is to measure and prevent contaminant release in aquatic environments which can result in harmful ecological impacts. Additionally, if toxicity is present, it is required of the discharger to identify, reduce, and eliminate it from occurring further (i.e., additional chemical and physical treatment). In cases of confirmed toxicity, identifying and reducing which contaminants are likely responsible of the toxicity can prove to be difficult with the conventional approaches.

The field of ecotoxicology relies heavily on biomonitoring assays to measure and predict adverse effects to aquatic populations. Environmental protection agencies, in most of the western world, have legislations which routinely regulate effluents and chemical production to evaluate potential environmental impacts (Koch and Ashford 2006). Examples include the National Pollution Discharge Elimination System (NPDES), the Toxic Substances Control Act's (TSCA) chemical substance inventory, the Federal

Insecticide Fungicide and Rodenticide Act (FIFRA), chemical and product registered Safety Data Sheets (SDS), the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) and the International Maritime Dangerous Goods (IMDG) code, all of which utilize some form of live aquatic animal toxicity testing for regulatory purposes. While there are differences on agencies' guidelines across borders, there exists a unified aim to reduce and eliminate anthropogenic effects on the aquatic environment through the use of toxicity testing (Norberg-King et al. 2018).

Alternatives to animal testing in ecotoxicology

Tiered testing approaches for chemical registration, risk assessment, compliance monitoring, and fate of contaminants incorporate aquatic toxicity testing with both vertebrate and invertebrate *in vivo* models (USEPA 1996a; USEPA 2002a). It's estimated that around one hundred million vertebrate animals are used worldwide annually for experimental and regulatory purposes (Taylor et al. 2008). Over 50% of these animals are mice, while fish account for around 10%, a figure that has been continuing to increase over the years (European Commission 2007). For instance, in the United States (US) alone, 3 million fish are used every year for Whole Effluent Toxicity (WET) testing (Tanneberger et al. 2013). Russell and Burch's *The Principles of Humane Experimental Technique 1959* was pivotal in emphasizing a need for alternative approaches to animal testing and introducing the concepts of the "3Rs" (reduction, replacement, and refinement). For the past three decades public and private interest has increased the development of *in vitro* animal alternatives (i.e., isolated vertebrate cells, biochemical tests and computer models) to *in vivo* toxicity testing (Lillicrap et al. 2016). Major drivers

include the ethical concerns of animal use and the desire for a reliable and cost-effective high throughput assay.

One example of this was the European Commission's (EC) 2013 ban on testing on animals of chemicals and ingredients for cosmetics and personal care products. A process which took approximately 20 years of debate between regulators, academia and industry and was successful due to the development of efficient *in vitro* alternatives and by sustained public pressure (Vogel 2009; Daniel et al. 2018). Currently, most regulatory methods in ecotoxicology require vertebrate animals such as fish and use lethality (lethal concentration of 50% population; LC_{50}) as the preferred measured end point to assess acute toxicity. Regulatory agencies methods also include chronic endpoints (inhibition/effect concentration of 25% population: IC_{25}/EC_{25}) when assessing chemical and effluent toxicity, with test duration commonly being one to three weeks. These test methods are popular for the ease of execution and interpretation, but do not necessarily generate useful information to predict the substance's mode of toxic action.

In vitro alternatives to whole animal testing allow the study of the mechanisms of toxicity of chemicals in a more ethical and cost-effective manner. Few *in vitro* methods have been approved for ecotoxicology purposes and there is a need to further identify which model best fit for regulatory purposes. Moreover, there exists the need to determine if these alternative models can be developed to predict and measure toxicity like whole animal toxicity tests with reliable and reproducible results.

In vitro models in regulatory toxicology

As previously discussed, regulatory agencies continue to implement new methods aimed at reducing the use of vertebrate animals in toxicity testing. The US Environmental Protection Agency (USEPA) has initiated the Toxicity Forecaster (ToxCast) and Aggregated Computational Toxicology Resource (ACToR). These consist of *in vitro* high throughput screening and computational toxicology approaches to rank and prioritize chemical toxicity (Richard et al. 2016). The USEPA has also emphasized *in vitro* testing for the regulatory sector by including the use of New Approach Methods (NAMs) to aid in moving away from mammalian testing by 2035 (USEPA 2021). Additionally, Toxicology in the 21st century (Tox21, www.tox21.gov) has shown advancements with cytotoxicity assays in the field of ecotoxicology (Krewski et al. 2010; Richard et al. 2016), with the whole effluent toxicity (WET) testing sector being of strong interest (Finlayson et al. 2022). To date, animal alternatives have not been incorporated into the USEPA's standardized WET regulatory methods (USEPA 2002a; USEPA 2002b). Other organizations, including the Alternatives Research and Development Foundation (ARDF), Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and European Centre for Validation of Alternative Methods (ECVAM), are pursuing new advancements in toxicity testing to identify and validate *in vitro* alternatives.

Currently, two advanced *in vitro* animal alternative models have been standardized for routine testing of effluents and chemicals. The first was recently accepted by the International Organization for Standardization (IS0) (ISO 21115:2019) and the Organisation for Economic Co-operation and Development (OECD) (OECD 2021) which

utilizes the rainbow trout (*Oncorhynchus mykiss)* gill cell line, RTgill-W1 (Bols et al. 1994). RTgill-W1 has been extensively used for research purposes and serves as an ideal replacement to determine acute toxicity of chemicals and effluents to fish (Lee et al. 2009). Ultimately, round robin testing of the RTgill-W1 cells, confirmed high repeatability (low intralaboratory variability) and high reproducibility (low interlaboratory variability) for the determination of fish acute toxicity (Fischer et al. 2019).

The second method approved as an alternative to the fish acute toxicity testing is the fish embryo acute toxicity test (FET). This was approved by both the OECD and by the ISO, using an assay that exposes fish embryos for 96 and 48-hour (h), respectively (OECD 236; ISO 15088 (ISO 2007; OECD 2013)). The OECD approval process took nearly a decade and involved two phases. Phase one aimed to evaluate the transferability and the intra- and interlaboratory reproducibility of the FET after exposure to seven chemicals. Phase two built on this by testing reproducibility with the addition of 13 chemicals and covering specific areas of use (i.e., chemicals, pharmaceuticals, pesticides, and biocides) (OECD 2012). This process resulted in the approval of the use of the FET test to replace the acute *in vivo* acute toxicity test (OECD 1992) for regulatory testing (Busquet et al. 2014). The following sections describe in detail these approved alternative model systems and their application in regulatory ecotoxicology.

Fish embryo tests

The FET can be defined as an alternative to animal testing based on the convention that embryos are not protected animals up to a certain stage of development. The

OECD's definition of a protected animal is any living vertebrate, other than man, at the point in which it becomes capable of independent feeding. Therefore, organisms at embryonic and eleuthero-embryonic stages, where the organism is still using the yolk sac as an energy source, are not protected animals. The US Office of Laboratory Animal Welfare (OLAW) sets a more stringent definition for fish and considers hatching as the point at which organisms are protected (Halder et al. 2010).

The FET has gained promising strides both in Europe with the use of zebrafish (*Danio rerio*) FET as an alternative to acute fish testing, and with the USEPA's use of fathead minnows' (*Pimephales promelas*) larval survival and teratogenicity tests and chronic toxicity (USEPA 2002b). The FET is also utilized for several regulatory biomonitoring protocols including the IMDG Code, FIFRA, and WET testing methods to detect and measure toxicity. Further investigation is needed to determine if FETs could build upon USEPA's regulatory guidelines of chemical and WET testing.

The FET allows teratogenic observations to be made on the organism at early stages of development. Embryos are gathered post fertilization from tanks holding spawning adult breeders and then exposed to aqueous solutions to determine toxicological effects. Viability of each embryo is visually observed with a microscope at 24-h intervals over the test duration (acute at 48 and 96-h post fertilization) to measure the toxicity markers which include: i) coagulation of the embryos; ii) lack of somite development; iii) non-detachment of the tail, and; iv) lack of heartbeat which are used as endpoints for mortality and also to generate LC_{50} values (Figure 1).

From an ethical standpoint, the FET does not truly take on the concept of live animal toxicity testing replacement, but rather significantly reduces the number of living animals used and provides an approach to measure additional toxicological endpoints (Strähle et al. 2012) compared to current toxicity testing methods. The idea of fish embryo and larvae welfare has been considered when used in toxicity testing. For instance, it has been found that 120-h post-fertilization fish larvae responded to noxious stimuli and that the response was relieved with pain reducing drugs (Lopez-Luna et al. 2017). Moreover, although the FET assays have shown to be an effective predictive methods of sublethal toxicity of chemicals (Rawlings et al. 2019) and effluents (Kamelia et al. 2019), they require husbandry practices of breeding adult vertebrate test specimens which can have ethical and cost implications.

Regardless, the ability to measure more sensitive sublethal effects through FET novel assays are worth investigating to potentially facilitate more precise predictions of adverse effects to aquatic populations (Birke and Scholz 2019). Examples include the use of genotoxic (Kosmehl et al. 2004; Lourenço et al. 2017), gene expression (Roush et al. 2018), and more detailed histopathological (Hoon et al. 2016; Babić et al. 2017) and developmental (Krzykwa et al. 2019) endpoint markers to measure toxicity. While FETs with zebrafish have been used extensively for the OECD methodology, the fathead minnow is a favored aquatic toxicity specimen by the USEPA. Looking at a more suitable species of FET for applied regional regulatory mandates may be more appropriately suited for geographical use (Braunbeck et al. 2005). A fathead minnow FET could help transition towards *in vitro* chemical registration and WET testing procedures in the US similar to OECD guidelines (Schirmer et al. 2008).

Use of isolated fish cell lines

As previously mentioned, the use of fish cells have been studied and proposed as an alternative to acute fish toxicology testing (Segner 2004; Schirmer 2006; L.E. Lee et al. 2008). Primary cultures are isolated cells from tissues and organs taken directly from whole organisms (Schnell et al. 2016). Most cells derived from a primary culture will eventually become senescent and die. However, if a primary culture starts to proliferate *in vitro* and can be divided and propagated into new culture flasks, it becomes a cell line (Bols et al. 1994). If a cell line can be propagated for a limited time, it is finite, or if can be propagated indefinitely it becomes an immortal or continuous cell line (Figure 2).

Cell lines serve as an ideal replacement to whole animal testing in toxicology, as once the cell lines have been established, there is no further need for live animals. Cell lines can be frozen indefinitely and thawed as needed. Several cell lines are readily available through commercial purchase (Vo et al. 2014). Mammalian cell lines are finite or continuous whereas most fish cell lines appear to be continuous (Bols et al. 2005). Fish cell lines have been successfully isolated from several fish species and various tissue types. Along with the RTgill-W1 cell line, RTgutGC (Kawano et al. 2011) and RTL-W1 (Lee et al. 1993) have been characterized from the intestine and liver of rainbow trout respectively (Figure 3). All three models have shown to be promising alternatives to current whole fish assays with an emphasis on tissue specific mechanism of toxicity. However, further research is warranted to determine if specific cell lines display advantages or limitations for ecotoxicological applications.

Gill Cell Line: RTgill-W1

RTgill-W1 cells have been used for 185 total publications (ScienceDirect search for RTgill-W1 cells, February 2, 2023) and was shown as an appropriate model for measurement of fish acute toxicity based on the premise that impairment of gill tissue after acute toxicant exposure is linked with fish death. Previous studies using RTgill-W1 cells have correlated *in-vitro/in-vivo* toxicity to metals, organic, fragrance chemicals and specific effluent chemicals of concern (Dayeh et al. 2005; Tanneberger, Knöbel, Frans J M Busser, et al. 2013; Natsch et al. 2018; Scott et al. 2021). Thus, RTgill-W1 cells represent an ideal alternative model which can link cellular gill toxicity with fish mortality.

Liver Cell Line: RTL-W1

The RTL-W1 cell line, derived from the rainbow trout liver, has been studied for several aquatic toxicology studies, with 420 publications (ScienceDirect search for RTL-W1 cells February 2, 2023). RTL-W1 cells have shown cytochrome p450 activity and have been used extensively for the study of biotransformation, bioconcentration and hepatic toxicity of chemicals (Lucila E.J. Lee et al. 1993; Stadnicka-Michalak et al. 2018).

Intestine Cell Line: RTgutGC

The RTgutGC cell line isolated from the rainbow trout intestine has been studied in 61 publications (ScienceDirect search for RTgutGC cell line, February 2, 2023) and is relevant to study chemical uptake and toxicity via the dietary route (Kawano et al. 2011). It has shown to conserve several features of the polarized intestinal epithelium *in vivo*

including apical expression of tight junction proteins and basolateral expression of the Na/K-ATPase (Minghetti et al. 2017).

Scope of Research

As mentioned earlier, cell lines and FETs offer both advantages and limitations that that warrant further investigation. Therefore, the following chapters are aimed at building on the current *in vitro* methods to facilitate their incorporation and validation into regulatory toxicity testing applications. Specifically for cell lines, care should be taken when manipulating the exposure media for cytotoxicity assays. Cell lines are normally cultured in Leibovitz's L-15 complete media containing salts, vitamins, amino acids, and supplemented proteins from fetal bovine serum (FBS) that can complex with chemicals and thus reduce bioavailability. To help prevent this interaction, an exposure media (L-15/ex) has been used extensively for cytotoxicity assays. L-15/ex medium has identical composition than Leibovitz's L-15 complete medium excluding amino acids, proteins and vitamins (Schirmer et al., 1997). Previous studies have shown that RTgill-W1 cells can tolerate a range of osmotic (hypoosmotic and hyperosmotic) exposure conditions when cultured as a monolayer on multiwell plates in L-15/ex (Scott et al. 2021). However, RTgill-W1 cells cultured in traditional flat bottom wells lack the ability to tolerate direct exposure to freshwater or seawater samples.

In order to overcome this limitation, Chapter II looked at advancements of using permeable membranes, (i.e., transwell inserts) which may facilitate the formation of a polarized epithelium allowing a more physiological representative testing model. When cultured on transwell inserts, cell lines have shown to develop a tight epithelium

potentially allowing direct water sample exposures on the apical surface (Lee et al. 2009). Therefore, we investigated if a tight epithelium using RTgill-W1 cells is truly developed to allow direct exposure of water samples. This consisted of various osmotic challenges using freshwater, saltwater, and mannitol all with and without reference toxicants. Mannitol is an inert sugar that is used in physiological studies to manipulate the medium osmolarity without affecting cell metabolism (Aronson 2007).

Previous studies have shown that cell lines such as RTgill-W1 have been used to facilitate detection of specific toxicants of concern through mechanistic and morphological effects such as metabolic activity and cell vacuolization, respectively, of WET chemicals of concern (Dayeh et al. 2005; Dayeh et al. 2009; Scott et al. 2021). Additionally, studies have utilized FETs to identify WET chemical toxicity and found them to be more sensitive compared to fish *in vivo* (Jeffries et al. 2014; Jeffries et al. 2015; Krzykwa et al. 2019; Krzykwa et al. 2021). Therefore, in order to determine which model may predict acute toxicity *in vivo*, cytotoxicity and teratogenicity assays (FETs) were conducted for Chapter III, to compare sensitivities and predict toxicity to fish using a list of common WET chemicals (Scott et al 2021).

Ultimately, *in vitro* alternatives could be highlighted for their use in replacing and reducing the need for live fish for toxicity testing if they prove successful as predictive models in real exposure sample scenarios. The majority of toxicity studies are based on single chemical exposures whereas studies looking at realistic and environmentally relevant chemical mixtures are lacking (McCarty and Borgert 2006; Millstone and Clausing 2023). Moreover, studies investigating concurrently the use of fish cell lines, embryos, and fish larvae to determine sensitivity comparisons and correlate toxicity of

actual wastewater samples are lacking. Therefore, we compared side by side the use of RTgill-W1 cells, fish embryos and larvae for WET testing using a wide range of real effluent samples. Additionally, Toxicity Identification and Reduction Evaluation (TIE/TRE) strategies are important components of WET testing, and applicability of the three models could further highlight their use in toxicant identification and reduction. While this approach may favor identification in less complex sample matrices, effluents tend to be more difficult due to their variability and broad spectrum of possible toxicants. Therefore, building on Chapter III, Chapter IV utilized actual wastewater samples to determine sensitivity differences between different models and correlation of FET and RTgill-W1 cytotoxicity assay with the fish acute toxicity assay aimed at *in vitro-in vivo* predictability.

Another consideration relative to the use of fish cell lines is that different cell lines may differ in toxicity sensitivity of certain chemicals and their sensitivity to chemical toxicity may differ to that of whole animals. This could be the case when the mechanism of toxic action of a chemical in an animal involves the inhibition of a specific receptor or pathway not presented in the cell line. For example, it was shown that neurotoxicants are not inducing toxicity in RTgill-W1 cells (Tanneberger et al. 2013; Scott et al. 2021). Various cell lines may experience reduced sensitivity to chemicals mode of toxic action and to exposure conditions, which should be considered when assessing the chemical bioavailability and toxicity. Importantly, investigating specific toxicological end points (i.e., cytotoxicity and gene expression) could be used to detect specific toxicants or highlighting adverse effects to specific tissue types increasing the TIE/TRE capabilities of fish cell lines. Therefore, Chapter V investigated sensitivity of

RTgill-W1, RTL-W1 and RTgutGC cells to specific WET chemicals of concern to determine a best fit for specific chemicals of concern. Cytotoxicity and gene expression assays were used to study cell sensitivity and their relationship to specific classes of chemicals.

Specifically, the overall aim of our research proposes to evaluate the available animal alternative assays and their ability to identify and predict chemical and effluent toxicity more efficiently than current *in vivo* methods. Importantly, there is a need to determine which model is best suited for routine applications and serve as a valid alternative to current *in vivo* methods. Moreover, studies such as this are needed to develop further these novel technologies to improve the efficiency in term of volume and sensitivity of toxicity testing which untimely will result in a better identification of situations of environmental concern and better protection of our environment.

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TABLES AND FIGURES

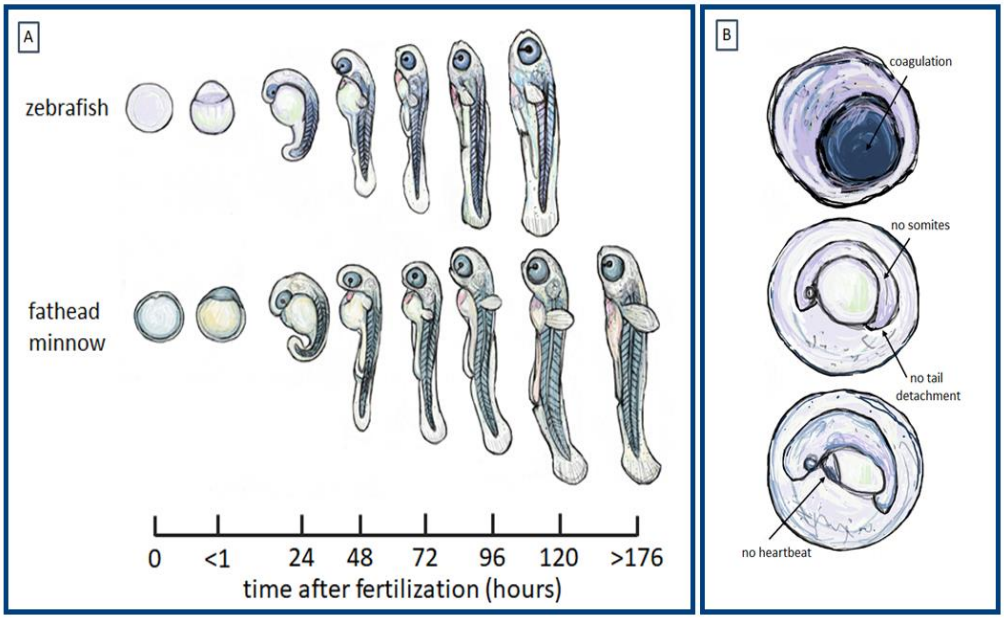

Figure 1: Panel A illustrates the zebrafish (*Danio rerio*), and the fathead minnow (*Pimephales promelas*) developmental stages relevant for embryo toxicity and teratogenicity assays. According to the OECD, fish embryos/larvae can be used for toxicity testing until the animal is capable of independent feeding, occurring approximately at 120 and < 176, post fertilization for the zebrafish and fathead minnow, respectively. Panel B illustrates the measured toxicological endpoints for the zebrafish FET (i.e., coagulation of the embryos; lack of somite formation; non-detachment of the tail; lack of heartbeat), which are measured at 24 h intervals until test termination. [Scott and Minghetti 2020]

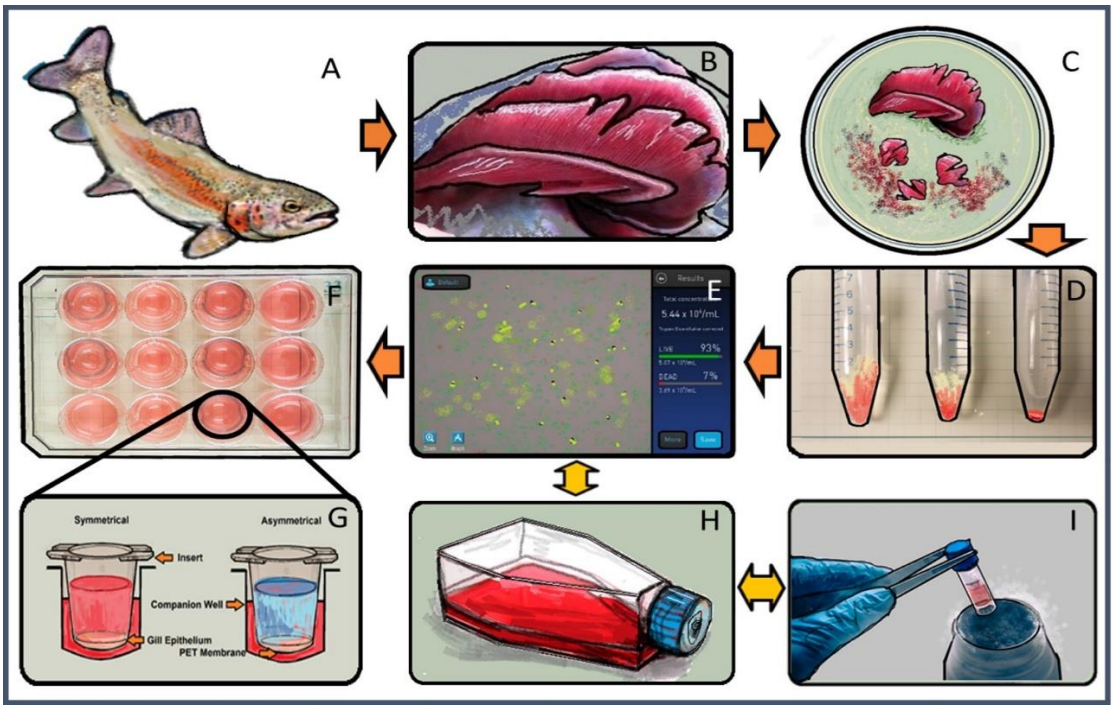

Figure 2: Preparation of gill cell cultures from rainbow trout (*Oncorhynchus mykiss)*. A-C) Gill arches are excised, gill filaments are separated and washed in phosphate buffer. D) Gill cells are washed repeatedly with antibiotic and anti-fungal solutions and dissociated from each other using a cell strainer and enzymatic digestion (i.e., trypsin digestion); E) Cell viability and total numbers are evaluated using the trypan blue exclusion assay with a hemocytometer or automatic cell counter. F) The primary gill cell culture system is generated by seeding two primary gill cells cultures (steps A to E) on a Polyethylene Terephthalate (PET) porous membranes in two consequent days; G) Double seeded primary gill cultures form a tight epithelium when cultured in symmetrical conditions for 5-7 days and when a TEER of above 5000 ohms*cm^2 is reached cells can be maintained in asymmetric conditions and tolerate direct exposure to fresh water; H) A gill cell line was isolated using a similar protocol (A to E). Primary gill cells were cultured as monolayers on culture flasks and passaged several times until the RTgill-W1 cell line was isolated; I) RTgill-W1 cells can be cryopreserved and thawed when needed for cytotoxicity assays and other applications. [Image: Scott and Minghetti 2020].

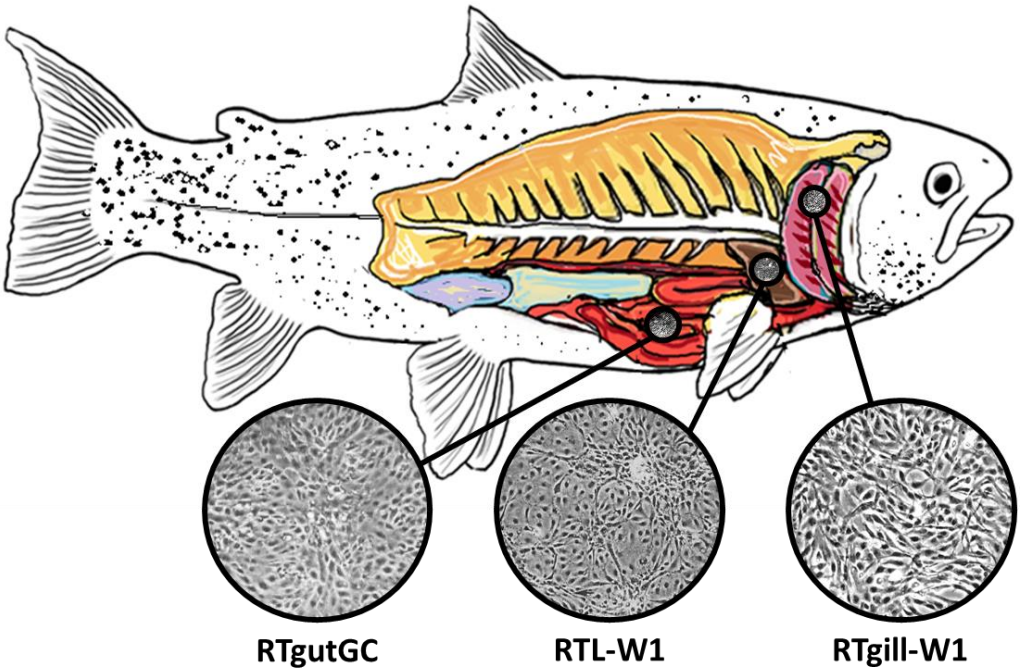

Figure 3. Illustration of three rainbow trout (Oncorhynchus mykiss) cell lines commonly used for aquatic toxicity testing of chemicals and effluents. Cell lines were derived from primary tissues of gill (RTgill-W1; Bols et al. 1994), liver (RTL-W1; Lee et al. 1993), and intestine (RTGutGC; Kawano et al. 2011). [Image: Scott and Minghetti 2020].

CHAPTER II

CHARACTERIZATION OF RTGILL-W1 CELLS EPITHELIAL DEVELOPMENT ON TRANSWELL INSERTS: EVALUATION OF OSMOTIC AND TOXIC CHALLENGES

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Justin Scott,[a]* and Matteo Minghetti[a]

[a]Integrative Biology, Oklahoma State University, Stillwater, Oklahoma, USA
*Corresponding author

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ABSTRACT

RTgill-W1 cells cannot be directly exposed to freshwater (FW) or seawater (SW) due to osmotic stress. Adjustments of exposure solutions are needed, but these might reduce the bioavailability and toxicity of pollutants. To facilitate cell polarization and allow direct exposure of water samples cells were cultured on transwell inserts. Monolayer formation was measured by trans-epithelial electrical resistance (TEER) and apparent permeability (P_{app}) assay. At 14 days both TEER and P_{app} indicated the lowest permeability. Cell viability showed that cells can tolerate apical FW with complete medium (L-15/FBS) in the basolateral compartment but SW reduced cell viability. However, when reference toxicants silver nitrate and sodium dodecyl benzene sulfonate were added no toxicity was detected. Increased osmolality in the apical side and presence of proteins indicated diffusion from the basolateral to the apical side. Thus, reduced toxicity was likely caused by complexation with media salts and amino acids. A protein and amino acid free exposure medium (L-15/ex) was applied in the basolateral compartment. However, FW exposures with basolateral L-15/ex resulted in reduced cell viability. To reduce osmotic stress, mannitol was added to apical FW maintaining basolateral L-15/ex which improved cell viability and allowed detection of silver toxicity. Finally, RTgill-W1 did not show normal tight junction protein (ZO-1) immunocytochemical staining which fits with the formation of a leaky epithelium. Overall, culturing of RTgill-W1 cells on transwell inserts allowed direct exposure to mannitol FW medium but showed a reduce sensitivity to toxicants. Thus, exposure on flat bottom wells is recommended for routine toxicity testing

INTRODUCTION

Over the past three decades *in vitro* animal alternatives, such as cell lines, continue to be pursued as an application for routine toxicity testing procedures. The use of *in vitro* cell line based alternatives allows for the identification of chemical mode of toxic action, high throughput approaches, and reduces the need for live animals (Lillicrap et al. 2016). The use of fish cell lines has been extensively studied and proposed as an alternative to acute fish toxicity testing (Segner 2004; Schirmer 2006). Recently, the International Organization for Standardization (ISO) and the Organisation for Economic Development (OECD) accepted an animal alternative method which utilizes the rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1, for acute toxicity testing of chemicals and effluent water samples (ISO 2019; OECD 2021). Previous studies have shown correlations between RTgill-W1 cells effective concentration 50% (EC50) and lethal concentration 50% (LC50) for a range of chemicals (Tanneberger, Knöbel, Frans J M Busser, et al. 2013; Natsch et al. 2018; Fischer et al. 2019; Scott et al. 2021). Moreover, RTgill-W1 cells were successfully used to test toxicity of whole effluent water samples from a paper mill (Dayeh et al. 2002). However, the effluents were supplemented with salts to maintain the exposure solution isosmotic to RTgill-W1 cells. While the ISO/OECD methods have proven useful in predicting toxicity, further studies are necessary to understand chemical interactions occurring in the exposure medium.

Fish cell lines are normally cultured in Leibovitz's L-15 medium, which contains salts, vitamins and amino acids and is supplemented with fetal bovine serum (FBS) which contains proteins. All these components can complex with chemicals and thus reduce bioavailability of pollutants present in the exposure solution. Specifically, the presence of

organic and inorganic anions such as cysteine and chloride in the exposure solution reduce the toxicity of metals present in water via complexation (Oldham et al. 2023). Moreover, the salt composition of exposure media could also affect the toxicity of organic pollutants such as sodium dodecyl sulphate (SDS) and perfluorooctane sulfonic acid (Rocha et al. 2007; Jeon et al. 2010). To prevent and or limit similar interactions, an exposure medium (L-15/ex) has been developed for cytotoxicity assays. L-15/ex medium has identical composition to Leibovitz's L-15 commercial medium excluding amino acids and vitamins that are removed (Schirmer et al., 1997). Moreover, it was shown that reducing the salt concentration in L-15/ex, resulting in a hypoosmotic exposure, can increase the sensitivity of RTgill-W1 cells to metals (Scott et al. 2022).

Previous studies have shown that RTgill-W1 cells can tolerate a relatively wide range of osmotic conditions (100 – 550 mOsm/kg) (Lee et al. 2009; Yue et al. 2015; Scott et al. 2021), but they cannot tolerate exposure of true freshwater (FW) and saltwater (SW) conditions (i.e., below 10 and about 1000 mOsm/kg, respectively) in traditional flat bottom wells (Scott et al., 2021). Interestingly, Lee et al., (2009) showed that RTgill-W1 cells cultured for 28 days (d) on transwell inserts can tolerate direct exposure to FW but no direct measurement of cell viability or epithelium tightness was reported.

Culturing of primary rainbow trout gill cells on permeable membranes such as transwell inserts has shown to form a tight epithelium with trans epithelial electrical resistance (TEER) above 20,000 ohms which is similar to that in intact fish gill *in vivo* (Wood et al. 2002; Schnell et al. 2016). Moreover, the primary gill cell model has been used to determine the toxicity of contaminated waters via direct exposure (i.e., direct application of the water sample on the *in vitro* fish gill epithelium (Minghetti et al. 2014;

Schnell et al. 2015). Therefore, advancements of the *in vitro* method using transwell inserts may facilitate the formation of a polarized epithelium in RTgill-W1 cell lines and possibly allow direct exposure to environmental waters. The role of the culturing substrate should be investigated in RTgill-W1 cells to determine if a tight epithelium is developed on transwell inserts permitting direct exposure of water samples. Importantly, determining if the need to adjust osmolality is necessary and to evaluate if toxicity of reference chemicals is affected in transwell inserts.

To evaluate the features of RTgill-W1 cells cultured on transwell inserts, cells were cultured for up to 28-d on transwell inserts and epithelium tightness was evaluated by measuring the TEER and the apparent intrinsic permeability (P_{app}). TEER allows the quantitative measurement of the resistance of ion current across the epithelium in cell culture models (Srinivasan et al. 2016). Transcellular resistance is created across the apical and basolateral membrane, while paracellular resistance is a result of cell to cell contact (Chen et al. 2015). The P_{app} method uses the fluorescent dyes, Lucifer Yellow (LY) potassium salt (520 Daltons) and Rhodamine B isothiocyanate–Dextran (Rho) (10,000 Daltons) to determine paracellular permeability between apical and basolateral compartments (Eckert et al. 1999). Thus, by increasing cells confluency and tight junction protein expression the epithelium tightness is increasing and an increase in TEER and decrease in P_{app} should be measured (Minghetti et al., 2017).

In our study, the role of media composition on RTgill-W1 cell viability, epithelium tightness and sensitivity to toxicants was evaluated. Specifically, focus was placed on the development of conditions that allow direct exposure to FW and SW samples. This included using L-15/ex and L-15/FBS media in symmetrical and asymmetrical

combinations between apical and basolateral compartments and use of mannitol, an inherit sugar actin as an osmotic effector, used to manipulate the medium osmolality (Aronson 2007). To further asses the applicability of RTgill-W1 cells cultured on transwell inserts for cytotoxicity assays, reference toxicants such as silver nitrate and sodium dodecyl benzene sulfonate were used. Tight junction proteins have been confirmed through mRNA expression in RTgill-W1 cells (Trubitt et al. 2015; Fuchylo et al. 2022), but have not been characterized by immunocytochemistry. Thus, the presence of tight junctions were investigated through immunocytochemical staining based on previous methods (Minghetti et al. 2017). The overall goal of the present study was to determine if RTgill-W1 cells cultured on transwell inserts can form a tight epithelium which is able to tolerate direct aqueous sample exposure (i.e., FW and SW reconstituted water) for chemical toxicological analysis.

MATERIALS AND METHODS

Cell culture techniques

RTgill-W1 cells were purchased from ATCC® (CRL-252) and were cultured in 75 cm^2 cell culture flasks (Greiner Bio-One, Kremsmunster, Austria) with Leibovitz's L-15 (Invitrogen, Thermofisher, Waltham, MA, USA) supplemented with 5% Fetal Bovine Serum (FBS; F6178, Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (5,000 units ml^{-1} penicillin and 5 mg ml^{-1} streptomycin; Sigma-Aldrich, St. Louis, MO, USA) at 19 ºC in normal atmosphere. For immunochemistry comparisons, RTgutGC, cells were kindly donated by Professor Kristin Schirmer (EAWAG, Switzerland) and cultured similarly to RTgill-W1 cells but with 1% gentamicin (Gibco,

Thermofisher, Waltham, MA, USA). Media changes were performed weekly, and cells were maintained up to approximately 80-90% confluency in culture flasks. Prior to seeding in transwell inserts, cells were washed twice with Versene, (Gibco, Thermofisher, Waltham, MA, USA) then detached using 0.25% trypsin solution (Gibco, Thermofisher, Waltham, MA, USA) in Phosphate Buffer Solution (PBS; Invitrogen, Thermofisher, Waltham, MA, USA). Cell counting and viability were conducted using an automated cell counter (Countess II, Thermofisher, Waltham, MA, USA). Viability was determined by using the Trypan blue exclusion assay and only cells with viability greater than 90% were used for transwell inserts seeding.

Optimization of cell seeding

Transwell inserts were conditioned with 100 µl of complete media 2 hours (h) before cell seeding. Two seeding densities were selected for optimization, based on previous studies (Georgantzopoulou et al. 2018), and adjusted for a 24-well transwell insert (0.33 cm^2 polystyrene; Greiner Bio-One Thincert, Kremsmunster, Austria) to allow cell polarization and development of a tight monolayer. Cells were initially seeded with L-15/FBS symmetrically in the apical compartment at 165,000 cells/ml (150,000 cells/cm^2) and 82,500 cells/ml (75,000 cells/cm^2) in 24-well transwell insert for up to 28-d. Based on the observed ideal seeding density, cells were seeded into transwell inserts and incubated at 19 ^oC, in the darkness in culture media to allow cell attachment and development of a confluent monolayer on the transwell inserts. Weekly media changes of both apical and basolateral medium were performed using L-15/FBS. TEER was the first endpoint measured for optimization, followed by P_{app} and finally cell viability, cytotoxicity, and imaging upon optimization determination. All optimization

measurements and cytotoxicity assays were performed in at least triplicate, on separate days and with cells of different passages.

Cell culture and exposure media composition

At the set time point of 7, 14, 21 and 28-d after seeding, cells were washed twice with the exposure medium to be applied. Apical exposure media consisted of L-15/ex, USEPA moderately hard freshwater (FW) and salt water (SW) (USEPA 2002), the mannitol media (Table 1). The mannitol media (M-150/ex and M-300/ex) was the FW medium supplemented with mannitol (Sigma-Aldrich, St. Louis, MO, USA), to adjust the osmolality to 150 or 300 mOsm/Kg. The basolateral medium was L-15/FBS or L-15/ex (see specific figures for details). Osmolality of all media was measured using an osmometer (Vapro 5600, Wescor) and pH meter (Lab 870; SI Analytics).

Moreover, to evaluate if diffusion of protein from the basolateral to the apical compartment was occurring, we have measured protein in the apical compartment. Proteins are not present in any of the apical exposure media, but they are present only in L-15/FBS. The modified Lowry assay (Pierce™, ThermoFisher, Waltham, MA, USA) was used to measure protein concentration in the medium. Briefly, in 14-d old transwell inserts the medium was aspirated from both apical and basolateral compartment and washed twice with PBS. After, the exposure conditions were applied for 24-h an aliquot of 200 µl of the apical compartment was removed for protein determination following the manufacturer's instructions for the modified Lowry protein assay (Thermofisher, Waltham, MA, USA).

Cell imaging

Cell monolayer development was observed in RTgill-W1 cells using two fluorescent dyes that stain cell membranes (CellMask™ Green, Thermofisher) and nuclei (NucBlue™ Live ReadyProbes™, Thermofisher). Briefly, cells were washed with respective exposure media and then incubated with 7.5 μg/ml of CellMask™ Green and 4 μM of NucBlue™ was added to the respective exposure media following manufacturer's instructions and added apically to the cells and allowed to incubate for 10 minutes (Geppert et al. 2016). Cells were then washed twice with PBS and cell membrane and nuclei were imaged using a plate reader (Cytation 5; Biotek) using the GFP (469/525) and DAPI (377/447) filters, respectively.

Tight junction staining procedures followed methods previously described by Minghetti et al., 2017. Briefly cells were washed with PBS and fixed with 3.7 % paraformaldehyde (Sigma-Aldrich, USA) in PBS. Cells were then permeabilized with 0.2 % Triton (Sigma-Aldrich, USA) and blocked with Image-iT™ (Molecular Probes, Invitrogen, USA) solutions. The primary monoclonal antibody, ZO-1 conjugated to Alexa Fluora® 488 (Molecular Probes, Invitrogen, USA) diluted in 1 % goat sera with 0.05 % Triton in PBS was applied at 5 μg/ml to the cells and incubated overnight at 4 °C. Cell nuclei and f-actin staining were conducted using NucBlue™, (Invitrogen, Thermofisher, USA) and Rhodamine phalloidin (Molecular Probes, Invitrogen, USA), respectively following manufacturer instructions. Finally, cells were washed with PBS and then embed with embedding medium (ProLong Antifade Kit, P7481). Imaging was conducted using a multiwell plate reader (Cytation 5; Biotek) using the DAPI, GFP and

RFP (531/593) filters for nuclei, ZO-1 and actin, respectively. RTgutGC cells were also stained as a comparison and prepared as described previously in Minghetti et al., 2017.

Trans epithelial electrical resistance (TEER)

TEER was measured using a Voltohmmeter with chopstick electrodes (EVOM2 with STX2, World Precision Instruments, Florida). Electrodes were placed simultaneously into the apical and basolateral chambers and allowed to stabilize before recording the TEER value ($\Omega * cm^2$). A transwell inserts without cells with respective media was used for blank subtraction of TEER values for transwell inserts with cells. TEER values were recorded at 1, 3, 7, 14, 21, and 28-d post seeding in L-15/FBS or after 24-h of specific exposure. Values were calculated by the following equation:

$$TEER\ (\Omega\ cm^2) = (\Omega\ cell\ monolayer - \Omega\ blank\ filter) \times filter\ surface\ area\ (cm^2)$$

Permeability assay

Apparent permeability (P_{app}) values were recorded in RTgill-W1 monolayers cultured on transwell inserts for at 1, 3, 7, 14, 21, and 28-d post seeding and cultured in L-15/FBS complete medium and at 24 h post experimental media exposures. Cells were exposed to both FW (~10 mOsm/kg) and SW (~1000 mOsm/kg) in monolayers cultured for 7, 14 and 21-d and P_{app} values were recorded. LY and Rho salts were purchased from Thermofisher. A working volume of 100 µg/ml LY and 1.4 mg/ml Rho in L-15/ex was added to the apical side of the transwell inserts. Apical and basolateral medium was removed from both compartments and 300 µl of LY/Rho working stock was added to the apical chamber and 1000 µl of L-15/ex to the basolateral of each transwell inserts. The plate was placed on an orbital shaker and rocked at 100 rpm. The fluorescence of both

dyes was measured every 15 to 30 min up to 2-h. 50 µl samples were taken from the basolateral compartment in triplicate and transferred to a 96-well plate and 150 µl of fresh L-15/ex medium was replaced. Fluorescence was measured simultaneously at an excitation/emission wavelength of 420/520 nm for LY and 520/580 nm for Rho, respectively for each time point. Pure L-15/ex medium served as the blank. The permeability coefficient is calculated using the following equation:

$$P_{app}\ [cm/s] = (dM_{baso} / dt * 1) / (A * CD0)$$

With dM_{baso} /dt being the change of mass in the basolateral compartment over time, *A* being the surface area of the membrane in cm^2 and *CD0* being the initial concentration in the donor compartment. The fluorescence of the samples was converted into concentration using the calibration curve and the following equation:

$$concentration\ [\mu g/ml] = (fluorescence - Y\ intercept) / slope$$

Transferred mass was recalculated in nmol and normalized to the surface area of the inserts in cm^2 using the following equation:

$$mass\ [nmol/cm^2] = (mass\ [\mu g]) / (MW\ [g/mol] * 1000) * 1) / (A/cm^2)$$

Mass in $nmol/cm^2$ was then plotted against the time in min to calculate P_{app} (cm/s).

Chemical analysis

The reference toxicants silver nitrate ($AgNO_3$) and sodium dodecylbenzene sulfonate (Linear Alkylbenzene Sulfonate; LAS) were purchased from Sigma-Aldrich. Chemicals are listed in Supplementary Information (Supplementary Information; Table S1), and include category numbers, purity, and source. A master stock was made by dissolving chemical salts into ultrapure water (16–18 MΩ cm^{-1} Barnstead GenPure, Thermofisher) and then by mixing aliquots into their respective media to achieve desired concentrations for exposures. The amount of water used to dissolve chemicals never exceeded 10% of the total volume in L-15/ex. Exposure concentrations of $AgNO_3$ and LAS consisted of the EC50 and EC99 that was previously found in RTgill-W1 monolayers cultured on flat bottom wells (Scott et al. 2021).

Exposure concentrations for silver were 500 and 1500 ng/ml and for the LAS concentrations, 4000 and 8000 ng/ml. These exposure concentrations were chosen due to a previous study which determined that silver nitrate is seven fold less toxic in RTgutGC cultured on transwell inserts compared to cells cultured on flat bottom wells (Minghetti et al. 2017) which would reduce toxicity to ~ EC_{10} and EC_{15}. Both low and high concentrations were confirmed analytically at test initiation and termination. Analytical procedures and their quality control criteria for the cytotoxicity assays followed previous methods (Scott et al. 2021). Concentrations of both chemicals were confirmed to be within 10% recovery (97% for silver as ion and 93% for LAS) and therefore not adjusted from nominal concentrations for reporting results.

Multiple endpoint viability assay and chemical exposures

Viability assays were conducted on cell monolayers cultured on transwells as previously described (Minghetti and Schirmer 2019) and consisted of one blank (i.e., an empty, cell free insert), three synthetic water treatments (i.e., synthetic moderately hard and artificial SW) three L-15/ex controls, and two L-15/FBS controls. L-15/ex serves as the negative control medium for cytotoxicity assays during exposure periods. L-15/FBS serving as a secondary negative control and $AgNO_3$ and the LAS exposures act as a positive control. Before the cytotoxicity assay was performed, the exposure solution was aspirated, and cells monolayers were washed twice with the respective exposure media. The exposure conditions were applied on the transwells for 24-h in the dark at 19 ^{o}C.

The multiple endpoint viability assay relies on the application of three commercially available dyes which are alamarBlue™ (AB; Invitrogen, Thermofisher, Waltham, MA, USA), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Thermofisher, Waltham, MA, USA), and 3-amino-7-dimethylamino-2-methylphenazine hydrochloride or Neutral Red (NR; Sigma-Aldrich, St. Louis, MO, USA) which are indicators of cell metabolic activity, cell membrane integrity and lysosome integrity (Schirmer et al., 1997). The dyes were then added based on their recommended protocols and ran using a multiwell plate reader (Cytation 5; Biotek) (Scott et al., 2021). Viability for each media condition tested was reported as % viability based on the blank corrected FU of the untreated cells (i.e., L-15/ex control).

Statistical analysis

Statistical analysis was performed using GraphPad Software (Version 8.0, San Diego, CA, USA). All data sets were measured for normality distribution using either Shapiro-Wilk, Anderson-Darling, and Kolmogorov-Smirnov test. Where needed, data was transformed using log transformation to improve normality. TEER, P_{app}, and viability endpoints, used to compare the sensitivity of RTgill-W1 cell monolayer cultured on transwell inserts to media exposures (FW, SW, M150 and M300) and to toxicants ($AgNO_3$ and LAS), were analyzed using a one-way ANOVA and post hoc Tukey's test and compared to control's using a one-way ANOVA and post hoc Dunnett's test. Cell viability was analyzed comparing respective control media using a student's t-test and one-way ANOVA and post hoc Dunnett's test. Individual endpoints at different time points were compared using a one-way ANOVA and post hoc Tukey's test. All statistical analysis tests were performed with alpha equal to 0.05.

RESULTS

Optimization, epithelium tightness, and imaging

Seeding density.

Microscopy analysis confirmed that RTgill-W1 cells formed a confluent monolayer at 14-d with an initial seeding density of 165,000 cells/ml (150,000 cells/cm^2) and 82,500 cells/ml (75,000 cells/cm^2). When cells were seeded at a density of 150,000 cells/cm^2, the entire cell epithelia partially or completely detached and lifted from the transwell inserts membrane at approximately 14 to 21-d (Data not shown). The lower

seeding density of 75,000 cells/cm^2 was chosen for the remaining treatments and 14-d was selected as optimal incubation time as they did not detach and formed a confluent monolayer (Figure 1A) however, some gaps and folding formed at 28-d (Supplementary Information; Figure S1).

TEER analysis.

In RTgill-W1 cells cultured in L-15/FBS symmetrically TEER values were shown to increase significantly from day 1 to day 3 and then again from day 7 to day 14 but then remained stable (at ~ 20 Ω*cm^2) until day 28 ($p < 0.001$; one-way ANOVA Tukey's multiple comparison test; alpha = 0.05; Figure 2). At day 7, 14 and 28 cells were exposed to L-15/ex, FW or SW for 24h. Exposure to apical L-15/ex did not induce a change in TEER in comparison to control (i.e., cells cultured symmetrically with L-15/FBS). Exposure to apical FW showed slightly higher TEER values at day 7 and 14 but the increase in TEER was significant at only day 28 ($p = 0.0073$; one-way ANOVA Dunnett's multiple comparison test; n = 3; alpha = 0.05). Exposure to SW induced a trend toward a lower TEER, however values were not statistically different from control.

Permeability assay.

Overall, and similarly to the TEER, P_{app} indicated a tightening of the epithelium for cells cultured in L-15/FBS from day 1 to day 3 maintaining similar tightness up to 21-d (Figure 3). Additionally, when challenged with a 24-h apical L-15/ex, FW or SW, the tightness (P_{app}) in cells cultured for 14-d did not vary from controls. Both Lucifer Yellow and Rhodamine-Dextran assays indicated a significantly different P_{app} at 1-d compared to

all other timepoints thereafter (LY: $p = 0.0012$; RD: $p = 0.0025$; one-way ANOVA Tukey's multiple comparison test; alpha = 0.05; n = 3).

Cell viability after FW and SW challenge maintaining basolateral L-15/FBS

When challenged to a 24-h apical FW exposure, the monolayers cultured for 7, 14, 21 and 28-d did not show a statistically significant difference in cell viability (i.e., geometric mean of metabolic activity, cell membrane integrity and lysosomal integrity) (Figure 4A). However, when looking at metabolic activity only, viability was lower than in controls (i.e., cells maintained in apical L-15/ex and basolateral L-15/FBS) in monolayer cultured for 21 and 28-d (Student's t-test; $p < 0.05$; n = 3). Moreover, in 21-d monolayers metabolic activity was lower than in 14-d monolayers ($p = 0.0252$; one-way ANOVA Tukey's multiple comparison test; alpha = 0.05; n = 3). In monolayers challenged to SW there was a reduction in the geometric mean of the three viability endpoints in 7-d to 14-d monolayers and an increase in viability in 21 and 28-d monolayers (Figure 4B). Moreover, 21 and 28-d monolayers exposed to SW showed an unusually high membrane integrity (Student's t-test; $p < 0.05$; n = 3). Therefore, based on TEER, P_{app} and viability results all further assays were performed in RTgill-W1 monolayers cultured for 14-d.

Cell morphology and osmolality

Morphology was observed at low (4x) and high magnification (20x) in cells cultured for 14-d, and cells appeared to be slightly swollen after FW exposure (Figure 1B) and shrunk and rounded after SW exposures (Figure 1C). Additionally, osmolality of the apical medium was measured after 24-h of exposure for both FW and SW at 171 ± 9

and 673 ± 5 mOsm/kg, respectively, indicating mixing between the apical and basolateral compartments.

Cytotoxicity of silver and LAS with Ex_A/FBS_B and FW_A/FBS_B exposure conditions

Cells exposed to low and high concentrations of silver and maintained in Ex_A/FBS_B and FW_A/FBS_B did not show any toxic effects compared to the control (Figure 5). However, a significant increase of metabolic activity occurred for the low LAS exposure in FW_A/FBS_B ($p = 0.0175$) and of membrane integrity for the low ($p = 0.0006$) and high LAS ($p = 0.0024$) exposure in FW_A/FBS_B compared to control (Student's t-test; alpha=0.05; n = 3). Overall, cells exposed to low and high LAS concentrations in FW_A/FBS_B showed a significant increase in cell viability (geometric mean) of 170 ± 52 % ($p = 0.0023$) and 159 ± 44 % ($p = 0.0105$), respectively, compared to their controls (Student's t-test; alpha=0.05; n = 3)..

Cell viability after apical L-15/ex, FW and mannitol challenges maintaining basolateral L-15/ex

Cells exposed to L-15/ex symmetrically (Ex_A/Ex_B) showed a significant decrease in lysosomal integrity compared to Ex_A/FBS_B control (One-way ANOVA, Dunnett's multiple comparison test; $p = 0.0098$; n = 3), but not overall based on the geometric mean of all toxicity endpoints (Figure 6). In cells exposed to FW_A/Ex_B metabolic activity and lysosomal integrity significantly decreased ($p = 0.0237$ and $p = 0.0191$, respectivelly), compared to the Ex_A/FBS_B control. Additionally, osmolality of the apical side was measured for Ex_A/Ex_B and FW_A/Ex_B and was found to be 302 ± 5 and 159 ± 9 mOsm/kg, respectively after 24 hours of exposure, indicating mixing between compartments for

apical FW exposures. Supplementation of mannitol (M150 and M300) to FW brought the viability back to Ex_A/FBS_B (Figure 6; One-way ANOVA, Dunnett's multiple comparison test; p = 0.0098) and Ex_A/Ex_B control levels (Figure 7; Student's t-test; alpha = 0.05; n = 3).

Cytotoxicity of silver in cells exposed in FW_A/Ex_B supplemented with mannitol

No toxicity was observed for any of the endpoints in cells exposed to low silver concentration in cells exposed in $M150_A/Ex_B$ but some (~20% reduction in lysosomal integrity) toxicity was detected in cells exposed in $M300_A/Ex_B$. For the high silver exposures, lysosomal integrity was significantly reduced in cells exposed in $M150_A/Ex_B$ and in $M300_A/Ex_B$ compared to their respective controls (one-way ANOVA Dunnett's multiple comparison test; $p< 0.05$; alpha = 0.05; n = 3).

Tight junction ZO-1 protein staining.

Imaging of the tight junction protein ZO-1 in RTgill-W1 cells cultured on transwell inserts for 14-d did not show a typical continuous protein staining in the cell periphery (Figure 8A). Conversely, RTgutGC cells cultured on transwell inserts for 14-d showed specific ZO-1 staining (Figure 8B).

DISCUSSION

Monolayer formation and epithelial tightness

The present study has shown that RTgill-W1 cells can be cultured on transwell inserts for up to 28-d in symmetrical conditions and that the formation of a monolayer was optimal at 14-d based on TEER, P_{app} and viability values. Previous studies have used

shorter (2-3-d) seeding durations (Trubitt et al., 2015; Mandal et al., 2020), but in these studies RTgill-W1 cells were cultured in symmetrical conditions with L-15/FBS. Remarkably, previous studies that cultured RTgill-W1 cells on transwell inserts in asymmetrical conditions (i.e., with L-15/ex in the apical chamber) used similar seeding durations such as 10-d, (Georgantzopoulou et al. 2018), 14-d, (Brinkmann et al., 2020; Fuchylo et al., 2022) and up to 21-d (Martin et al. 2022).

Moreover, based on our results and the existing literature (Brinkmann et al. 2020; Fuchylo et al. 2022; Martin et al. 2022), the cell monolayer of 14-d and 21-d was leaky as shown by the relatively low TEER (~ 20 Ω*cm^2) and diffusion of salts and proteins from the basolateral compartment to the apical compartment. The leakiness of the RTgill-W1 cell monolayer is particularly evident when we compare the TEER values of RTgill-W1 cells to that of rainbow trout primary gill cell which is greater than 10,000 Ω*cm^2 when grown on transwell inserts (Minghetti et al. 2014; Schnell et al. 2016). It should be noted however, that the primary gill model is obtained using a double seeding technique (Schnell et al. 2016) and it is a multilayer of cells, whereas RTgill-W1 cells are normally cultured as a monolayer. Additionally, RTgill-W1 cells are seeded at a density of 105,263 cells/cm^2 on flat bottom wells for 48-h (Scott et al. 2021), the lower cell seeding density used in this study (75,000 cell/cm^2) may be contributing to a less tight monolayer on transwell inserts. However, at the higher seeding density, overcrowding and lifting of the monolayer was observed. Future studies could attempt the double seeding technique and also the use of fibronectin, previously shown to increase cell attachment to the transwell (Bentley and Klebe 1985; Drieschner et al. 2017).

Based on our imaging results, the tight junction protein ZO-1 was not expressed and localized normally in RTgill-W1 cells as seen in the intestinal trout cells, RTgutGC (Figure 8). It was previously shown by immunocytochemical staining that the tight junction proteins such as Claudins were expressed in rainbow trout primary cells grown on transwell inserts (Sandbichler et al. 2011). Moreover, Claudins 10a, 28b and 30 and ZO-1 were expressed at the mRNA level in RTgill-W1 cells (Georgantzopoulou et al. 2018; Brinkmann et al. 2020; Mandal et al. 2020; Fuchylo et al. 2022) but there is no evidence of Claudin 10a and Claudin 30 protein expression by western blot analysis (Trubitt et al. 2015) and therefore tight junction could be demonstrated only at the transcriptional level in RTgill-W1 cells. These results could explain why proteins and salts diffused from the basolateral to the apical chamber due to a leaky epithelium formed by RTgill-W1 cultured on transwell insets.

FW and SW direct sample exposures

It has been previously shown that RTgill-W1 cells cultured on regular polystyrene multiwell plates can tolerate osmolality ranges from 100 to 500 mOsm/kg (Scott et al., 2021). One of the main aims of this study was to evaluate if RTgill-W1 cells cultured on transwell inserts could tolerate a wider range of osmolarity and direct exposure to FW or SW. Our results show that RTgill-W1 cultured for 14-d on transwell inserts can tolerate direct exposure to a 24-h FW exposure as cells attachment, morphology (Figure 1), TEER (Figure 2), P_{app} (Figure 3) and cell viability (Figure 4A) was similar to that of controls. Moreover, considering the diffusion of salts and proteins from the basolateral side to the apical side, RTgill-W1 cells were exposed to ~ 160 mOsm/Kg in the FW exposure which is within the range of tolerance of these cells (Scott et al., 2021).

Conversely, cells did not tolerate as well 24-h SW apical exposure as cell morphology appeared rounded (Figure 1C), monolayers were more prone to detachment (Scott personal observation), the TEER was slightly lower than controls (Figure 2), and most importantly, the cell viability was affected by the SW exposure (Figure 5B). For SW exposures, cell viability was lower than controls in RTgill-W1 cultured for 7 and 14-d. In cells cultured for 21 and 28-d the cell viability was abnormally high, especially for cell membrane integrity. Visual observations of cells cultured for 21 and 28-d, showed the formation of fold like structures in the monolayer (Supplementary Information; Figure S1). Interestingly, similar formations were imaged by previous studies (Lee et al. 2009). The folded monolayer could thus result in higher fluorescence (from the CFDA-AM) due to the increased surface area. This supports the data of Lee et al., (2009) where RTgill-W1 cells cultured on inserts showed normal morphology when exposed to FW but a stressed (formation of vacuoles and rounded shape) in cell exposed to SW.

Based on cell physiological understanding in relation to osmoregulation these results can be explained by the uptake of water in FW exposures, and the loss of water in SW exposures to the cells. It has been well documented that euryhaline species transitioning between FW and SW conditions have adapted physiological and morphological responses across the gill membrane (Laurent and Perry 1991). However, *in vivo*, endocrine reposes such as cortisol secretion, have a key physiological importance in the adaptation to FW and SW (McCormick et al. 2020) and this effect was demonstrated previously *in vitro* with RTgill-W1 cells (Lee et al. 2009; Trubitt et al. 2015).

Evaluation of cytotoxicity in RTgill-W1 cultured on transwell inserts

RTgill-W1 cultured on polystyrene multiwell plates have shown to be able to respond to a broad range of chemicals including organic chemicals (Tanneberger et al., 2013), metals (Scott et al., 2021) similarly to fish. Thus, RTgill-W1 cells are an excellent alternative to fish to detect acute toxicity of chemicals. However, when cultured of transwell inserts, and using different exposure conditions (i.e., FW, M150, M300 and L-15/ex) RTgill-W1 cells showed only mild effects in response to silver and LAS. These effects could be attributed mainly to the diffusion of salts and proteins from the basolateral compartment containing compete medium (i.e., L-15/FBS) to the apical compartment. The lack of toxicity of silver can be explained by free ionic silver complexation with proteins and cysteine, found in L-15/FBS. Amino acids such as cysteine have been shown to complex with metals such as copper, cadmium, zinc and silver, which in RTgutGC cells resulted in a reduction of metal toxicity but not bioaccumulation (Oldham et al. 2023). Although the toxicity of organic chemicals such as the LAS seem to be less affected by salt and amino acid complexation (Scott et al. 2021), the culturing and exposure conditions of using transwell inserts seem to reduce toxicity. Previous studies that used the asymmetrical exposure conditions with L-15/ex in the apical compartment and L-15/FBS in the basolateral compartment have evaluated the toxicity of titanium and silver nanoparticles in RTgill-W1 cells cultured on transwell inserts (Georgantzopoulou et al. 2018; Martin et al. 2022). Remarkably, the toxicity of silver nanoparticles was significant in RTgill-W1 (Georgantzopoulou et al. 2018) which fits with previous studies showing that silver nanoparticles toxicity is less affected by salts and amino acids complexation than silver ions (Minghetti and Schirmer 2016).

Exposures of RTgill-W1 cells apically to FW and with L-15/ex in the basolateral compartment induced a reduction in cell viability (Figure 6) and affected monolayer attachment. The reduction in viability was most likely due to increased osmotic stress. Indeed, adding mannitol to the FW medium rescues the viability in RTgill-W1 cells. Therefore, RTgill-W1 cells grown on transwell inserts with L-15/ex in the basolateral compartment could not be utilized as a viable option for direct FW exposures without manipulation of the apical media osmolality to prevent osmotic stress. The mannitol media was used to ameliorate the osmotic stress while maintaining a low metal complexation in the exposure medium. Moreover, mannitol addition to the apical compartment and removal of L-15/FBS to the basolateral compartment increased the toxicity of silver. Median lethal silver toxicity to rainbow trout has been shown to range between 6.5-70 ng/ml (Bury et al. 1999). Previous cytotoxicity studies for RTgill-W1 cells exposed in L-15/ex media as a monolayer reported a silver EC50 of 117 ng/ml, well below our lowest exposures (Scott et al. 2021). The 1500 ng/ml of silver exposure with mannitol did show reduced viability of about 15-20% which is lower than the 99% reduction that we would expect in RTgill-W1 cells cultured in flat bottom wells (Scott et al., 2021). However, it was shown in RTgutGC cells that toxicity of silver is reduced of seven fold when cells are cultured on transwell inserts (Minghetti et al. 2017) which would explain the toxicity level measured in our study (i.e., ~EC15). This may be due to the ability of the cells to pump silver into the basolateral compartment reducing toxicity to the cells as previously seen (Minghetti and Schirmer 2019).

CONCLUSION

Based on the current study, RTgill-W1 cells can be cultured for up to 28-d in symmetrical complete medium on transwell inserts. Measurement of TEER, P_{app}, viability, and imaging showed that 14-d is optimal for exposure in asymmetrical conditions with manipulated medium (e.g., L-15/ex and FW). RTgill-W1 cells could tolerate direct exposure of true FW but not of SW medium. However, diffusion of salts and protein from the basolateral compartment to the apical compartment might have ameliorated the osmotic stress and inhibited the toxicity of silver. By removing protein and amino acids for the basolateral compartment, silver induced a toxicity level similar to what we would expect at this exposure conditions. Moreover, the use of a mannitol-based media has shown to be a promising alternative to L-15 based exposure media (L-15/ex) reducing complexation with salts while reducing osmotic stress.

In terms of limitations, the transwell insert culturing method requires more time than the seeding on flat bottom wells (i.e., 1-2-d vs 14-d) and, although this method might be more physiologically relevant, possibly allowing cell polarization, the reduced toxicity levels measured and more laborious method makes this method unsuitable for routine toxicity testing. Furthermore, the costs associated with increased time and use of transwell inserts should be considered. Compared to the approved ISO and OECD cytotoxicity methods (ISO 2019; OECD 2021), utilizing transwell inserts does not improve the use of RTgill-W1 cells for cytotoxicity testing and as an alternative to whole animal testing. Our study suggests that the use of transwell inserts with RTgill-W1 cells may be more suited for physiological studies rather than routine toxicity testing as seen with the standardized monolayer approach.

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TABLES AND FIGURES

Table 1. Composition of the exposure media.

Inorganic salt/component (mM)	L-15/ex	M-150/ex	M-300/ex	Freshwater (FW)	Saltwater (SW)
Cl^-	145.4	4.08	4.08	4.08	544.1
Ca^{2+}	1.34	1.99	1.99	1.99	9.98
Mg^{2+}	1.92	0.5	0.5	0.5	54.31
Na^+	142.5	0.77	0.77	0.77	468.9
K^+	6.28	0.08	0.08	0.08	10.74
PO_4^{3-}	1.78	0	0	0	0
SO_4^{2-}	0.85	0.5	0.5	0.5	27.69
Na-Pyruvate	5.08	5.08	5.08	5.08	5.08
Galactose	5.07	5.07	5.07	5.07	5.07
Mannitol	0	0.14	0.3	0	0
Ionic strength (mmol/kg)	149.8	8.03	8.03	8.03	565.3
Osmolality (mOsm/kg)	300	150	300	10	1000
pH (su)	6.96	7.03	7.03	7.03	8.12

Ionic strength calculated using Visual MINTEQ, version 3.1. Media osmolality and pH were measured using an osmometer and a pH meter, respectively.

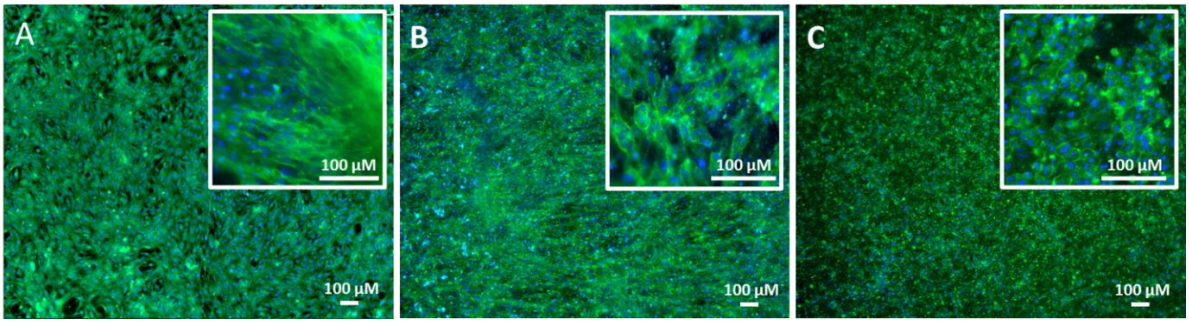

Figure 1. Images show RTgill-W1 cell monolayer cultured on transwell inserts for 14 days (seeded at 75,000/cm^2) using the 4x objective. Control (i.e., in L-15/FBS) A, challenged with a 24-hour apical exposure to freshwater B and saltwater C. Insets are obtained using a 20x objective. Complete culture media L-15/FBS was maintained in the basolateral compartment. RTgill-W1 cell nuclei are stained blue using NucBlue™ and cell membranes are stained green using CellMask™.

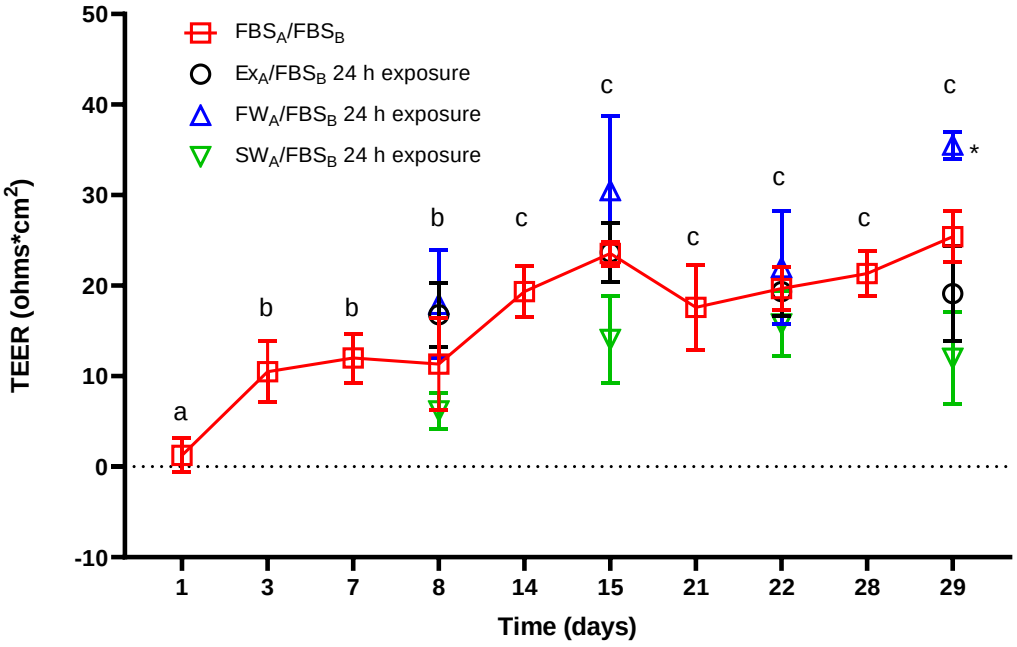

Figure 2. Transepithelial electrical resistance (TEER) values in RTgill-W1 cells cultured from 1 to 28 days in symmetrical complete media, L-15/FBS (FBS_A/FBS_B). Subscript letters A and B represent apical and basolateral compartments, respectively. 24-hour exposures were conducted with asymmetrical media conditions, L-15/Ex (Ex_A/FBS_B), freshwater (FW_A/FBS_B), and saltwater (SW_A/FBS_B) at day 7, 14, 21 and 28. Different lowercase letters denote significant differences among timepoints based on TEER values (one-way ANOVA Tukey's multiple comparison test; alpha = 0.05; n = 3). Asterisks denote a significant difference in TEER between cell monolayers exposed to control conditions FBS_A/FBS_B and experimental conditions (one-way ANOVA Dunnett's multiple comparison test; alpha = 0.05; n = 3).

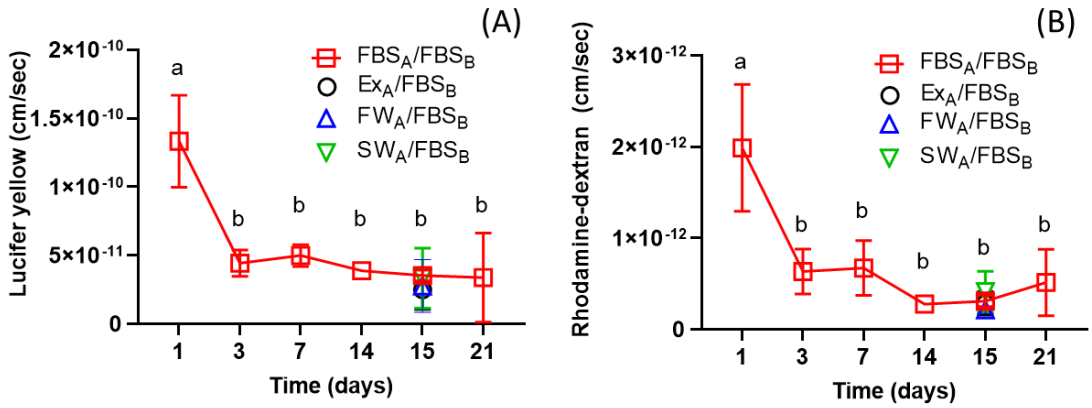

Figure 3. Permeability assay (P_{app}) values in RTgill-W1 cells cultured on transwell inserts in complete media, L-15/FBS (FBS_A/FBS_B) for Lucifer yellow (A) and Rhodamine-dextran (B) from 1 to 21 days. 24-hour exposures were conducted with asymmetrical media conditions, L-15/ex (Ex_A/FBS_B), freshwater (FW_A/FBS_B), and saltwater (SW_A/FBS_B) in monolayers seeded for 14 days. Different lowercase letters denote significant differences among timepoints based on P_{app} values (one-way ANOVA Tukey's multiple comparison test; alpha = 0.05; n = 3). No statistical difference was observed in the P_{app} of cells exposed for 24-hours to FW, SW or L-15/ex compared to the control L-15/FBS symmetrical (one-way ANOVA Dunnett's multiple comparison test; alpha = 0.05; n = 3).

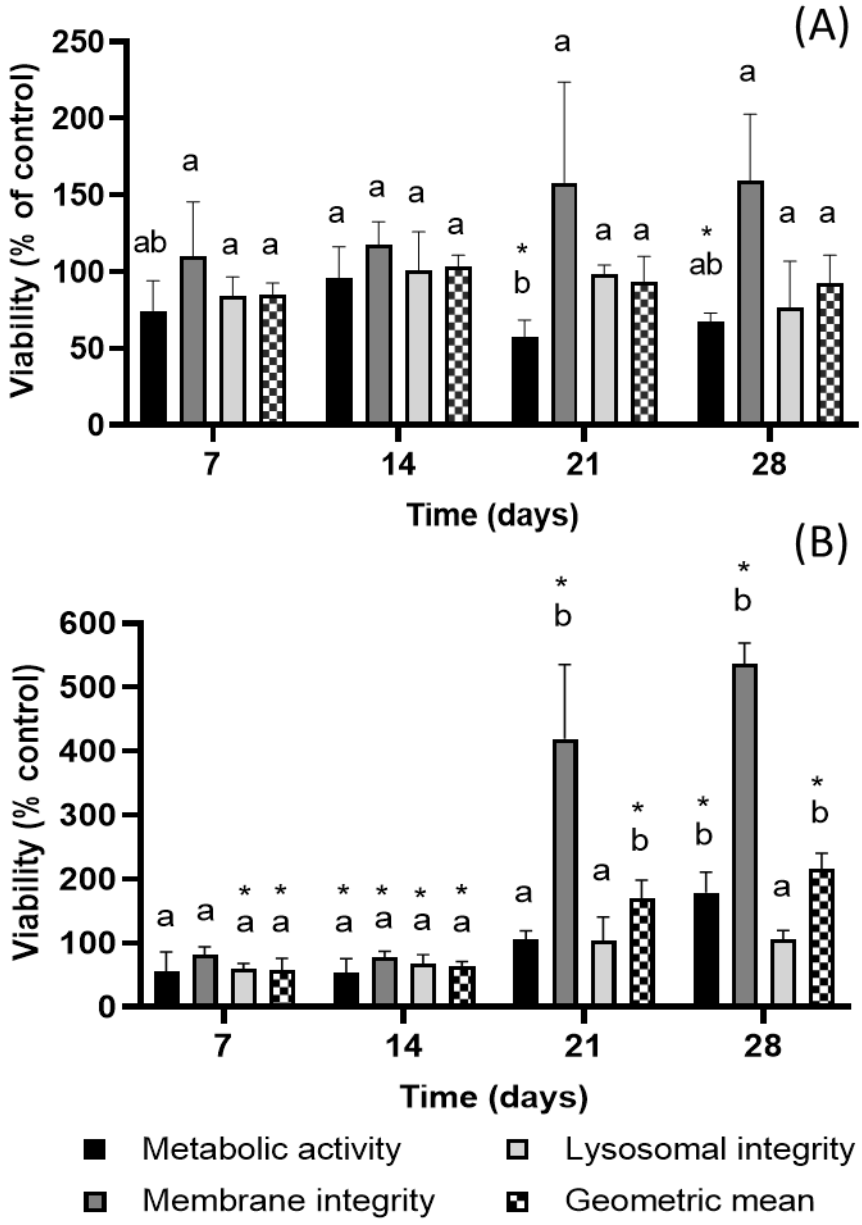

Figure 4. Cell viability in RTgill-W1 cells exposed for 24-h to apical freshwater (A) or saltwater (B) with L-15/FBS in the basolateral compartment. Viability results are reported as percentage viability based on the fluorescent units of the control (i.e., cells exposed to apical L-15/ex with L-15/FBS in the basolateral compartment). Different lowercase letters denote significant differences of individual toxicity endpoints among timepoints based on viability values (one-way ANOVA Tukey's multiple comparison test; alpha = 0.05; n = 3). Asterisks denote significant differences of individual toxicity endpoints compared to control (Student's t-test; alpha = 0.05; n = 3).

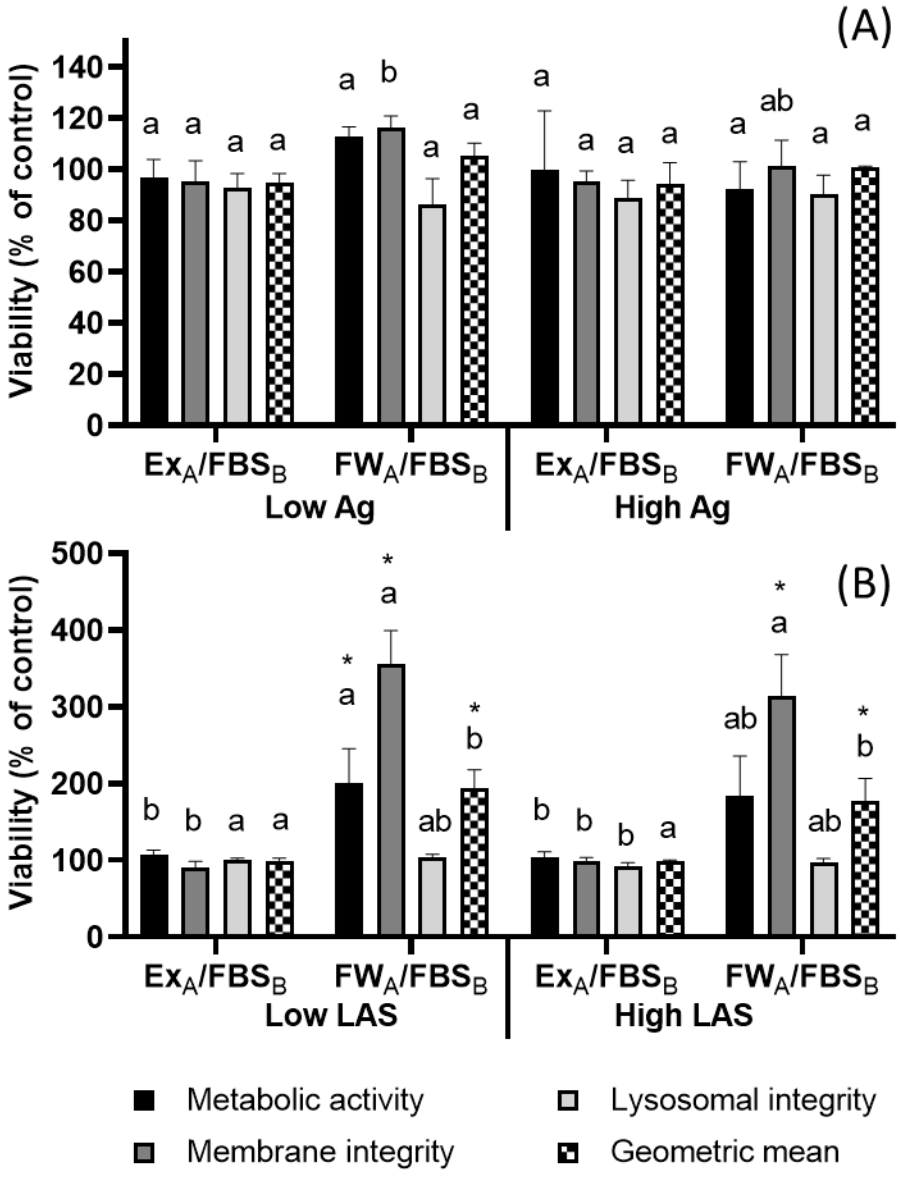

Figure 5. Cell viability in RTgill-W1 cells exposed apically to low (120 ng/ml) and high (240 ng/ml) silver as ion (A) and low (4000 ng/ml) and high (8000 ng/ml) linear alkyl sulfonate (LAS) (B) dissolved in L-15/ex (Ex_A) and freshwater (FW_A) with L-15/FBS in the basolateral compartment (FBS_B). Viability results are reported as percentage viability based on the fluorescent units of the L-15/Ex control (Ex_A/FBS_B). Different lowercase letters denote significant differences among individual endpoints based on viability (one-way ANOVA Tukey's multiple comparison; alpha = 0.05; test n = 3). Asterisks denote significant differences of individual endpoints compared to Ex_A/Ex_A control (Student's t-test; alpha = 0.05; n = 3).

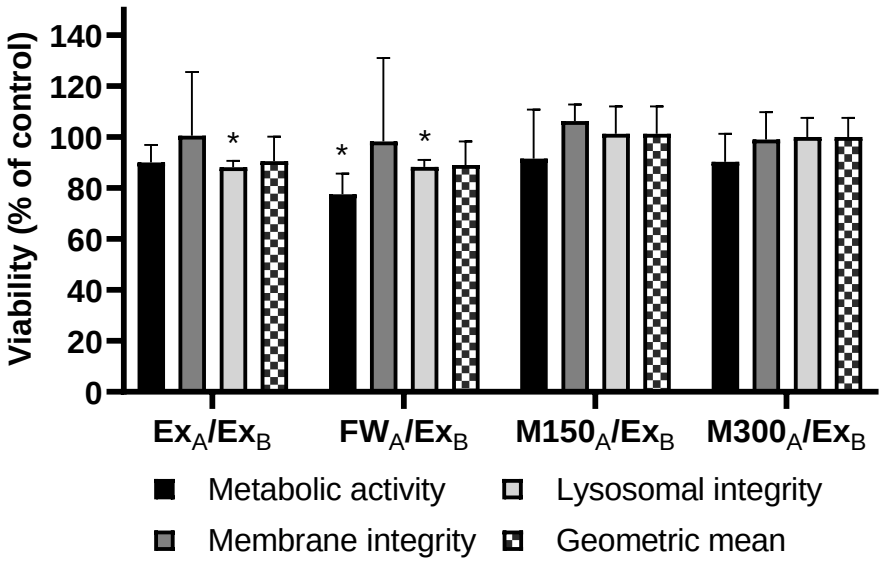

Figure 6. Cell viability in RTgill-W1 cells seeded for 14-d and exposed for 24-h to L-15/ex symmetrically or freshwater in apical with L-15/ex in the basolateral compartment. Viability results are reported as percentage viability based on the fluorescent units of the control (symmetrical; Ex_A/FBS_B). Asterisks denote significant differences with respective control (Student's t-test; alpha = 0.05; n = 3).

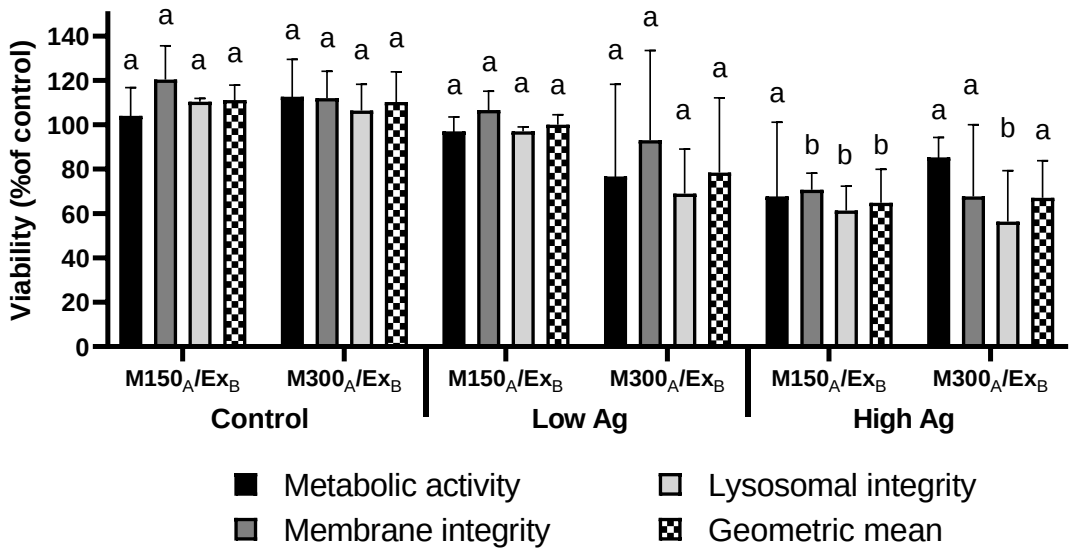

Figure 7. Cell viability in RTgill-W1 cells seeded for 14-d and exposed for 24 hours apically to mannitol hypoosmotic (150 mOsm/kg; M150) and isosmotic (300 mOsm/kg; M300) media and low (500 ng/ml) and high (1500 ng/ml) silver concentrations maintaining L-15/ex in the basolateral compartment (Ex_B). Viability in apical M150 and M300 is reported as percentage of symmetrical L-15/ex and control no statistical difference was found between controls (Student's t-test; alpha = 0.05; n = 3). Viability of low and high apical silver concentrations are compared to respective mannitol controls. Different lowercase letters denote significant differences in specific endpoints (e.g. metabolic activity) between silver treatments and respective controls (i.e., M150 and M300; one-way ANOVA Dunnett's multiple comparison test, alpha = 0.05; n = 3).

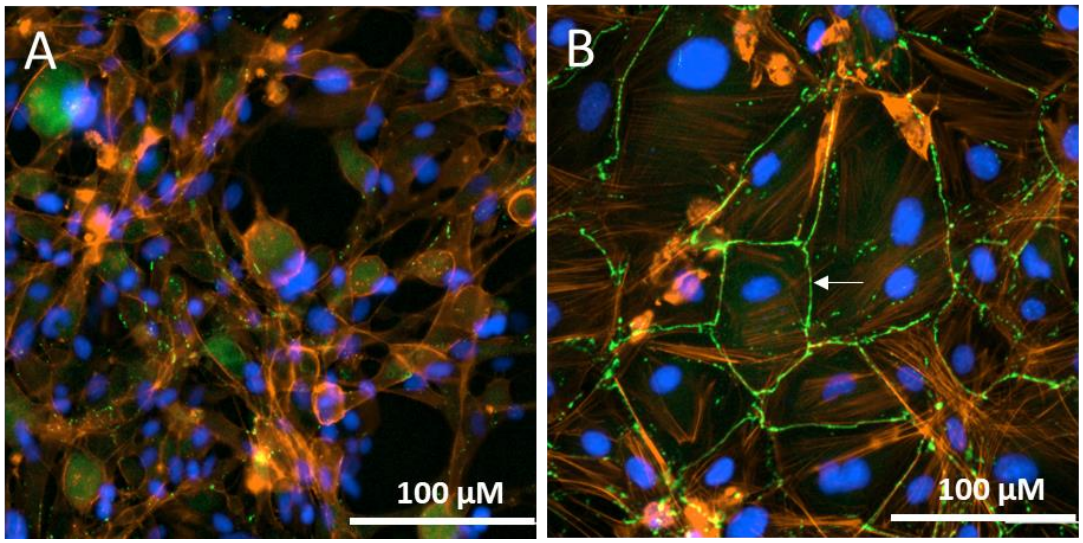

Figure 8. Images show RTgill-W1 (A) and RTgutGC (B) cell monolayers grown on transwell inserts for 14-d. Tight junction proteins were stained green with ZO-1 conjugated to Alexa Fluora® 488 indicated by the arrow. Cell nuclei were stained blue using NucBlue™ and f-actin stained red using Rhodamine phalloidin.

SUPPLEMENTARY MATERIALS

Table S1. Reference toxicant chemical salt information.

Chemical Name	CAS	Purity	Source
Sodium dodecylbenzene sulfonate (LAS)	25155-30-0	Technical Grade	Millipore Sigma
Silver nitrate	7761-88-8	≥ 99%	Millipore Sigma

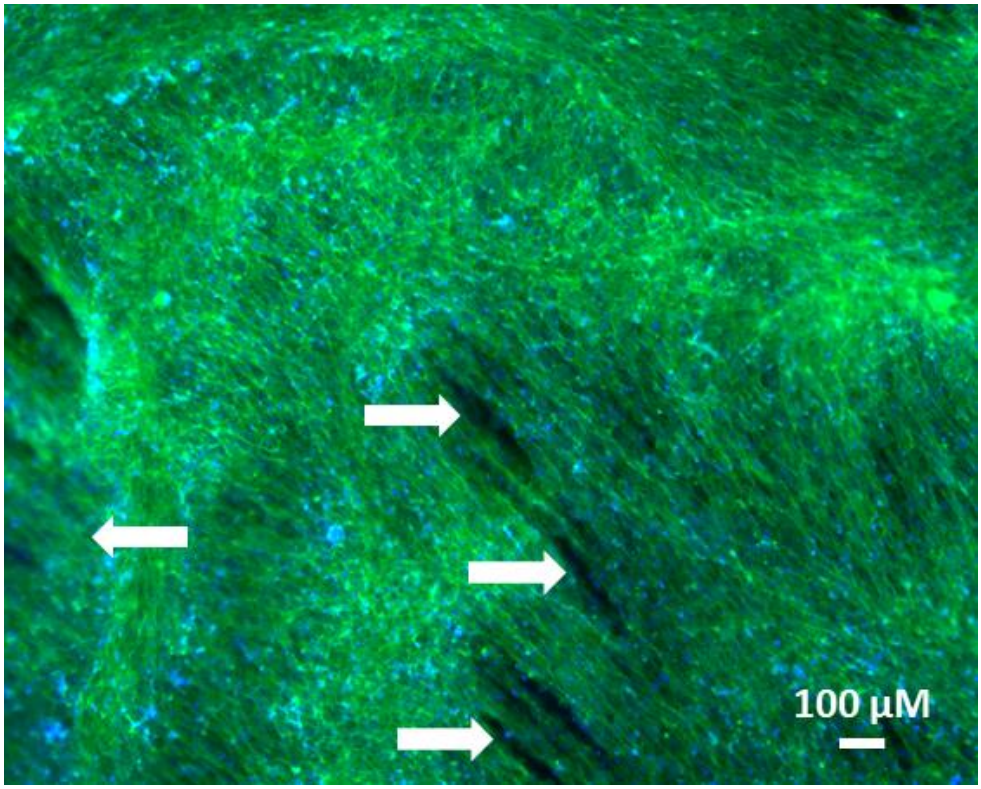

Figure S1. Image show RTgill-W1 cell proliferation and monolayer development at 28 days cultured in symmetrical L-15/FBS media (75,000 cells/cm^2 initial seeding). RTgill-W1 cell nuclei are stained blue using NucBlue™ and cell membranes are stained green using CellMask™. Arrows indicate lifting and gap formation of monolayer.

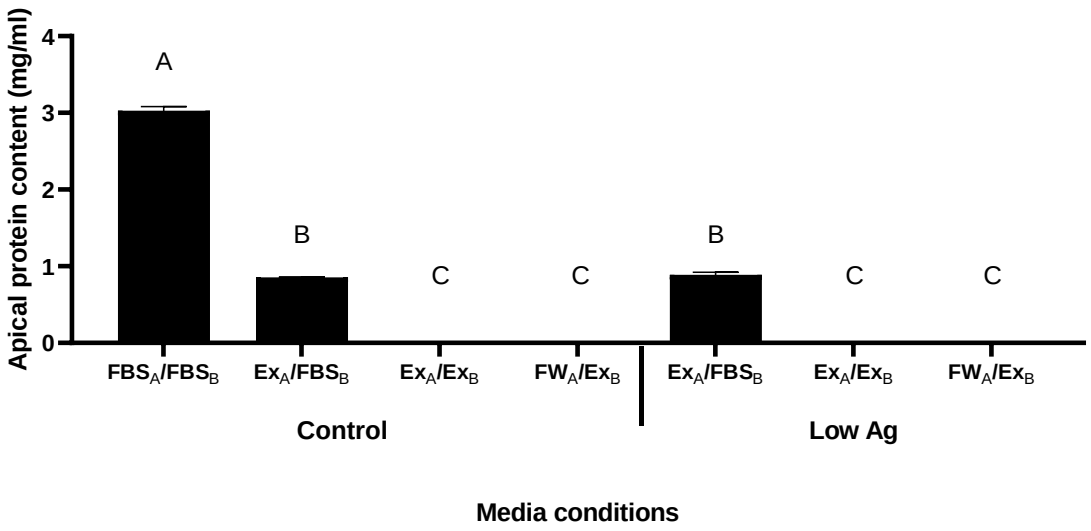

Figure S2. Protein content measured from the apical compartment of RTgill-W1 cells cultured on transwells for 14 day at 24 hour after exposure to media conditions. Subscript letters A and B represent apical and basolateral media compartments, respectively. Different uppercase letters denote significant differences among protein content ($p < 0.05$; one way ANOVA Tukey's multiple comparison test n = 3).

CHAPTER III

FISH EMBRYO ACUTE TOXICITY (FET) TESTING AND THE RTGILL-W1 CELL LINE AS IN-VITRO MODELS FOR WHOLE EFFLUENT TOXICITY (WET) TESTING: AN *IN VITRO*/*IN VIVO* COMPARISON OF CHEMICALS RELEVANT FOR WET TESTING.

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Justin Scott,[a,b],* Ryan Grewe,[a,b] and Matteo Minghetti[a]

[a]Integrative Biology, Oklahoma State University, Stillwater, Oklahoma, USA
[b]Cove Environmental, Stillwater, Oklahoma, USA

KEYWORDS: Aquatic toxicology; *in vitro* toxicology; predictive toxicology; toxic identification evaluation; whole-effluent toxicity testing.

ABSTRACT:

The fathead minnow (Pimephales promelas) Fish Embryo Acute Toxicity (FET) test was compared to the fish gill cells (RTgill-W1) *in vitro* assay and to the fish larvae acute toxicity test with the purpose to evaluate their sensitivity for whole effluent toxicity (WET) testing. The toxicity of 12 chemicals relevant for WET testing was compared as proof of principle. The concentrations lethal to 50% of a population (LC50) of embryos was compared to that in fish larvae and to the effective concentration 50% (EC50) in RTgill-W1 cells from previous literature. Along with traditional FET endpoints (coagulation, somite development, tail detachment and heartbeat), cardio toxicity was evaluated for WET applicability. Heart rate was measured at LC20 and LC50 values of six sub selected chemicals (Cd, Cu, Ni, ammonia, 3, 4-dichloraniline and benzalkonium chloride). Additionally, the toxicity of cadmium and nickel was evaluated in RTgill-W1 cells exposed in a hypoosmotic media to evaluate the effect that osmolarity may have on metal toxicity. A significant correlation was found between the FET and larvae LC50 values, but not between the RTgill-W1 EC50 and FET LC50 values. While sensitivity to Ni and Cd were found to increase with hypoosmotic conditions for FET and RTgill-W1 cells, a correlation was only found with the removal of Ni from the analysis. Cardio toxicity was shown in 3 of the 5 sub selected chemicals (Cd, Cu, and 3,4-dichloroaniline). Overall, FETs were less sensitive than larvae and more sensitive than RTgill-W1 cells to the WET chemicals, but both *in vitro* alternative models have shown good predictability of toxicity in fish, *in vivo*.

INTRODUCTION

In the United States (US), whole effluent toxicity (WET) tests rely heavily on the use of fish larvae to determine and predict harmful effects of wastewater to aquatic populations. Acute 96-hour fish tests are routinely used to measure the concentration lethal to 50% of a fish. While fish larvae offer a standardized approach for aquatic toxicity testing, alternative assays utilizing fish embryo acute toxicity (FET) tests population (LC50) for reporting purposes of effluent dischargers (USEPA 2002a). FETs and rainbow trout (*Oncorhynchus mykiss*) cell lines such as RTgill-W1, have been proposed as animal alternatives for regulatory use of effluent testing and chemical registration (OECD 2013; OECD 2021). Several regulatory biomonitoring protocols currently use fish embryos to measure toxicological and teratogenic effects including the International Organization for Standardization (ISO) International Maritime Dangerous Goods (IMDG) Code, the Federal Insecticide Fungicide Rodenticide Act (FIFRA), the Organization for Economic Co-operation and Development (OECD) and the US Environmental Policy Act (USEPA) (Scott and Minghetti 2020). Fish embryos have been approved for WET testing in Germany (Embry et al. 2010), but have seen little application in WET testing in the US. For the USEPA methods fathead minnow embryos are typically only used in chronic teratogenicity testing for site specific and water quality criteria investigations (USEPA 2002b).

Additionally, RTgill-W1 cells have been recognized as an animal alternative to acute fish testing through organizations such as OECD and the U.S. Department of Health and Human Service's National Toxicology Program but have not been considered for regulatory applications such as WET testing. Therefore, this study focused primarily

at determining the applicability of FET tests for routine USEPA acute WET testing, as well as looking at comparisons between FET tests and RTgill-W1 cells, incorporating previous findings of exposure conditions for cytotoxicity (i.e. hypoosmotic conditions) (Lee et al. 2009; Scott et al. 2021). Moreover, to date, FET tests have not been incorporated as an approved alternative method for acute WET testing in routine regulatory guidelines by the USEPA.

Although zebrafish is a commonly used species for research proposes and it is an established model to study fish development (OECD 2013), the USEPA guidelines utilize the fathead minnow in WET testing, as it is more of a geographically relevant species to the US (USEPA 2002b). Therefore, utilizing the fathead minnow for FET tests will serve as a more suitable species of fish for WET testing. Previous studies using fathead minnow FET tests showed good comparisons to zebrafish FETs for the reference chemicals ammonia and 3,4 dichloroaniline (Jeffries et al. 2014) as well as mock effluents simulating municipality and oily effluents (Jeffries et al. 2015).

Based on OECD's concept, a protected animal is any living vertebrate, other than man, capable of independent feeding (Halder et al. 2010). Therefore, fathead minnow embryos would not be considered living organisms until 176.5 hours post fertilization (hpf), at which they exogenously feed (USEPA 1996b). As such, FET tests and fish cell lines are both considered alternative models in toxicity testing, and their use can help to reduce the number of live animals used routinely in acute fish tests. In addition to the ethics of using fewer live animals, and the reduction in space and volume associated with alternative tests, cell lines and FET tests can provide insight into tissue specific and developmental toxicity, respectively. Thus, by incorporating cell line testing and FET

tests into acute WET testing guidelines, more ethical practices may also provide additional mechanistic toxicological information. The latter being particularly important for toxicity identification and reduction evaluation (TIE/TRE) strategies where classes of chemicals and toxicants of concern are difficult to specify in complex effluent matrices.

Similarly, to the larvae test, the FET test measures lethality through visual observation but with a focus on developmental markers throughout the test duration. For FET tests observations are conducted at 24-hour (h) intervals to determine the impact of chemicals on fish development. Standard toxicity endpoints include: i) coagulation of the embryo; ii) lack of somite development; iii) non-detachment of the tail, and; iv) lack of heartbeat which is used as endpoint of mortality and to generate LC50 values. Moreover, additional endpoints to measure more sensitive sublethal effects through FET novel assays would be important to potentially facilitate more precise predictions of adverse effects to aquatic populations (Birke and Scholz 2019). For instance, FET tests have shown to be an effective predictive method of sublethal toxicity of chemicals (Rawlings et al. 2019) and effluents (Kamelia et al. 2019), and have shown that heart rate can be good markers of toxicity for metals (Krzykwa et al. 2019). Therefore, in this study, we investigated if heart rate measurement could be incorporated as a more sensitive endpoint for FET tests in WET testing applications.

To determine if fathead minnow FET tests serve as a suitable alternative for acute WET testing, in the present study we measured and compared toxicity of 12 chemicals relevant for WET testing as proof of principle. The overall goal of this study was to determine how well the FET tests can predict acute toxicity in fish by correlating FET test LC50 values with LC50 values obtained from fathead minnow 96-h LC50 literature

results (Scott et al. 2021). Few animal alternative methods have been approved for ecotoxicological purposes and there is a need to identify which models serve as a best fit for regulatory purposes. For instance, the cellular effective concentration 50% (EC50; i.e., cytotoxicity) of RTgill-W1 cells has recently been shown to correlate with lethal concentration to 50% of fathead minnow populations (LC50) to common WET chemicals (Scott et al. 2021) and a broad range of organic chemicals (Tanneberger, Knöbel, Frans J.M. Busser, et al. 2013; Fischer et al. 2019).

Sample manipulation (i.e., supplementation of the sample with salts and sugars) is needed for compatibility with RTgill-W1 cytotoxicity assays. Specifically, the control and exposure medium used for cytotoxicity assays is manipulated to match the salt composition of Leibovitz's L-15, the medium routinely used to culture fish cell lines (Scott et al. 2021). Such exposure medium is called L-15/ex. L-15/ex is similar to Leibovitz's L-15 but without vitamins and amino acids and is isosmotic to the cells (300 mOsm/kg) to reduce osmotic stress. However, the addition of salts to allow isosmotic exposure conditions may result in a reduced bioavailability and toxicity of certain chemicals such as metals via complexation with anions or competition with cations (Ibrahim et al. 2020). Thus, metal complexation with anions and competition with cations may reduce the sensitivity of RTgill-W1 to metal toxicity and potentially affect their ability to detect toxicity in whole effluents contaminated with metals.

Previous studies have shown RTgill-W1 cells to tolerate hypoosmotic conditions at approximately 150 mOsm/kg compared to their isosmotic conditions of 300 mOsm/kg (Scott et al. 2021). Therefore, the toxicity of a subset of chemicals (cadmium and nickel) was tested in RTgill-W1 cells in a media with lower salt concentrations to determine if

the use of hypoosmotic versus isosmotic media affects the sensitivity of RTgill-W1 cells to metals in comparison to FET tests and larvae.

MATERIALS AND METHODS

Effluent toxicants

Inorganic metal and metalloid salts were purchased from Sigma-Aldrich and included: arsenic (III) oxide, cadmium chloride, chromium (VI) oxide, copper (II) sulfate pentahydrate, nickel (II) sulfate hexahydrate, sodium selenite, silver nitrate, and zinc sulfate heptahydrate. The organic compounds 3,4 dichloroaniline (3,4 DCA), benzalkonium chloride (BAC, classified as a Quaternary Ammonium Compound; QAC is 74.5% benzyldimethyldodecylammonium chloride, 22.3% benzyldimethyltetradecylammonium chloride), sodium dodecylbenzene sulfonate (Linear Alkylbenzene Sulfonate; LAS), and the volatile compound, ammonium chloride was also purchased from Sigma-Aldrich. Inorganic salts and polar organics were dissolved in ultrapure water (16–18 MΩ cm^{-1} Barnstead GenPure, Thermofisher) while 0.5 % v/v ultrapure water and dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used to dissolve non-polar organic chemicals.

All toxicants stock solutions were diluted in reconstituted synthetic moderately hard water for FET tests or in L-15/ex medium (ISO 2019) for RTgill-W1 cells. Reconstituted moderately hard water and L-15/ex medium chemical composition can be found in Table 1. Chemicals are listed in Table S1, and include category numbers, purity, and source. The concentration ranges (in µg/L as ion) for each chemical tested to the FET was: Ag, 21-170; As, 980-93,015; Cd, 9.7-4,640; Cr, 4,633-70,272; Cu, 93-11,280; Ni,

112-5,600; Se, 1,135-25,750; Zn, 880-14,300; BAC, 1,840-5,760; LAS, 92-19,600; ammonia (unionized), 71-4,928; and 3,4 DCA 100-500,000. For RTgill-W1 osmotic study concentrations ranges (in µg/L as ion) was: Cd, 254-157,607 and Ni, 4,565-780,334. In the present study, chemical exposures required higher than environmentally relevant concentrations in order to generate dose response curves for all three models. For metals and metalloids, an aliquot of exposure solution was acidified immediately after preparation to 5% nitric acid final concentration and measured using inductively coupled plasma mass spectrometry (ICP-MS; ICAP RQ, Thermofisher) analysis. The LAS was measured using a modified methylene blue standard method (Chitikela et al. 1995) in L-15/ex medium through chloroform extraction and measured at absorbance of 652 nm (Cytation 5 multiwell plate reader; BioTek). BAC was extracted using a 3:2 L-15/ex: acetonitrile liquid-liquid extraction followed by the addition of NaCl to aide in partitioning. The recovered acetonitrile extract was analyzed by ultra-high-performance liquid chromatography (UHPLC) following previous methods (Martínez-Carballo et al. 2007)) representing benzyldimethyldodecylammonium (BAC r=12) and benzyldimethyltetradecylammonium (BAC r= 14) degradation. Concentrations were calculated using the average concentration of the products. 3,4-DCA was analyzed by ultra-high performance liquid chromatography (UHPLC) using a solid phase extraction (Sep-Pak® C18 cartridge, Waters Corporation) and methanol elution following previous methods (Primel et al. 2007). Total ammonia was measured before and after exposure using ultraviolet-visible spectrophotometry (UV/Vis; Hach DR/2500, 115 Vac Hach) analysis at an excitation/emission value of 694 and 515 nm, respectively. Chemical

concentrations were measured before (time 0 hours – T_0) and at test take down (time 96 hours – T_{96}) to confirm toxicant stability in solution (Table 2).

Culturing of embryos

All animal husbandry, and FET tests were conducted at Cove Environmental's aquatic toxicity laboratory (Stillwater, OK) from spawning brood stock, following USEPA guidelines (USEPA 2002a). Briefly, sexually mature adult fathead minnows were cultured in source water from City of Stillwater (OK, USA). Water was carbon and ultraviolet light filtered and adjusted to meet proper USEPA water quality guidelines. Brood stock were cultured in recirculating systems and maintained at a temperature of 25 ± 1 °C under a 16:8-h light:dark photoperiod, between 50-100 footcandles. Cut polyvinyl chloride (PVC) pipe sections were used as spawning substrates and fertilized embryos were removed from spawning tanks within one hour of lights turning on and checked for their cell stage under a phase contrast light microscope (Olympus CKX53) equipped with a high-resolution camera (Olympus EP50). Less than 32-cell stage embryos were manually removed from spawning substrates, rinsed with synthetic moderately hard water, and used immediately for effluent toxicant exposures.

FET tests

FET tests were conducted using previous study methods (Jeffries et al. 2015) using the fathead minnow. Each condition (i.e. one concentration of a single chemical plus a positive and a negative control) consisted of 20 embryos at $\leq$ 32-cell stage (Figure 1A). The embryos were placed into the well of a 24-well plate (one egg per well) containing 2.5 mL of exposure solution. The remaining 4 wells on each plate were used as internal

negative controls. A negative control (USEPA synthetic moderately hard water) and a positive control (EC50 of 3,4 DCA) plate were included with each test. FETs exposure solutions were renewed (80% removal of existing volume) every 24-h and incubated at 25 ± 1 ºC with a 16:8 h light: dark photoperiod between 50 to 100 ft-c throughout the 96-h duration. Measured toxicity endpoints (determined for LC50 values) consisted of i) coagulation of the embryos; ii) lack of somite formation; iii) non-detachment of the tail; and, iv) lack of heartbeat (Figure 1). These endpoints were evaluated under a phase contrast light microscope (Olympus CKX53) equipped with a high-resolution camera (Olympus EP50) at 24-h intervals until test termination at 96-h. Lethality was determined if at least one endpoint was observed throughout test duration.

Cardio toxicity was determined at 96-h test take down through observation under the microscope. The video recordings of embryos heartrate were counted using a tally counter as beats per minute. Cardio toxicity was not factored into final LC50 values and correlations but analyzed on a subset of chemicals: ammonia, Cd, Cu, Ni, BAC and 3,4 DCA. The LC20 and LC50 values established in the current study were used as target concentrations in cardio toxicity tests and compared to controls (synthetic water). Heart rate (bpm) was recorded on remaining living embryos at the end of each test. FET test quality control criteria followed OECD Guidelines 236 and consisted of ≥ 90% survival in the negative controls. Analytical recovery values are reported in Table 2. All chemical values excluding ammonia and benzalkonium chloride at FET test take down were within 10 % recovery from nominal concentrations.

RTgill-W1 cells and cytotoxicity assays

Cytotoxicity assays were conducted for 3,4 DCA and cadmium to determine EC50 values in isosmotic and hypoosmotic conditions, respectively following the multiple viability endpoint assay (ISO 2019). Briefly, RTgill-W1 cells were cultured in 75 cm^2 cell culture flasks (Greiner Bio-One, Kremsmunster, Austria) with Leibovitz's L-15 (Invitrogen, Thermofisher, Waltham, MA, USA) supplemented with 5% Fetal Bovine Serum (FBS; F6178, Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (5,000 units ml^{-1} penicillin and 5 mg ml^{-1} streptomycin; Sigma-Aldrich, St. Louis, MO, USA) at 19 ºC in normal atmosphere. Cells were seeded into 24 well plates (Greiner Bio-One, Kremsmunster, Austria) at a seeding density of 150,000 cells/mL (78,947 cells/cm^2) and placed into 24 multiwell plates (Greiner Bio-One, Kremsmunster) and incubated at 19 ºC, in the darkness for 48 h to allow development of a confluent monolayer.

Before exposure, cells were washed with respective hypo- (150 mOsm/kg) and isosmotic (300 mOsm/kg) medium and then exposed to chemical concentrations in respective media (isosmotic for Cd, Ni, and 3,4-DCA and hypoosmotic for Cd and Ni) for 24 h in the dark at 19 ^{o} C. After 24 h of exposure, cells were washed again, and the multiple endpoint viability assay was performed. The multiple endpoint viability assay relies on the application of three commercially available dyes which are alamarBlue (AB; Invitrogen, Thermofisher, Waltham, MA, USA), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Thermofisher, Waltham, MA, USA), and 3-amino-7-dimethylamino-2-methylphenazine hydrochloride or Neutral Red (NR; Sigma-Aldrich, St. Louis, MO, USA). These dyes are used as indicators of cell metabolic activity,

membrane integrity, and lysosomal integrity, respectively (Lee et al. 2008; Minghetti and Schirmer 2016b). Chemical concentrations, positive control (3,4-DCA) and negative control (L-15/ex) were run in triplicate wells.

Statistical Analysis

RTgill-W1 EC50s were generated previously and the *in vivo* LC50s were obtain from the literature and are described in Scott et al. (2021). Larvae 3,4 DCA LC50 values were obtained from the previous study by Jeffries et al. (2014). FET test LC20 and LC50 and RTgill-W1 EC50 values were determined by the non-linear regression sigmoidal dose-response curve fitting module using the hill slope equation. Correlation data was found to be parametric through base-10 logarithm transformations using Kolmogorov-Smirnov test. Therefore, parametric analysis with no weighing was applied, and a two tailed Pearson's r coefficient test was performed. Ranking of FET test chemical toxicity data was also found to be parametric through base-10 logarithm transformations using Anderson-Darling test and a post hoc Tukey's multiple comparison was conducted. Embryo cardio toxicity data was found to be non-parametric, and a Kruskal-Wallis and post hoc Dunn's multiple comparison test was conducted. All experiments were repeated at least 3 times on different days with cells of different passage and embryos from different days. All statistical analysis tests were performed with alpha equal to 0.05 using GraphPad Software (Prism version 9.0).

RESULTS

Embryo toxicity

Lethal concentration values.

LC20 and LC50 values were calculated from dose-response curves for all 12 chemical exposures of FET tests (Figure 2). Toxicity ranking of chemicals for the FET tests can be found Table 3 and are ordered from most toxic to least toxic as: Ag > Cu > NH_3-N > Ni > Cd > Zn > BAC > LAS > 3, 4-DCA > Se > Cr > As, based on LC50 values. Coagulation and lack of heartbeat was the most common observed lethality marker (data not shown). For copper and ammonia, lethality occurred primarily upon hatching before test take down at 96 h. Embryo hatch rate success was not determined from the current study's results. Figure 3 shows a representational image of embryo development for the control and the subset of 5 chemicals (Cu, Cd, ammonia, 3,4-DCA, and BAC) throughout the 96-h test period at the respective LC50 values of each chemical.

Embryo cardio toxicity.

Cardio toxicity results are shown in Figure 4 and compare embryo LC20 and LC50 values to control based on heart rate (beats/min). Average heart rate values were found to be statistically different from control for the following exposure scenarios: Cd LC20 ($p = 0.0321$) and Cd LC50 ($p < 0.0001$), Cu LC50 ($p < 0.0001$), 3,4-DCA LC20 ($p < 0.0001$) and 3,4-DCA LC50 ($p < 0.0001$) and, BAC LC20 ($p < 0.0001$) and LC50 ($p = 0.0002$). Average heart rate values were statistically different between their respective

chemical LC50 to LC20 values for Cd ($p = 0.0066$) and Cu ($p = 0.0165$). For 3,4 DCA cardio toxicity, the LC20 was significantly lower than all other chemical LC20s. For Cd cardio toxicity, the LC50 was lower than all other chemicals, however only significantly lower than ammonia, Ni, and BAC LC50 values.

Model correlations

Fathead minnow FET tests and larvae LC50 values correlated significantly (Figure 5A Pearson's R correlation coefficient, $p = 0.0061$). Embryos were more sensitive than larvae to Cu, ammonia, Ni, Cd, and Zn. The embryo Ni LC50 values were one order of magnitude lower than the larvae. Conversely, the embryo BAC LC50 value was one order of magnitude higher than that of the larvae. FET LC50 and RTgill-W1 cell values did not indicate a correlation between the results as shown in Figure 5B (Pearson's R correlation coefficient, $p = 0.241$). Between embryos and RTgill-W1 cells, embryos were more sensitive to Ag, Cu, Ni, Cd, LAS, 3, 4-DCA, and Se. The embryo LC50 value from Ni was more than one order of magnitude lower than that of the RTgill-W1 cells, whereas the LC50 values from As was more than one order of magnitude higher in embryos than RTgill-W1 cells. However, when Ni was removed from the analysis, a significant correlation was found between FETs and RTgill-W1 cells (Pearson's R correlation coefficient, $p = 0.0242$).

Role of media composition on RTgill-W1 cell sensitivity

Hypoosmotic conditions increased sensitivity in RTgill-W1 cells for Cd and Ni. Hypoosmotic conditions for Ni indicated a decrease in EC50 values from 1709 ± 328 to 123 ± 3 mg/L, and Cd was seen from 8154 ± 540 to 1570 ± 51 µg/L. However, when

these values were calculated into the present study, the results did not indicate a change in correlation between RTgill-W1 cells and the FET tests as shown in Figure 5C (Pearson's R correlation coefficient, $p = 0.0822$).

DISCUSSION

Toxicity model correlations

Based on the correlation between embryo LC50 values and larvae LC50 values, the embryos and larvae showed similar sensitivity to many of the chemicals tested. This indicates that FET tests may offer a viable alternative to larvae testing to many effluent toxicants. These trends become more evident when the chemicals are grouped by classes and their toxic mechanism of action. Of the chemicals that were more toxic to larvae than embryos, only the QAC, (BAC) and 3,4-DCA showed an increased toxicity greater than one order of magnitude. This difference in sensitivity could be due to the protective effect of the chorion present in the embryo. Although there have been no studies determining the protective effects of the chorion on BAC specifically, Braunbeck *et al.* (2005) found that in zebrafish, removing the chorion increases toxicity of 4-chloroaniline. This indicated that the chorion may protect the embryo from some organic compounds. Interestingly, Olivares et al. (2016) saw a similar increase in embryo toxicity to As when the chorion was removed. Chorion protection may also, therefore, explain why the embryos were less sensitive than the RTgill-W1 cells to As. For this reason, Henn and Braunbeck (2011) suggest that removing the chorion may improve the representativeness of FET tests for some chemicals, especially those that are large or have been shown to accumulate in the chorion. However, this procedure may increase the technical difficulty

of trying to incorporate FET tests to routine WET testing, and that it would not be suitable for all exposure scenarios or chemicals.

Furthermore, FET tests focus primarily on exposure at the embryonic developmental stage (< 96-h) but may incorporate eleuthero embryonic stages where premature hatching may occur as seen in our results. Krzykwa et al., (2021) used up to 120-h to allow eleutheroembryo exposures for fathead minnow as the chorion is an important defense for the developing embryo and eleutheroembryos have potential for an increase in chemical sensitivity. Future studies could determine if alternative exposure protocol is warranted (e.g., 48, 96 or 120-h exposure time) to ensure both in ovo and eleutheroembryo stages. For instance, the ISO FET method (ISO 2007) utilizes a 48-h duration which ensures exposure is only to the embryo and is currently used by Germany for regulatory chemical discharges and WET testing (Embry et al. 2010). Additionally, using a 48-h acute test duration could reduce WET test costs. Further studies would need to compare assay sensitivity side by side in fathead minnow FET tests with exposures done for 48, 96 and 120-h to determine if extending or reducing the acute exposure timeframe is warranted. Moreover, based on our findings, using recently fertilized fathead minnow embryos up to the 96-h timepoint, successfully predicts toxicity for 10 of 12 WET chemicals of concerns to larvae.

Ag, Cu, and ammonia were among the most toxic chemicals to fish embryos, RTgill-W1 cells, and fish larvae. Interestingly, the toxicity order of Ag > Cu > ammonia was consistent across all three models. In freshwater fish, both Ag and Cu can inhibit the Na-K/ATPase and disrupt osmoregulation, which may possibly explain why Ag and Cu were particularly toxic in all three models (Wood *et al*. 1999; Blanchard and Grosell

2006). Zn is another chemical that has the ability to cause osmoregulatory stress in fish (McRae *et al.* 2016). In this study, Zn was also consistent with regards to its toxicity to all three models. In hypoosmotic exposure medium RTgill-W1 cell's sensitivity to Cd increased to concentrations similar to those of the embryo and larvae. This may be due to the reduction of salts in the exposure media, especially Ca which has been previously shown to reduce toxicity in zebrafish (*Danio rero*) embryos (Meinelt et al. 2000) and rainbow trout juveniles (Pascoe et al. 1986). In a previous study, RTgill-W1 cells were also shown to be more sensitive to Ni in hypoosmotic conditions (Scott et al. 2021), however for Ni, embryos and larvae are shown to be more sensitive than RTgill-W1 cells in hypoosmotic conditions.

The overall low correlation between the sensitivities of embryos and RTgill-W1 cells may in part be explained by a matrix effect in the cell exposure media. Ni toxicity has been shown to decrease with higher water hardness, and possibly also due to complexation with chloride (Bielmyer et al. 2013). The comparatively high salt concentration of the medium used in the RTgill-W1 cultures may result in a reduction of chemical (i.e., metals) bioavailability via complexation with anions or competition with cations. This may explain why RTgill-W1 cells were less sensitive than the embryos for Ni and Cd. For instance, dissolved Ca has been shown to protect from both Ni and Cd toxicity (Richards and Playle 1999; Blewett and Leonard 2017; Ibrahim et al. 2020).This may also be the case with 3, 4-DCA which could be having reduced bioavailability in a high salt isosmotic exposure medium and should warrant further investigation for organic exposures. This would theoretically shift the sensitivity more towards the embryos, which are exposed in a media with extremely low osmolarity compared to the cells.

Decreasing the osmolarity of the RTgill-W1 media did increase the sensitivity of the cells to Ni and Cd. Therefore, according to our data, exposure of real effluent samples at hypoosmotic conditions (i.e. 50% of the salts added) would represent a more sensitive exposure set up for RTgill-W1 cells cytotoxicity assays as seen with Cd and Ni. Incorporation of this modification of current ISO and OECD guidelines would improve WET cytotoxicity assays methods using RTgill-W1 cell lines.

FET tests and cardio toxicity

Cardio toxicity (i.e., heart rate) was measured with the goal of establishing an additional endpoint for FET tests in response to WET chemicals used in the present study. Overall, heart rate offers an empirical and easily quantifiable endpoint for cardio toxicity to be incorporated into the standard four endpoints for WET testing. Embryos exposed to Cd, 3,4-DCA, and BAC had significantly lower heart rates at both LC20 and LC50 concentrations, while those exposed to Cu had significantly lower rates at LC50 concentrations. Cardio toxicity as heart rate and pericardial swelling have been shown to be viable endpoints for fathead minnows in previous studies for 3,4-DCA and Cd (Krzykwa et al. 2019; Krzykwa et al. 2021). Similarly, Gondek (2010) found that BAC can cause cardiac deformations and pericardial swelling in medaka (*Oryzias latipes*) embryo. Pericardial swelling was seen in the present study for both BAC and 3, 4-DCA exposures (Figure 3). Pericardial swelling has also been previously seen in zebrafish (*Danio rerio*) for 3, 4-DCA exposures (Scheil et al. 2009).

Interestingly, some studies suggest that Cu exposure can cause increases in heart rate in fish embryo, specifically zebrafish (Johnson et al. 2007) and medaka (Barjhoux et

al. 2012). However, this was not seen in our results with fathead minnows, where only a decrease in heart rate was measured for Cu exposures. In the present study, embryos exposed to Ni and NH_3-N did not have significantly different heart rates than controls. Cd toxicity was also a strong indicator of cardio toxicity and has previously been shown to affect calcium metabolism (Wright et al. 1985) of juvenile striped bass (*Morone saxatilis*) which is vital in its role of cardiovascular health and physiological components. Moreover, Cd^{2+} was shown to induce inhibition of the L-type calcium current for ventricular myocytes (Haverinen et al. 2021) in rainbow trout (*O. mykiss*). From these results the addition of cardio toxicity endpoints in FET tests for WET testing could prove beneficial as a quantifiable endpoint to serve as a forensic component in identifying specific toxicants of concern as seen with Cd.

Future progress and applications

From an ethical standpoint, the FET test does not truly address the concept of live animal toxicity testing replacement as the cell line does, but it can serve to reduce the number of living animals used and provides an approach to measure additional toxicological endpoints compared to the fish acute toxicity test (OECD 236; USEPA 2002a). Furthermore, it can provide insight into the toxicity of substances at sensitive developmental stages in fish. However, consideration must still be given to the organism's wellbeing when using embryos in toxicity testing. For instance, it has been found that 120-hpf fish larvae responded to noxious stimuli and that the response was relieved with pain reducing drugs (Lopez-Luna et al. 2017). While this timepoint exceeds our current study, further insight is needed into the neurological development and pain sensitivity of the embryos up to 96-h of development. Moreover, while the FET tests

have shown to be an effective predictive method of sublethal toxicity of chemicals, they still require husbandry practices of breeding adult vertebrate test specimens which can have ethical and cost implications.

Advanced assays could provide more information to toxicological insults of effluents but may not be ideal for routine WET methods due to costs and duration. Examples such as the comet assay has also been used with FET tests for whole sediments (Kosmehl et al. 2006), sewage effluents (Babić et al. 2017), and uranium mining effluents (Lourenço et al. 2017). Growth hormone inhibition and stress induced gene expression have been suggested and warrant additional biomarkers to be studied (Kroon et al. 2017). Importantly, there is a need to build on FET test chemical exposure data and comparing lethal to sublethal exposures of FET tests with fish larvae (Roush et al. 2018). Advanced toxicological endpoints may not be feasible in current WET testing facilities, but the present study has shown the feasibility of possibly incorporating the four traditional endpoints and cardio toxicity into WET testing.

Additional developmental endpoints such as hatch success (Roush et al. 2018) have also been suggested to broaden the current developmental endpoints. Moreover, extending the exposure time to 120-h would potentially increase the sensitivity of this assay extending the time of exposure post hatching. The limitation of the 96-h FET test using fathead minnows is that embryos remain in ovo for the duration of the test exposure, and others that hatch prematurely. More research is needed to compare systematically 96 vs 120-h exposure sensitivity of fathead minnow embryo for acute WET exposures. As suggested, modifying the exposure length to 120-h would allow hatching timepoints to be observed. As mentioned earlier a systematic evaluation of the

test duration should be further investigated to optimize the FET tests into current WET methods. Furthermore, there may be ways to reduce the overall time and labor involved in FET testing when applied to WET testing. In the present study, coagulation and lack of heartbeat were the most common endpoints measured. In fact, one of these endpoints was measured in all cases where mortality occurred (i.e. no other endpoints occurred without either coagulation or lack of tail detachment as well). Therefore, it may be more feasible to focus specifically on these two endpoints, rather than evaluating all 4 traditional endpoints throughout the test for WET testing. Additionally, pericardial edema, and cranial and tail malformations, and premature hatching were observed (Figure 3), which could be incorporated as lethal or sublethal predictive endpoints in this assay.

For RTgill-W1 cells, further studies towards WET applications should include hypoosmotic conditions which have shown to increase the sensitivity of the cytotoxicity assay. Ultimately, actual effluent and wastewater samples will need to be utilized to determine how successful the alternative assays are of predicting acute toxicity to the larvae assays. Our results suggest that embryos and RTgill-W1 cells may act as better surrogates than larvae to test chemical mechanism of toxicity (i.e., cardio toxicity and subcellular endpoints, respectively). Future studies can expand on the relationship between specific mechanisms of toxicity and the toxicological predictability among larvae and alternative tests.

CONCLUSION

Ultimately, larval assays would only be used as a final step needed for confirming toxicity when alternative models are unable to detect failures (i.e., toxicity) potentially due to target organ toxicity and or physiochemical properties of toxicants. In

addition, incorporation of these models into current WET testing guidelines could be considered for intralaboratory and interlaboratory studies, such as round robin testing to determine how well the approach fits into current WET testing applications. This should include the use of actual wastewater and proficiency testing samples. If incorporated appropriately, cytotoxicity and FET tests may have the ability to help reduce the large-scale use of live fish testing for acute WET testing as well as introduce advanced toxicological screening and identification capabilities.

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STATEMENTS AND DECLARATIONS

Competing Interest:

The authors have no known conflict of interest that could have influenced the reporting of data in this article**.**

Authors Contribution: Justin Scott: Conceptualization; Investigation; Data curation, Formal analysis; Writing—original draft. Ryan Grewe: Investigation; Validation; Writing—review & editing. Matteo Minghetti: Conceptualization; Supervision; Validation; Writing—review & editing.

Animal Research (Ethics): Fathead minnow embryos were obtained from Cove Environmental's aquatic toxicity laboratory (Stillwater, OK) and cultured from spawning brood stock following USEPA guidelines (USEPA 2002) and Oklahoma State University Institutional Animal Care and Use Protocol (IACUC-22-45).

TABLES AND FIGURES

Table 1. Composition of hard synthetic water and L-15/ex media.

Inorganic salt/component (mM)	Hard synthetic water	L-15/ex Hypoosmotic	L-15/ex Isosmotic	L-15/ex Hyperosmotic
Cl^-	8.16	72.7	145.4	218.1
Ca^{2+}	3.98	0.67	1.34	2.01
Mg^{2+}	1	0.96	1.92	2.88
Na^+	1.54	71.25	142.5	213.75
K^+	0.16	3.14	6.28	9.42
PO_4^{3-}	0	0.89	1.78	2.67
SO_4^{2-}	1	0.425	0.85	1.28
Na-Pyruvate	0	5.08	5.08	5.08
Galactose	0	5.07	5.07	5.07
Ionic strength (mmol/kg)	15.7	76.3	149.8	220.3
Osmolality (mOsm/kg)	18	150	300	450
pH (su)	7.62	7.18	7.21	7.43

Ionic strength calculated using Visual MINTEQ, version 3.1. Osmolality and pH were measured by Vapro® Vapor Pressure Osmometer (Model5600, ELItech group, South Logan, UT, USA) and SI Analytics pH meter (SI Analytics, College Station, TX, USA), respectively.

Table 2. Analysis of chemical concentrations for FET exposure samples

Chemical	Initial chemical stability	Final chemical stability
	% Mean ± sd	% Mean ± sd
Silver	91 ± 9	82 ± 6
Arsenic	101 ± 3	94 ± 5
Cadmium	99 ± 2	92 ± 4
Chromium	101 ± 13	105 ± 6
Copper	98 ± 10	102 ± 5
Nickel	108 ± 5	101 ± 4
Selenium	103 ± 7	109 ± 7
Zinc	108 ± 7	106 ± 17
Benzalkonium chloride	95 ± 2	89 ± 5
Ammonia	82 ± 8	50 ± 6
Sodium dodecylbenzene sulfonate	95 ± 3	91 ± 4
3,4 dichloroaniline	97 ± 9	78 ± 3

Table 3. Summary of chemical lethality concentration of 50% (LC50) values for fathead minnow (*Pimephales promelas*) fish embryo acute toxicity (FET) test (FET), RTgill-W1 cell effect concentration 50% (EC50) and larvae literature LC50 values.

Chemical	FET mean LC50 ± SD (µg/L)	RTgill-W1 geometric mean EC50 ± SD (µg/L)[a]	Larvae median LC50 (µg/L)[a]
Ag	48 ± 6[A]	117 ± 6	14
Cu	267 ± 41[B]	329 ± 50	427
NH_3-N (Unionized)	833 ± 147[C]	767 ± 169	1010
Ni	1443 ± 227[C]	1708625 ± 327870	28500
Cd	1471 ± 291[C]	8154 ± 540	3060
Zn	3085 ± 943[D]	2348 ± 305	4700
BAC	3451 ± 90[D]	1067 ± 93	280
LAS	3678 ± 477[D]	3955 ± 417	3400
3, 4 Dichloroaniline	6054 ± 898[E]	66068 ± 336	350[b]
Se	7979 ± 3041[F]	8232 ± 285	6250
Cr	40116 ± 4868[G]	6105 ± 433	36100
As	61533 ± 3819[H]	2396 ± 586	15900

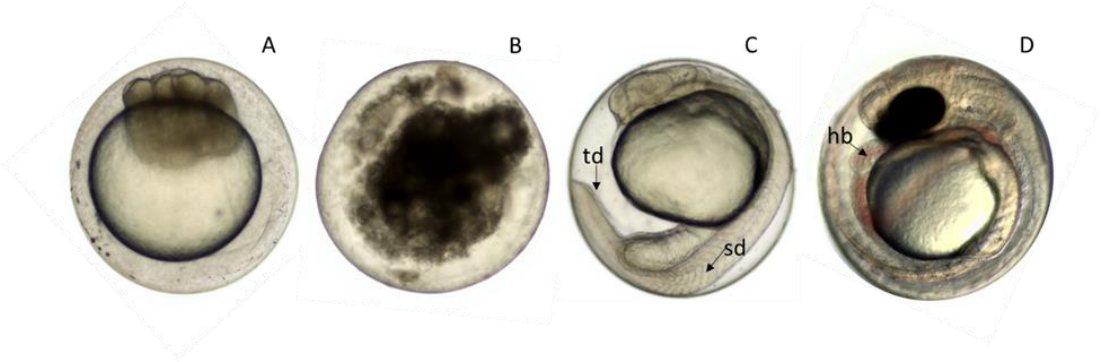

Figure 1. Development and morphological endpoints of the FET test using the fathead minnow (*Pimephales promelas*) starting with less than 32 cell stage embryos (A). Endpoints include the presence or absence of: coagulation (B), tail detachment (td) and somite development (sd) (C), and heartbeat (hb) (D).

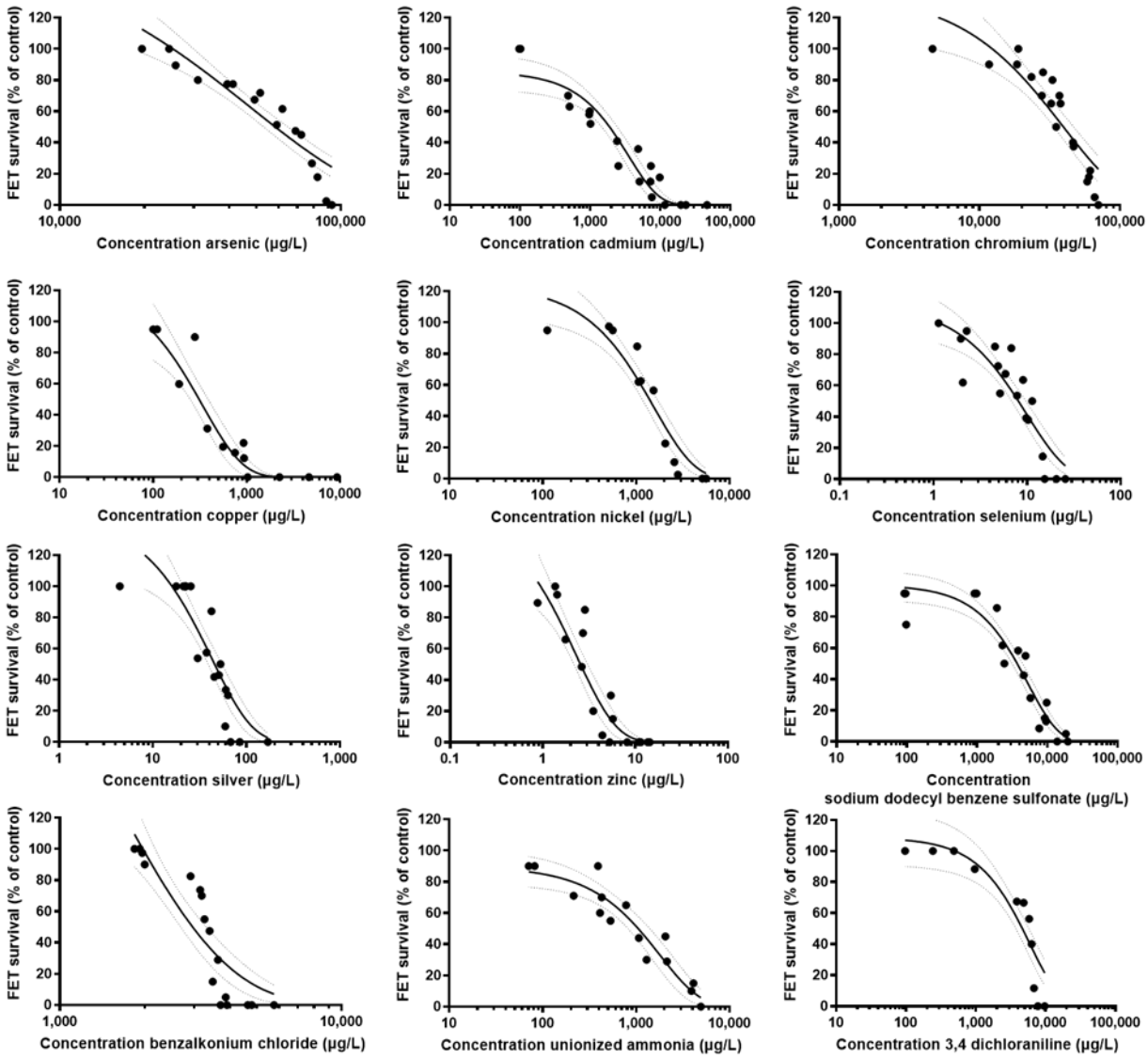

Figure 2. Dose response curves of selected chemicals in the fathead minnow (*Pimephales promelas*) fish embryo acute toxicity (FET) tests. Lethality is determined by presence of either coagulation, lack of tail detachment, somite development and heartbeat. Results from lethality assays are reported as percent survival based on the control. Values reported as mean (marker) and confidence intervals (dashed lines) of at least three independent experiments (n = 3).

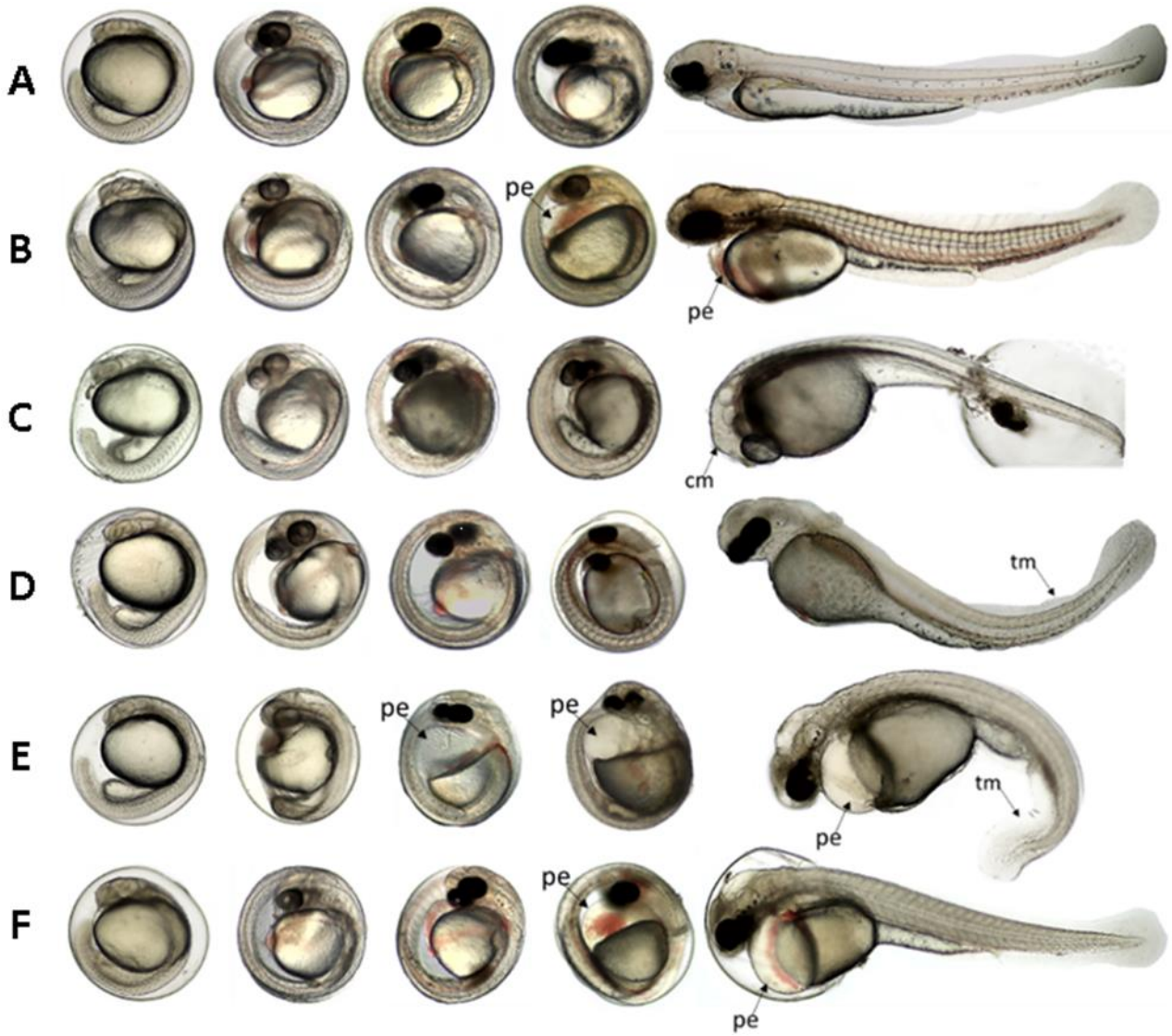

Figure 3. Images of the fathead minnow (*Pimephales promelas*) fish embryo test (FET) for A) control; B) copper; C) cadmium; D) ammonia; E) 3,4 dichloroaniline; and, F) benzalkonium chloride exposures at respective lethal concentrations of 50% population (LC50). Embryos are aged starting left to right: approximately 24, 48, 72, and 96-h prehatch (fully within chorion) and hatched, respectively. Teratogenic effects are indicated as pericardial edema (pe), cranial malformation (cm), and tail malformation (tm).

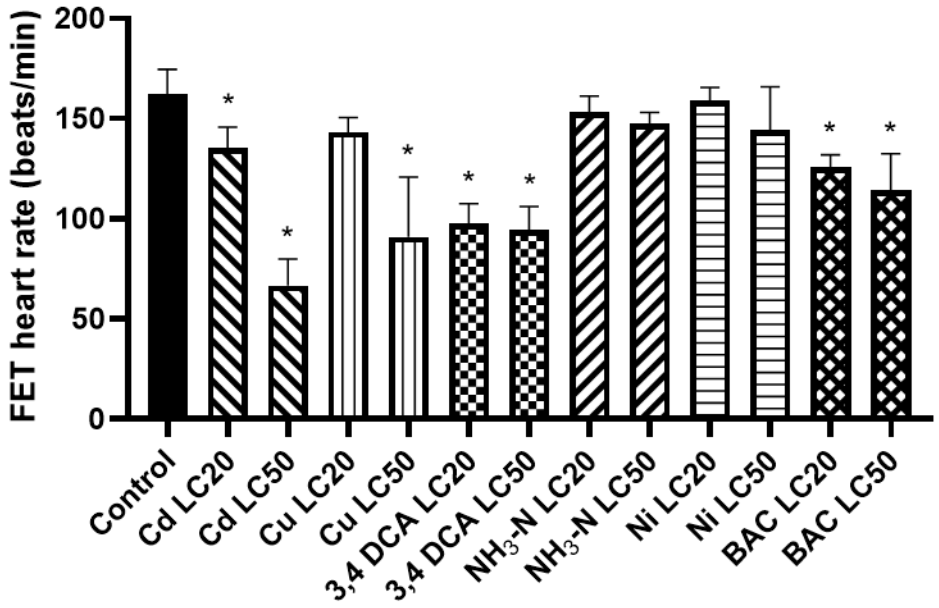

Figure 4. Cardio toxicity of the fathead minnow (*Pimephales promelas*) fish embryo acute toxicity (FET) test. Heart rate in beats per minute were measured for lethal concentration to 20 and 50% of population (LC20/LC50) for cadmium, copper, 3-4 dichloroaniline (DCA), ammonia (NH3-N), nickel, and benzalkonium chloride (BAC). *Statistical difference from control (Kruskal-Wallis, one-way analysis of variance, Dunn's multiple comparison test, alpha = 0.05).

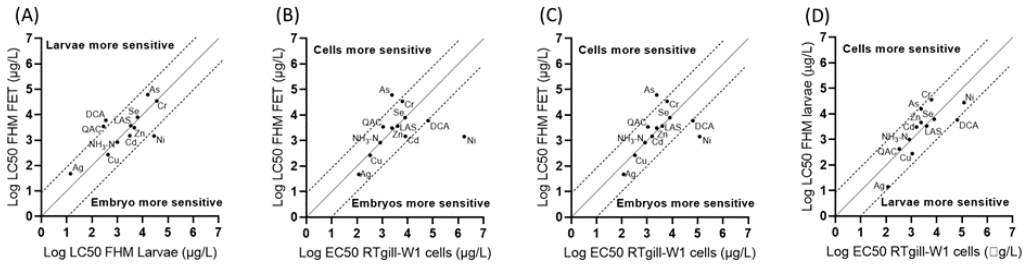

Figure 5. Correlation of LC50 values for the fathead minnow (FHM) fish embryo acute toxicity (FET) and larvae (A); FET LC50 and RTgill-W1 cell EC50 values at cellular isosmotic conditions (B); FET LC50 and RTgill-W1 cell EC50 values at cellular hypoosmotic conditions for nickel and cadmium (C); and larvae LC50 and RTgill-W1 cell EC50 values at cellular hypoosmotic conditions for nickel and cadmium (D). Solid line represents the line of unity and dashed lines one order of magnitude. Pearson R correlation coefficient analysis (two tailed test, alpha = 0.05) indicated a statistically significant correlation between FHM FET and larvae LC50 values (P value of 0.0016). FET LC50 and RTgill-W1 cell EC50 values did not show a correlation for isosmotic (P value of 0.241) and hypoosmotic conditions for nickel and cadmium (P value of 0.0822).

SUPPLEMENTARY MATERIALS

Table S1. List of whole effluent toxicity chemicals and specifications.

Chemical Name	CAS	Purity	Source
Sodium dodecylbenzene sulfonate	25155-30-0	Technical Grade	Millipore Sigma
Benzalkonium chloride[a]	63449-41-2	≥ 95%	Millipore Sigma
Ammonium chloride	12125-02-9	> 99.5%	Fisher Scientific
Silver nitrate	7761-88-8	≥ 99%	Millipore Sigma
Copper sulfate pentahydrate	7758-99-8	≥ 98%	Millipore Sigma
Cadmium chloride	10108-64-2	99.99%	Millipore Sigma
Zinc sulfate heptahydrate	7446-20-0	≥ 99%	Millipore Sigma
Sodium selenite	10102-18-8	≥ 98%	Millipore Sigma
Arsenic oxide	1327-53-3	≥ 99%	Millipore Sigma
Nickel sulfate hexahydrate	10101-97-0	≥ 98%	Millipore Sigma
Chromium oxide	1333-82-0	≥ 98%	Millipore Sigma
3,4-dichloroaniline	95-76-1	≥ 98%	Millipore Sigma

[a]Benzalkonium chloride is as 74.5% benzyldimethyldodecylammonium chloride, 22.3% benzyldimethyltetradecylammonium chloride.

CHAPTER IV

ALTERNATIVES TO FISH ACUTE WHOLE EFFLUENT TOXICITY (WET) TESTING: PREDICTABILITY OF RTGILL-W1 CELLS AND FATHEAD MINNOW EMBRYOS WITH ACTUAL WASTEWATER SAMPLES.

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Scott, Justin[ab#], Mortensen, Shannon[b], and Minghetti, Matteo[a].

[a]Oklahoma State University, Stillwater, OK, 74078, USA
[b]Cove Environmental, Stillwater, OK, 74075, USA

Corresponding author

ABSTRACT

Toxicity assays using fish cells and embryos continue to gain momentum as a more ethical and informative alternative to fish acute toxicity testing. The goal of our study was to test the accuracy of RTgill-W1 cells and the fathead minnow (Pimephales promelas) embryos to predict actual Whole Effluent Toxicity (WET) in the fathead minnow larvae. The three models were compared concurrently using samples of various origin and treatment type. Additionally, toxicity of reference toxicants (Cd, Cu, NH3-N, 3,4-dichloraniline, and benzalkonium chloride) spiked into a nontoxic wastewater was compared. Toxicity of reference toxicants was tested in isosmotic and hypoosmotic exposure media in RTgill-W1 cells. Embryos predicted 11 of the 14 wastewater samples toxic to the larvae. RTgill-W1 cells predicted toxicity of all 14 toxic samples and six samples that were nontoxic to larvae. Exposures in hypoosmotic medium significantly increased sensitivity of RTgill-W1 cells to all reference toxicants compared to exposures in isosmotic medium and showed toxicity levels similar to that in larvae. Thus, such exposure medium should be considered for aquatic toxicity testing applications. Overall, both gill cell and embryo models predicted toxicity of wastewater samples in larvae and demonstrated that they should be incorporated into regulatory WET testing.

KEYWORDS: Animal Alternatives, WET Testing, Cytotoxicity, FET, Acute Toxicity, *In vitro-In vivo* Correlations

SYNOPSIS: Alternative toxicity assays are now being considered for regulatory WET testing. This study reports the predictability of fish gill cells and embryo toxicity assays for fish acute toxicity tests.

INTRODUCTION

Within the field of aquatic toxicology, models such as cell-based cytotoxicity assays and Fish Embryo Acute Toxicity (FET) tests have been shown to represent valid alternatives and refinements to fish for toxicity testing (Tanneberger et al. 2013; Kamelia et al. 2019; Krzykwa et al. 2021; Scott et al. 2022b). *In vitro* animal alternatives have been highlighted for their ethical value, high throughput and mechanistic toxicological information (Lillicrap et al. 2016). Both, cell-based cytotoxicity assays and FET tests comply with the concepts of the 3Rs (replacement, refinement, and reduction) for animal testing and possess distinct strengths and limitations (Scott and Minghetti 2020). Movement away from vertebrate testing has been emphasized by the regulatory sector including the United States Environmental Protection Agency (USEPA) which promote the use of New Approach Methods (NAMs) to move away from mammalian testing by 2035 (USEPA 2021).

Additionally, Toxicology in the 21st century (Tox21, www.tox21.gov) has shown advancements with cytotoxicity assays in the field of ecotoxicology (Krewski et al. 2010; Richard et al. 2016), with the whole effluent toxicity (WET) testing sector being of strong interest (Finlayson et al. 2022). Specifically, guidelines utilizing the rainbow trout (Oncorhynchus mykiss) gill cell line, RTgill-W1 (ISO 2019; OECD 2021) and FET tests (ISO 2007; OECD 2013) have been developed for chemical and effluent toxicological analysis building on this concept. To date, animal alternatives have not been incorporated into the USEPA's standardized WET regulatory methods (USEPA 2002a; USEPA 2002b). Importantly, with the USEPA commitment to eliminating mammalian animals for toxicity tests (USEPA 2021), a need to reduce and replace the use of fish for aquatic

toxicity testing still exists. Therefore, considering the substantial number of fish used for WET testing (Norberg-King et al. 2018) it is imperative that these animal alternative assays are validated and incorporated into current WET testing guidelines.

The immortal cell line, RTgill-W1 serves as a true replacement method by eliminating the need of continued culturing and use of fish specimens for routine testing and is commercially available (ATCC® CRL-2523; DSMZ® ACC-899). Previous studies with RTgill-W1 cells have predicted toxicity to fish of various classes of chemicals (Tanneberger et al. 2013; Natsch et al. 2018; Fischer et al. 2019) including chemicals that are often found in effluent samples (Scott et al. 2021). Moreover, RTgill-W1 cells can be used to study the mechanism of toxicity of chemicals through subcellular endpoint measurements. For instance, it was shown that ammonia induces a specific induction of lysosome activity which can be used to identify the presence of ammonia in the effluent sample (Dayeh et al. 2009; Scott et al. 2021).

However, exposure of RTgill-W1 cells to effluent samples requires addition of salts to reduce osmotic stress, which may reduce bioavailability of chemicals present in the effluent sample (Scott et al. 2021; Scott 2022). FET tests reduce the number of fish used for analysis and have shown to identify toxicants in water through standardized developmental markers (OECD 2013) and more sensitive endpoints, such as heart rate, eye development, and growth (Krzykwa et al. 2019; Krzykwa et al. 2021; Scott et al. 2022). It was also shown that the toxicity of classes of chemicals present in effluent samples correlates significantly between the fathead minnow embryos and larvae (Scott et al. 2022). However, FET tests still require husbandry of breeding stocks and sacrificing specimens at test termination. Moreover, FET tests use a 96-h timepoint, whereas the

RTgill-W1 cell method uses a 24-h time point, thereby not reducing test duration when compared to the 96-h larvae assay.

While there is a large body of research on the toxicity of single chemical exposures to fish cells (Tanneberger et al. 2013; Natsch et al. 2018; Fischer et al. 2019; Scott et al. 2021), fish embryos (Embry et al. 2010; OECD 2012; Jeffries et al. 2015; Klüver et al. 2015) and fish larvae (Holcombe et al. 1983; Schirmer et al. 2008; Tanneberger et al. 2013; Scott et al. 2021), studies looking at the toxicity of realistic and environmentally relevant chemical mixtures are lacking (McCarty and Borgert 2006; Millstone and Clausing 2023). Even more studies investigating and comparing the toxicity of real effluent samples between *in vitro* and *in vivo* models are lacking. Therefore, in this study, we have compared side by side the use of RTgill-W1 cells, fathead minnow embryos and larvae for WET testing using a wide range of real effluent samples.

Our main goal was to determine the predictability of the acute toxicity detection of RTgill-W1 cells and FET in comparison to a standard USEPA freshwater fish model, the fathead minnow (Pimephales promelas). Additionally, RTgill-W1 cells have shown increasing sensitivity to WET chemicals of concern using hypoosmotic exposure conditions (150 mOsm/kg) (Scott et al. 2021), therefore, we also investigated if RTgill-W1 cells exposed in hypoosmotic wastewater samples would increase sensitivity to a subset of toxic chemicals in comparison to isosmotic samples.

MATERIALS AND METHODS

Wastewater sample preparation

Various wastewater treated and untreated, grab and composite samples were used from either influent, mid-operation, or pre outfall discharge effluents (Table 1). Sample collection, preservation and hold times followed closely USEPA acute WET methods (USEPA 2002a). Additionally, pH, dissolved oxygen (DO) and total residual chlorine of the wastewater samples was measured following WET method sample acceptance guidelines. Alkalinity and hardness were also measured for additional WET sample composition. Support chemical analysis of WET samples can be found in Table 1. Samples were prepared for FET and larvae assays following closely USEPA acute WET testing guidelines (USEPA 2002a) and OECD methods for RTgill-W1 cytotoxicity assays (OECD 2021). Exposure samples were prepared by mixing wastewater samples with synthetic hard water to make dilution concentrations at 20, 40, 60, 80 and 100% of exposure sample for embryo and larvae assays. Synthetic hard water served as the negative control for the embryo and larvae. For RTgill-W1 cell cytotoxicity assays, an aliquot of each of the exposure sample concentrations and negative controls were then adjusted through salt addition to approximately 300 mOsm/kg following the OECD method (OECD 2021).

Toxicity of reference toxicants spiked in hypo- and isosmotic samples

Non-toxic effluent samples were spiked with reference toxicants to allow further measurement of toxicity markers (i.e., subcellular and embryo development) to be identified for specific chemicals of interest. Both isosmotic (300 mOsm/kg) and

hypoosmotic (150 mOsm/kg) conditions were used for cytotoxicity assays to further compare sensitivity of RTgill-W1 cells in comparison to fish embryos and larvae. The toxicants cadmium, copper, 3,4-dichloroaniline (DCA), ammonia, and the quaternary ammonium compound (QAC), benzalkonium chloride (BAC), were selected as proof of principle WET chemicals. The exposure concentrations for each chemical ranged from: Cd, 312.5 to 10,000; Cu, 62.5 to 2000; NH3-N (unionized), 0.044 to 1.4; 3, 4-DCA; 156 to 200,000; and BAC, 62.5 to 6000 ng/ml. Preparation of stock chemicals and confirmatory analysis and quality control of each matrix spike chemical followed previous methods (Scott et al. 2022)

RTgill-W1 cells and cytotoxicity assays

Cell culturing and cytotoxicity assays were conducted following the multiple viability endpoint assay as found in OECD guidelines (OECD 2021). Before exposure, cells were washed with respective hypoosmotic (150 mOsm/kg) or isosmotic (300 mOsm/kg) wash medium as described previously (Scott et al. 2021) and then exposed to sample concentrations for 24-h in the dark at 19 oC. After 24-h of exposure, cells were washed again, and the multiple endpoint viability assay was performed. The multiple endpoint viability assay relies on the application of three commercially available dyes which are alamarBlue (AB; Invitrogen, Thermofisher, Waltham, MA, USA), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Thermofisher, Waltham, MA, USA), and 3-amino-7-dimethylamino-2-methylphenazine hydrochloride or Neutral Red (NR; Sigma-Aldrich, St. Louis, MO, USA). These dyes are used as indicators of cell metabolic activity, membrane integrity, and lysosomal integrity, respectively (Lee et al. 2008; Minghetti and Schirmer 2016). Effluent samples at different concentrations,

positive control (3,4-DCA) and hypo- and iso negative controls (i.e., L-15/ex) were analyzed in triplicate wells. All cytotoxicity assays were repeated in triplicate on the same day.

Culturing of embryos and FETs tests

Fathead minnow embryos were obtained from Cove Environmental's aquatic toxicity laboratory (Stillwater, OK) and cultured from spawning brood stock following USEPA guidelines (USEPA 2002a) and Oklahoma State University Institutional Animal Care and Use Protocol (IACUC-22-45). Briefly, sexually mature adult fathead minnows were cultured in carbon and ultraviolet light filtered source water from City of Stillwater (OK, USA). Brood stock were cultured in recirculating systems and maintained at a temperature of 25 ± 1 °C under a 16:8-h light:dark photoperiod, between 50-100 footcandles. Recently fertilized embryos were removed from spawning tanks within one hour of lights turning on and checked for their cell stage under a phase contrast light microscope (Olympus CKX53) equipped with a high-resolution camera (Olympus EP50). Less than 32-cell stage embryos were manually removed from spawning substrates, rinsed with synthetic hard water, and used immediately for wastewater sample exposures. Fathead minnow FET tests were conducted using a method previously described (Scott et al. 2022).

Briefly, each FET WET test consisted of 1 embryo (< 32-cell stage) per well in a 24 well plate for each test condition (i.e., six sample concentrations, one synthetic hard negative control, and one 3, 4-DCA positive control plate for each wastewater sample). Each condition plate consisted of the treatment embryos (n = 20) and plate internal

negative control embryos (n = 4). Measured toxicity endpoints consisted of i) coagulation of the embryos; ii) lack of somite formation; iii) non-detachment of the tail; and iv) lack of heartbeat, which were also measured at 24-h intervals until test termination at 96-h. Lethality was determined if at least one endpoint was observed throughout test duration based on OECD 2013 guidelines. FET tests exposure solutions were renewed (80% removal of existing volume) every 24-h and incubated at 25 ± 1 ºC with a 16:8 h light: dark photoperiod between 50 to 100 ft-c throughout the 96-h duration. Embryos that did not show toxicity upon test takedown were euthanized following IACUC-22-45.

Culturing of larvae and toxicity tests

Fathead minnow larvae were cultured similarly to embryos up to collection upon fertilization. Embryos were then maintained until hatching and up to 14-d following USEPA guidelines (USEPA 2002) until WET testing use. Larvae environmental conditions were identical to brood stock in separate holding containers. Larvae were fed twice daily with Artemia nauplii and water quality checked daily. 14-d old larvae were then transferred to WET tests vessels. Each test consisted of 3 replicates of 10 larvae (n = 30) for each condition (e.g., one sample concentration, synthetic hard negative control, and 3, 4-DCA positive control). The measured toxicity endpoint (determined for LC50 values) consisted of mortality based on lack of response upon prodding. Larvae tests exposure solutions were renewed (80% removal of existing volume) every 24 h and incubated at 25 ± 1 ºC with a 16:8 h light: dark photoperiod between 50 to 100 ft-c throughout the 96-h duration. Larvae that did not induce toxicity upon test takedown were euthanized following IACUC-22-45.

Statistical analysis

RTgill-W1 cell effect concentration 50% (EC50), and embryo and larvae lethal concentration 50% (LC50) were determined by the non-linear regression sigmoidal dose-response curve fitting module using the Hill slope equation. The lowest observed effect concentration (LOEC) values for all three models were calculated using a one-way ANOVA post hoc analysis comparing the negative control to treatments (Dunnett's multiple point comparisons). For wastewater samples, concentrations are based on volume to volume (v/v) for calculations as percent total effluent sample. For reference toxicants toxicity spiked in hypo- and isosmotic conditions, concentrations are reported as ng/ml of chemical. For RTgill-W1 cell EC50 correlations, geometric means were calculated from all three cellular endpoints to compare with LC50 values of embryo and larvae, unless otherwise specified.

EC50 values calculated in RTgill-W1 cells exposed in hypo- and isosmotic media were compared to LC50 values for embryo and larvae using one-way ANOVA post hoc analysis (Tukey's multiple point comparisons). For correlation data, a two tailed Pearson's r coefficient test was performed. All experiments were repeated at least 3 times with each sample, with all three model's tests being set up on the same day immediately upon sample preparation. All statistical analysis tests were checked for normality and performed with alpha equal to 0.05 using GraphPad Software (Prism version 9.4). All data for EC50 and LC50 correlations, and hypo- and isosmotic comparisons were found to be normally distributed through D'Agostino and Pearson tests and data was ran using parametric analysis.

RESULTS AND DISCUSSION

Wastewater toxicity and correlations

Of the 28 wastewater samples shown in Table 1, 14 induced a toxic response in fish larvae populations (i.e., ≥ 50% lethality at the highest concentration). The RTgill-W1 cells detected toxicity (i.e., ≥ 50% inhibition or stimulation of cellular markers) in all the 14 samples deemed toxic by the larvae test (100% match) plus six more that did not induced toxicity in fish larvae (i.e., samples: 2, 3, 9, 13, 26, and 27). The FET tests detected toxicity in 11 out of the 28 wastewater samples, 9 out of the 14 deemed toxic by the larvae test, but detected toxicity in two additional samples (i.e., sample 26 and 27) that passed the larvae test but not the RTgill-W1 cytotoxicity test. No toxic effects were detected in any of the models for the negative control sample (i.e., synthetic hard water) and in all experiments the positive control induced the expected toxic effect. Dose response curves for wastewater samples comparing all three models can be found in Figure 1.

In this study, RTgill-W1 cells exposed to real effluents showed EC50 values that did not correlate to LC50 values in embryos (Figure 2A; $p = 0.3039$) and LC50 values in larvae (Figure 2B; $p = 0.6302$). This is due to a higher sensitivity in the cells for most of the WET samples (Table 2). While the toxicity of individual WET chemicals has previously been shown to correlate in RTgill-W1 cells and larvae (Scott et al., 2021), increased sensitivity may be occurring in chemical mixture scenarios of the WET samples (Hernando et al. 2005). Conversely, LC50 values in embryos correlated to LC50 values in larvae (Figure 2C; $p = 0.0463$) with only three samples being one order of

magnitude more sensitive in embryos and thus demonstrating the strong similarity between embryos and larvae in toxicity response to aqueous chemical mixtures as shown previously for single WET chemicals exposures (Scott et al. 2022). For the LOEC values, the number of samples identified as toxic were 25 for RTgill-W1 cells, 14 for embryos, and 16 for the larvae (Table 3). Interestingly, six of the highest toxic samples were found to have similar toxicity ranking based on the LOEC values for all three models (i.e., samples 4, 8, 15, 17, 23 and 24; Table 3).

Overall, RTgill-W1 cells were shown to be more sensitive in predicting acute toxicity in fish larvae than the embryo for WET testing, however embryos were able to show a similar magnitude of sensitivity to toxicants. The rationale for the stronger correlation between embryos and larvae could be explained by the use of the same species (i.e., fathead minnow) which could have a more similar sensitivity to WET toxicants. Previous studies have shown good correlation for acute toxicity of fathead minnow embryos and larvae (Jeffries et al. 2015; Scott et al. 2022). RTgill-W1 cells are from rainbow trout that have been shown to be more sensitive to a broad range of chemicals with varying toxic modes of action. Specifically, previous studies found that rainbow trout were more sensitive than fathead minnows to five chemicals (carbaryl, copper, 4-nonylphenol, pentachlorophenol, and permethrin) and showed a broad range toxic mode of action (Dwyer et al. 2005). In another study, rainbow trout were found to be more sensitive than fathead minnows to pesticides (Holcombe et al. 1982). For the samples that were more toxic to cells and embryos than larvae the type of wastewater sample and constituents of concern may give an explanation. For example, municipal and pulp and paper samples, where ammonia was present (data not shown) were shown to be

more sensitive in RTgill-W1 cells and embryos than fish likely due to the higher sensitivity of cells and embryos to ammonia (Dayeh et al. 2002; Brinkman et al. 2009; Scott et al. 2022). Additionally, the refinery and power wastewater samples, which can be associated with heavy metals and organic chemical toxicity in fish, were also found to be more toxic to the cells and embryos. While confirmatory chemical analysis was beyond the scope of the present study, future efforts are warranted to further identify which constituents are present and if mechanistic toxicity can be correlated.

Additionally, factors such as water chemistry variability (i.e., hardness, pH, temperature, etc.) between the model's exposure methods may play a role on toxicity. Specifically, competition with cations, complexation with anions and chemical ionization in solution might affect chemical bioavailability and toxicity (Niyogi and Wood 2004; Qin et al. 2010). For instance, in a previous study, ammonia toxicity was shown to be affected by temperature, thus rainbow trout assays conducted at 12 °C showed less toxicity than fathead minnows assays, conducted at 20 °C, resulting in fathead minnows being more sensitive (Wang et al. 2007). However, in our results, when water chemistry and test parameters are standardized to USEPA WET guidelines (2002) for the embryo and larvae and to the OECD (2021) guideline for RTgill-W1 cells, the cytotoxicity assays were overall more sensitive to the wastewater samples than the fish acute tests. Specifically, with water chemistry being similar, excluding osmolality and temperature in RTgill-W1 cells assays, the cells were able to successfully predict toxicity to all samples found acutely toxic to larvae. If available, future studies incorporating the use of fathead minnow gill cells could be applied to determine if toxicant sensitivity is similar to that of embryo and larvae within the same species.

RTgill-W1 cytotoxicity endpoints and responses

For RTgill-W1 cells, specific endpoints were useful in highlighting toxicity of specific wastewater sample types and were found to be more prevalent than others. For instance, EC50s for lysosomal integrity were detected for most of the samples (19/28), followed by metabolic activity (15/28) and then by membrane integrity (9/28) for wastewater samples. The stimulation effect of lysosomal integrity was found to be more specific to all pulp and paper samples wastewater samples (23-29) but it was also found in a municipal (8), a industry (12) and a desalination sample (13). Indeed, all seven pulp and paper samples induced lysosomal activity. This occurrence has previously been shown to be associated with ammonia toxicity and pulp and paper effluent samples (Dayeh et al. 2009; Scott et al. 2021). Metabolic activity stimulation effects also occurred in cells exposed to municipal and industry samples (6, 8, and 12) and membrane integrity stimulation in municipal and desalination samples (2 and 13) but such effect could not be linked to a specific toxicant.

As mentioned earlier, while it is beyond the scope of the present study, future efforts could build on toxicity identification evaluations (TIE) to aid in forensically identifying either classes or specific toxicants of concern in wastewater samples. This would include determining if the multiple viability endpoint assay can identify chemicals toxic mechanism of action which can be linked to wastewater sample matrices. Cytotoxicity assays could also offer better understanding of long-term toxic insults and be applied to chronic WET assays. As seen with the LOEC results (Table 3), RTgill-W1 cells were more sensitive to WET toxicants and were able to predict toxicity to larvae. Previous studies have investigated the use of mammalian cell lines to predict chronic

effects and warrant the use of fish cell lines for chronic WET testing (Massalha et al. 2018). Moreover, future studies could determine if the current multiple viability assay or other unique cellular endpoints (i.e., enzymatic responses, cell proliferation, and apoptosis) could be used to correlate with chronic effects. Previous studies have used fish cell proliferation to predict reduced fish growth (Stadnicka-Michalak et al. 2015) and warrants further investigation for application into WET testing such as the chronic 7-day fathead minnow survival and growth test (USEPA 2002b).

Embryo teratogenicity endpoints and responses

For embryos, coagulation followed by lack of heartbeat were the only endpoints observed for lethality prediction in the FET assay for EC50 calculations. As seen in previous studies (Krzykwa et al. 2021; Scott et al. 2022), the chorion has a protective role in exposures to chemical toxicity and was also shown in our current results. In fact, embryos that hatched before 96-h were more likely to die due to lack of heartbeat for exposure at the eleutheroembryo stage (data not shown). Therefore, the reduced predictability of FETs to determine larvae toxicity in our study could be explained by this characteristic. Previous studies have investigated the removal of the chorion to allow increased sensitivity to embryo stages for metals (Olivares et al. 2016) and organics (Henn and Braunbeck 2011). However, this process would most likely complicate the application into WET testing and not represent true exposure scenarios of the embryo at a physiologically relevant developmental stage.

Acute WET tests use 1 to 14-day (d) hatched larvae which eliminates early exposure to eleutheroembryo and embryo stages. Future studies could look to expand the

fathead minnow FET test duration to 120-h post fertilization to allow eleutheroembryos exposure as seen previously (Krzykwa et al. 2019; Krzykwa et al. 2021). Ultimately, the current FET test method which acts as an acute timepoint, provides teratogenic endpoints that serve as sublethal effects common for chronic test durations (USEPA 2002c). Additionally, the sublethal endpoint of heart rate has previously been shown to successfully predict acute toxicity to WET chemicals of concern (Scott et al 2022) and could be considered for incorporation into acute and chronic WET testing.

Toxicity of spiked hypo- and isosmotic effluent samples in RTgill-W1 cells

Previous studies have highlighted the use of hypoosmotic conditions to increase the bioavailability and sensitivity of RTgill-W1 cells for WET chemicals of concern (Scott et al. 2021). Indeed, toxicity was shown to increase in hypoosmotic wastewater samples compared to their isosmotic counterparts. Specifically, hypoosmotic conditions were shown to significantly increase sensitivity in RTgill-W1 cells for all chemicals excluding BAC (Cd, $p < 0.0001$; Cu $p < 0.0001$; NH3-N $p < 0.0001$; 3, 4-DCA, $p < 0.0001$; BAC $p > 0.9999$; one-way ANOVA, post hoc Tukey's multiple comparison, alpha = 0.05) when compared to isosmotic conditions (Figure 3). RTgill-W1 cell dose response curves for chemicals spiked in hypo- and isosmotic wastewater samples can be found in Figure 4. EC50 and LC50 value comparisons for hypo- and isosmotic can be found in Supporting Information (Table S2).

When comparing the toxicity of the spiked chemicals between RTgill-W1 and embryo, exposures in hypoosmotic medium showed higher sensitivity for ammonia ($p = 0.0001$), and BAC ($p < 0.0001$), 3,4-DCA was equally toxic ($p = 0.0968$), and Cu and Cd

were less toxic ($p = 0.0006$ and $p = 0.0011$, respectively). Compared to the larvae, RTgill-W1 exposed in hypoosmotic medium showed similar sensitivity to Cd ($p = 0.6679$), Cu ($p = 0.1349$) and BAC ($p = 0.8891$) but were more sensitive to ammonia ($p = 0.0076$) and less to 3,4-DCA ($p < 0.0001$). For Cu and Cd in L-15/ex isosmotic medium, the calcium concertation is higher (1.34 mM) than hypoosmotic medium (0.67 mM) and calcium has been shown to protect against divalent metal toxicity (Franklin et al. 2005; Walker et al. 2008). Moreover, as previously shown, an increase in calcium concentration in the exposure medium results in a decrease in toxicity and bioavailability for some metals such as Cu and Cd (Oldham et al. 2023). Additionally, increasing salinity has shown to decrease ammonia toxicity (Bucking 2017) and organic toxicity (Rocha et al. 2007) and could explain RTgill-W1's increased sensitivity in hypoosmotic media. Overall, when comparing cells exposed in hypoosmotic conditions an increase in sensitivity was measured for four of the five WET chemicals of concern, with BAC showing similar sensitivity to larvae. Therefore, exposure in hypoosmotic condition should be included in RTgill-W1 cell assays for further implementation of WET testing procedures.

Advancements towards WET testing incorporation

Based on the present study results using actual wastewater samples of varying matrices, RTgill-W1 cell assays have shown to be effective and more sensitive in predicting WET in comparison to the fish embryos and larvae. Additionally, FET LC50 values showed a significant correlation with larvae LC50 values but did not improve on sensitivity of the model. Furthermore, the cytotoxicity assay results encourage moving away from the use of whole organisms (i.e., fish embryo and larvae) and using the gill

cell line as an initial phase of WET testing or potential pre-test screening of known toxicants of concern (i.e., metals and ammonia). However, previous studies have shown that certain chemicals do not respond to RTgill-W1 such as the neurotoxicant, permethrin or highly lipophilic compounds such as fluoranthene (Tanneberger et al. 2013; Scott et al. 2021) and caution should be taken if these chemicals are of concern. This concern can be extended also to the overall low sensitivity of FET tests to certain organic compounds (Klüver et al. 2015) and to the reduced toxicity due to the protection of the embryo's chorion to WET chemical insults. Therefore, whole organisms may be needed where there are concerns for false negatives due to specific chemicals (e.g., neurotoxicants or highly lipophilic chemicals). Furthermore, to cover the lack of sensitivity of RTgill-W1 cells to chemicals such as neurotoxicants, assay using brain cell cultures may bridge the gap (Sun et al. 2023). Based on the wastewater treatment facility type, the relevance to the effluent could warrant the use of the most appropriate model. A good example is that of municipality sewage or pulp and paper treatment facilities and the ubiquitous concern with the presence of unionized ammonia being the leading driver of toxicity to fish. RTgill-W1 cells have been shown to specifically detect the presence of ammonia toxicity and would be an ideal tool to detect toxicity in effluents coming from these facilities.

As mentioned earlier, the ability to measure specific chemicals and their respective concentrations in actual wastewater samples would have been necessary to identify chemicals of concern specific to the type of wastewater treatment facility. While additional analytical measurement was beyond the scope of the present study, future efforts could consider this for toxicity confirmation in relation to the model's toxic mechanism of action. In conclusion, multiple strengths and weaknesses exist for both

alternative models (i.e., RTgill-W1 cells and FET) and a best fit practice and/or a tier-based analysis should be considered. Overall, both animal alternatives have shown the ability to predict toxicity to actual wastewater samples and warrant further validation through intralaboratory and interlaboratory efforts such as round robin studies (Fischer et al. 2019). Moreover, RTgill-W1 cells were shown to have a higher predictability than embryos and further highlights their use not only as a more sensitive model but as a true replacement to the use of live fish. The findings from our study are encouraging and support the movement towards a more ethical, high throughput, and informative WET assay for regulatory applications.

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TABLES AND FIGURES

Table 1. Toxicity summary of wastewater exposure samples.

Sample Number	Sample Type	Induced toxicity ≥ 50% at highest concentration (Y/N)			
		RTgill-W1 Cell Endpoints Inhibited (MA, MI, LI)		FHM Embryo	FHM Larvae
1	Synthetic Control	N	-	N	N
2	Municipality A	Y	MI*, LI	N	N
3	Municipality B	Y	LI	N	N
4	Municipality C	Y	MI, LI	Y	Y
5	Municipality D	N	-	N	N
6	Municipality E	Y	MA*, MI, LI	Y	Y
7	Municipality F	N	-	N	N
8	Municipality G	Y	MA*, MI, LI*	Y	Y
9	Municipality H	Y	LI	N	N
10	Industry A	Y	MA	N	Y
11	Industry B	Y	MA, MI, LI	N	Y
12	Industry C	Y	MA*, LI*	N	Y
13	Desalination Facility A	Y	MI*, LI*	N	N
14	Desalination Facility B	N	-	N	N
15	Refining Facility A	Y	MA, LI	Y	Y
16	Refining Facility B	N	-	N	N
17	Refining Facility C	Y	MA, MI, LI	Y	Y
18	Refining Facility D	N	-	N	N
19	Power Facility A	Y	MA, MI, LI	N	Y
20	Power Facility B	N	-	N	N
21	Power Facility C	N	-	N	N
22	Power Facility D	N	-	N	N
23	Pulp and Paper A	Y	MA, MI, LI*	Y	Y
24	Pulp and Paper B	Y	MA, LI*	Y	Y
25	Pulp and Paper C	Y	MA, LI*	Y	Y
26	Pulp and Paper D	Y	MA, LI*	Y	N
27	Pulp and Paper E	Y	MA, LI*	Y	N
28	Pulp and Paper F	Y	MA, LI*	Y	Y
29	Pulp and Paper G	Y	MA, LI*	N	Y
30	Cd Matrix Spike Iso	Y	MA	Y	Y
31	Cd Matrix Spike Hypo	Y	MA, MI, LI	Y	Y
32	Cu Matrix Spike Iso	N	-	Y	Y
33	Cu Matrix Spike Hypo	Y	MA, MI, LI	Y	Y
34	3,4 DCA Matrix Spike Iso	Y	MA, MI, LI	Y	Y
35	3,4 DCA Matrix Spike Hypo	Y	MA, MI, LI	Y	Y
36	NH_3-N Matrix Spike Iso	N	-	N	Y
37	NH_3-N Matrix Spike Hypo	Y	LI*	N	Y
38	QAC Matrix Spike Iso	Y	MA, MI, LI	N	Y
39	QAC Matrix Spike Hypo	Y	MA, MI, LI	N	Y

MA – metabolic activity; MI – membrane integrity; LI – lysosomal integrity
*Indicates Viability Endpoint Stimulation Effect
Y indicates the sample induced toxic response more or equal to EC50/LC50; N indicates response less than EC50/LC50

Table 2. RTgill-W1 cells EC50 and the fathead minnow embryo and larvae concentration LC50 values.

Sample Number	Sample Type	RTgill-W1 Cells EC50 (% Sample)				FET LC50 (% Sample)	Larvae LC50 (% Sample)
		Metabolic Activity	Membrane Integrity	Lysosomal Integrity	Geometric Mean		
1	Syn Control	>100	>100	>100	>100	>100	>100
2	Municipal A	>100	>100	41.4 ± 11.3	NC	>100	>100
3	Municipal B	>100	>100	63.8 ± 35.5	NC	>100	>100
4	Municipal C	>100	73.4 ± 19.1	3.42 ± 3.62	12.5 ± 8.63	38.5 ± 1.85	49.7 ± 9.23
5	Municipal D	>100	>100	>100[b]	>100	>100	>100
6	Municipal E	63.9 ± 14.6[b]	31.12 ± 15.01	NC	44.1 ± 15.8	81.8 ± 1.96	74.1 ± 1.47
7	Municipal F	>100	>100	>100	>100	>100	>100
8	Municipal G	65.1 ± 17.7[a]	NC	NC	NC	41.3 ± 2.16	24.4 ± 2.92
9	Municipal H	>100	>100	15.7 ± 11.2	NC	>100	>100
10	Industry A	>100	56.7 ± 31.9	>100	NC	> 100	37.5 ± 3.88
11	Industry B	>100	9.77 ± 13.8	NC	NC	> 100	73.7 ± 9.38
12	Industry C	>100[b]	>100	67.0 ± 6.12[a]	NC	> 100	22.6 ± 3.02
13	Desalination A	>100	33.9 ± 8.89[a]	36.31 ± 3.95[a]	35.4 ± 4.04	>100	>100
14	Desalination B	>100	>100	>100	>100	>100	>100
15	Refinery A	57.2 ± 1.5	>100	NC	NC	43.8 ± 12.1	38.3 ± 10.8
16	Refinery B	>100	>100	>100	>100	>100	>100
17	Refinery C	4.7 ± 1.14	0.63 ± 0.52	7.72 ± 2.70	3.93 ± 2.6	57.9 ± 0.65	70.1 ± 5.23
18	Refinery D	>100	>100	>100	>100	>100	>100
19	Power A	18.7 ± 5.82	61.5 ± 9.34	14.3 ± 3.18	24.9 ± 0.9	>100	67.7 ± 1.35
20	Power B	44.7 ± 24.2[b]	>100	40.6 ± 13.5[b]	40.0 ± 3.36	>100	>100
21	Power C	>100	>100	>100	>100	>100	>100
22	Power D	>100	>100	>100	>100	>100	>100
23	Pulp Paper A	65.5 ± 15.47	22.9 ± 5.47	60.4 ± 24.4[a]	47.8 ± 9.25	45.5 ± 5.93	50.6 ± 6.65
24	Pulp Paper B	59.9 ± 17.9	40.1	45.8 ± 12.6[a]	48.6 ± 8.50	34.7 ± 9.85	75.4 ± 0.98
25	Pulp Paper C	46.8 ± 9.13	>100	39.2 ± 7.28[a]	42.9 ± 8.16	35.4 ± 4.94	66.27 ± 1.12
26	Pulp Paper D	42.3 ± 5.23	>100	47.21 ± 7.7[a]	44.6 ± 5.67	75.72 ± 12.1	>100
27	Pulp Paper E	48.5 ± 11.3	>100	40.5 ± 15.7[a]	43.1 ± 9.6	33.9 ± 11.4	>100
28	Pulp Paper F	69.6 ± 25.7	>100	39.5 ± 2.96[a]	54.9 ± 13.3	62.9 ± 17.4	89.8 ± 4.93
29	Pulp Paper G	71.9 ± 23.4	>100	35.8 ± 3.95[a]	61.6 ± 26.7	92.2	96.6 ± 5.66

For correlations with RTgill-W1 cells cytotoxicity assays if geometric mean was not calculated (NC) the most sensitive endpoint was used.

[a] Stimulation effect (< 150% stimulations)

[b] Intermediate dose response (i.e., non-monotonic dose response)

Table 3. RTgill-W1 cell and the fathead minnow (*Pimephales promelas*) embryo and larvae lowest observed effect concentration (LOEC) values.

Sample Number	Sample Type	RTgill-W1 Cells LOEC (% Sample)				FET LOEC (% Sample)	Larvae LOEC (% Sample)
		AB	CFDA	NR	GEO		
1	Synthetic Control	>100	>100	>100	>100	>100	>100
2	Municipal A	40	>100[a]	40	>100	>100	>100
3	Municipal B	100	60	100	100	>100	>100
4	Municipal C	>100	60	20	20	20	20
5	Municipal D	20[b]	>100[a]	40[ab]	20	>100	>100
6	Municipal E	20[b]	20	20	20	40	80
7	Municipal F	>100	>100	>100	>100	>100	>100
8	Municipal G	20[a]	20[ac]	20[ac]	20	40	40
9	Municipal H	20	>100	20	>100	>100	>100
10	Industry A	20	>100	80	>100	>100	40
11	Industry B	20	20	20	20	100	80
12	Industry C	40[ab]	>100[a]	>100[a]	>100	>100	40
13	Desalination A	20	60[a]	60[a]	20	>100	>100
14	Desalination B	40	>100	>100[a]	>100	>100	>100
15	Refinery A	20	>100	20	20	40	40
16	Refinery B	100	>100[a]	>100[a]	>100	>100	>100
17	Refinery C	20	20	20	20	20	20
18	Refinery D	100	>100	100	100	>100	100
19	Power A	20	20	20	20	40	80
20	Power B	20[b]	>100	20[b]	20	>100	>100
21	Power C	80	>100	>100	>100	>100	40
22	Power D	100	>100	>100	>100	>100	>100
23	Pulp Paper A	>100	40	80[a]	60	40	60
24	Pulp Paper B	40	>100	>100[a]	>100	40	40
25	Pulp Paper C	60	>100	>100[a]	>100	20	60
26	Pulp Paper D	40	>100	>100[a]	>100	20	>100
27	Pulp Paper E	60	>100	>100[a]	>100	20	>100
28	Pulp Paper F	40	>100	40[a]	>100	20	60
29	Pulp Paper G	40	>100	40[a]	>100	20	80

[a] Stimulation effect
[b] Intermediate dose response (i.e., non-monotonic dose response)

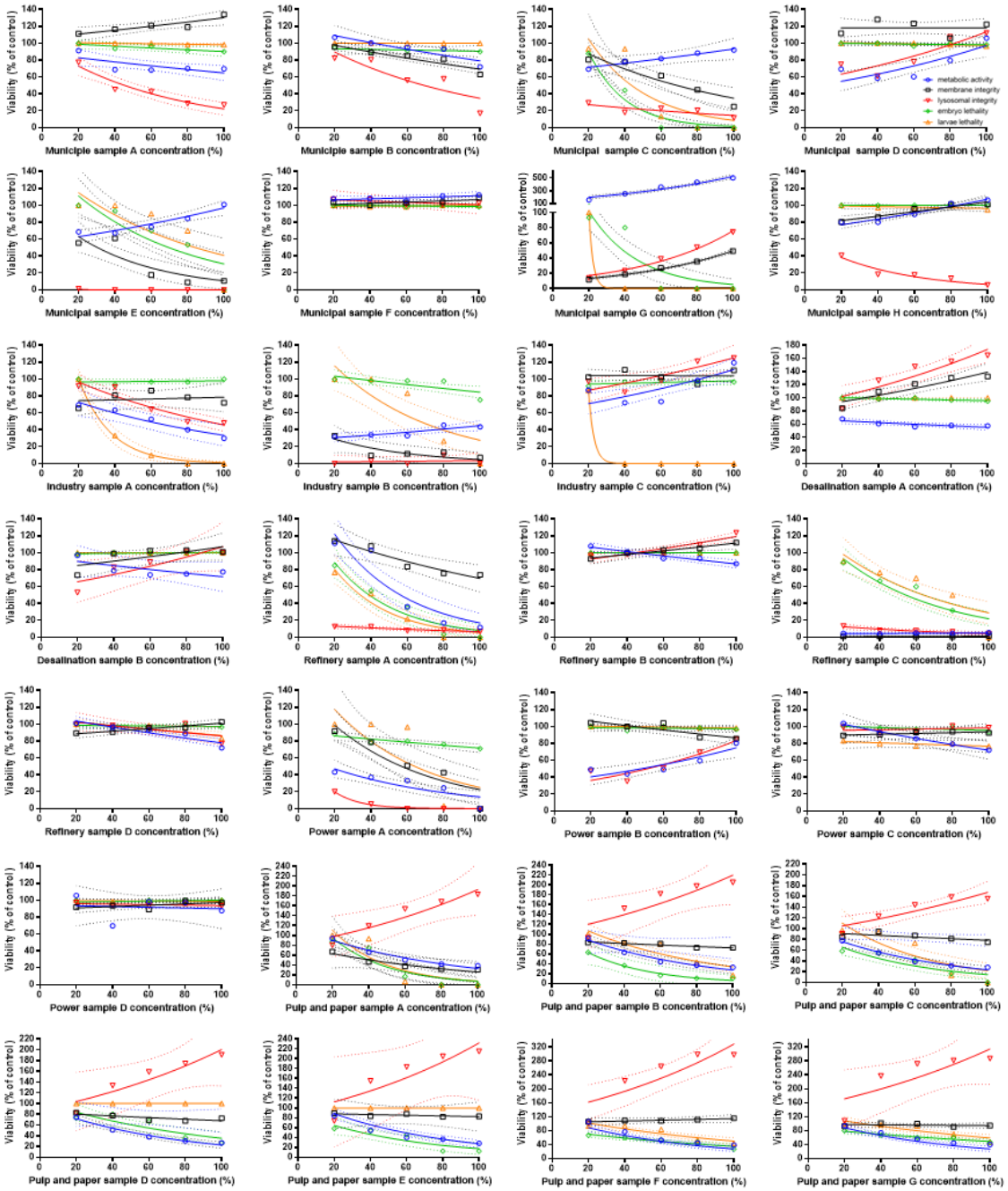

Figure 1. Wastewater samples dose response curves comparing RTgill-W1 cells and the fathead minnow embryo and larvae acute toxicity. RTgill-W1 cells indicate toxicity using the multiple viability assay for metabolic activity, membrane integrity, and lysosomal integrity and embryo and larvae using lethality. Responses are presented as percentage viability of control.

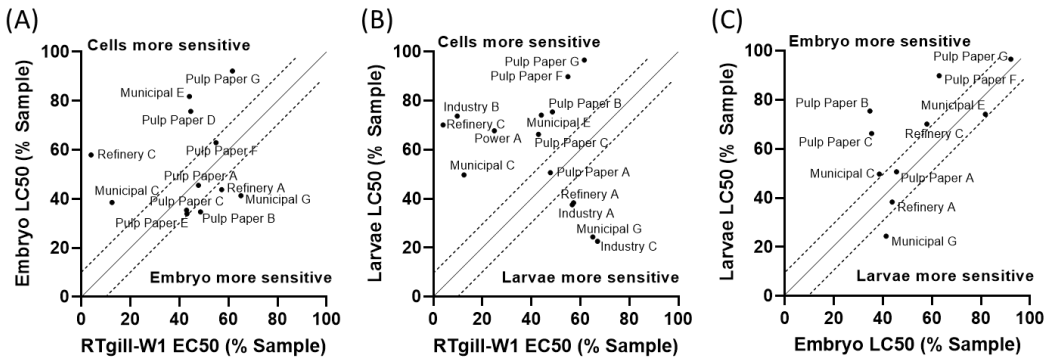

Figure 2. Correlation of wastewater sample's acute toxicity comparing RTgill-W1 cells EC50 and the fathead minnow's LC50 (A); RTgill-W1 cell's EC50 and larvae LC50 (B); and, larvae LC50 and embryo LC50 (C). A significant correlation did not occur for panel A (p = 0.3039) and B (p = 0.6302), and a significant correlation occurred for panel C (p=0.0463) (Pearson's r correlation coefficient, two tailed, alpha = 0.05).

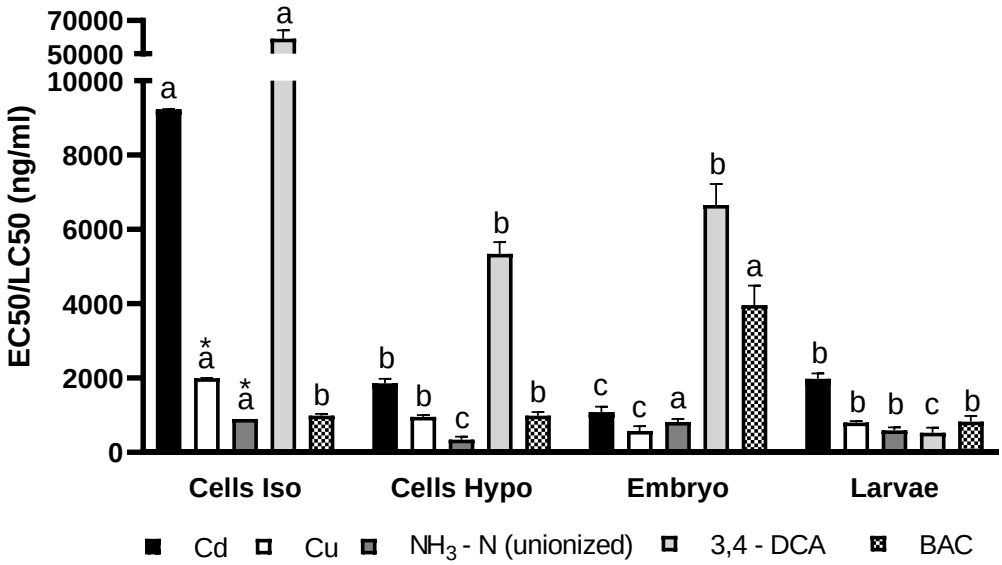

Figure 3. Comparison of geometric EC50 values RTgill-W1 cell viability assays at iso- (approximately 300 mOsm/kg) and hypoosmotic (150 mOsm/kg) conditions to LC50s for fathead minnow embryo and larvae assays. Dose response curves (see figure 4) were calculated from concentrations of cadmium, copper, unionized ammonia 3, 4-dichloraniline (DCA), and benzalkonium chloride (BAC) spiked into nontoxic wastewater samples and measured for acute toxicity at 24 h and 96 h for cells and fish, respectively. Lowercase letters indicate significant difference between models for each chemical (one-way ANOVA, post hoc analysis with Tukey's multiple comparison; n = 3, alpha = 0.05). Asterisk indicates that toxicity was > 2000 ng/mL for Cu and 900 ng/mL fir ammonia which was the highest concentrations tested (see Table S2).

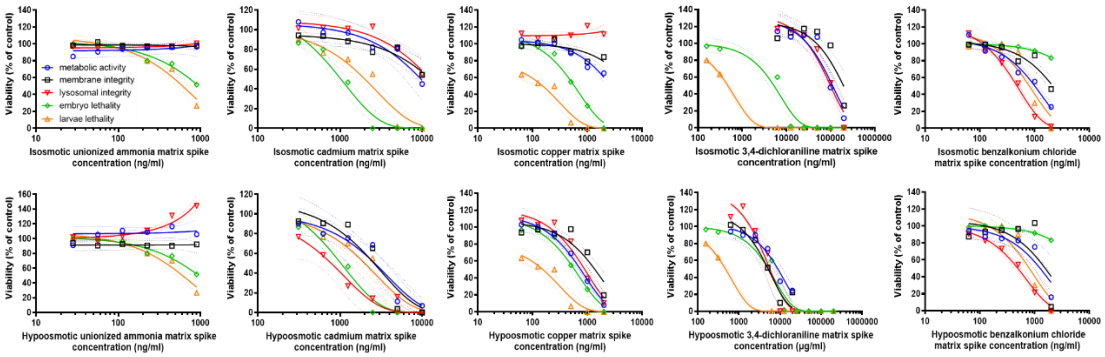

Figure 4. Hypo- and isosmotic dose response curves comparing RTgill-W1 cells and the fathead minnow embryo and larvae acute toxicity. RTgill-W1 cells indicate toxicity using the multiple viability assay for metabolic activity, membrane integrity, and lysosomal integrity and embryo and larvae using lethality.

SUPPLEMENTARY MATERIALS

Table S1. Analytical chemistry analysis of wastewater samples.

Sample Number	Sample Type	pH (s.u.)	Dissolved Oxygen (mg/L)	Conductivity (µs/cm)	Alkalinity (mg/L)	Hardness (mg/L)	Isosmotic Osmolality (mOsm/kg)
1	Synthetic Control	7.89	8.02	782	104	211	306
2	Municipal A	8.45	7.76	796	315	340	299
3	Municipal B	7.22	7.7	891	275	320	305
4	Municipal C	8.03	8.13	894	255	260	301
5	Municipal D	8.11	8.23	891	305	370	296
6	Municipal E	8.05	8.12	886	200	220	294
7	Municipal F	7.62	9.32	882	190	210	312
8	Municipal G	8.39	7.68	1133	115	310	315
9	Municipal H	7.66	8.15	1017	175	400	306
10	Industry A	8.46	7.31	1718	45	1130	325
11	Industry B	8.16	7.64	1127	40	1110	321
12	Industry C	7.9	4.56	1684	45	1190	319
13	Desalination A	7.89	6.4	3393	330	680	408
14	Desalination B	7.72	7.97	5960	185	2060	462
15	Refinery A	7.28	8.05	866	265	330	290
16	Refinery B	7.07	8.97	853	245	300	293
17	Refinery C	7.78	3.06	1351	350	380	311
18	Refinery D	7.41	9.16	993	175	370	309
19	Power A	6.79	10.12	5084	450	1450	319
20	Power B	6.83	9.27	4996	50	1620	315
21	Power C	6.78	9.62	5042	330	680	347
22	Power D	7.34	7.64	5295	45	1190	307
23	Pulp Paper A	9.18	7.31	3176	1380	170	321
24	Pulp Paper B	7.97	5.4	2836	1300	200	320
25	Pulp Paper C	8.47	7.7	2972	1230	760	323
26	Pulp Paper D	7.87	2.42	3077	1335	320	316
27	Pulp Paper E	7.83	3.29	2596	1105	330	319
28	Pulp Paper F	7.76	6.82	2864	1271	453	322
29	Pulp Paper G	7.92	7.13	2789	1209	367	321

Table S2. Comparison of EC50s for RTgill-W1 cell viability assays at iso- (300 mOsm/kg) and hypoosmotic (150 mOsm/kg) conditions to LC50s for fathead minnow embryo and larvae assays.

Sample	RTGill-W1 cells EC50 (ng/ml)				FET LC50 (ng/ml)	Larvae LC50 (ng/ml)
	AB	CFDA	NR	GEO		
Ammonia (unionized) matrix spike isosmotic	> 900	> 900	> 900	> 900	817 ± 79.2	596 ± 74.7
Ammonia matrix (unionized) spike hypoosmotic	> 900	> 900	344 ± 78.9	NC		
Cadmium matrix spike isosmotic	6468 ± 1653	12396 ± 4048.2	10250 ± 709	9227 ± 15.3	1083 ± 145	1979 ± 146
Cadmium matrix spike hypoosmotic	2841.3 ± 973	2483 ± 290.4	669 ± 162.4	1858 ± 119		
Copper matrix spike isosmotic	> 2000	> 2000	> 2000	> 2000	579 ± 129	805 ± 41.9
Copper matrix spike hypoosmotic	744 ± 16.4	1312 ± 151	887 ± 53.7	952 ± 51.7		
3,4-dichloroaniline matrix spike isosmotic	49517 ± 2027	85698 ± 12536	48926 ± 4053	59160 ± 5040	6658 ± 560	531 ± 133
3,4-dichloroaniline matrix spike hypoosmotic	8813 ± 2058	5263 ± 1386	5400 ± 214.3	6279 ± 214		
Benzalkonium chloride matrix spike isosmotic	978 ± 73.7	2016 ± 49.4	492 ± 28.5	990 ± 42.6	3964 ± 524	824 ± 155
Benzalkonium chloride matrix spike hypoosmotic	1258 ± 117	1638 ± 189	467 ± 70.5	986 ± 102		

[a] Stimulation effect
[b] Intermediate dose response (i.e., non-monotonic dose response)
NC – Not calculated

Table S3. List of whole effluent toxicity chemicals and specifications.

Chemical Name	CAS	Purity	Source
Benzalkonium chloride[a]	63449-41-2	≥ 95%	Millipore Sigma
Ammonium chloride	12125-02-9	> 99.5%	Fisher Scientific
Copper sulfate pentahydrate	7758-99-8	≥ 98%	Millipore Sigma
Cadmium chloride	10108-64-2	99.99%	Millipore Sigma
3,4-dichloroaniline	95-76-1	98%	Millipore Sigma

[a]Benzalkonium chloride is as 74.5% benzyldimethyldodecylammonium chloride, 22.3% benzyldimethyltetradecylammonium chloride.

ANALYTICAL METHODS

Matrix spike reference toxicants

Inorganic metal and metalloid salts were purchased from Sigma-Aldrich and included: cadmium chloride and copper (II) sulfate pentahydrate. The organic compounds 3,4 dichloroaniline (3,4 DCA), benzalkonium chloride (BAC, classified as a Quaternary Ammonium Compound; QAC is 74.5% benzyldimethyldodecylammonium chloride, 22.3% benzyldimethyltetradecylammonium chloride and ammonium chloride were also purchased from Sigma-Aldrich. Inorganic salts were dissolved in ultrapure water (16–18 MΩ cm^{-1} Barnstead GenPure, Thermofisher) while 0.5 % v/v ultrapure water and dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used to dissolve non-polar organic chemicals. All toxicants stock solutions were diluted in reconstituted synthetic moderately hard water for FET tests or in L-15/ex medium (ISO 2019) for RTgill-W1 cells. Chemicals are listed in Table S3, and include category numbers, purity, and source. For metals and metalloids, an aliquot of exposure solution was acidified immediately after preparation to 5% nitric acid final concentration and measured using inductively coupled plasma mass spectrometry (ICP-MS; ICAP RQ, Thermofisher) analysis. BAC was extracted using a 3:2 L-15/ex: acetonitrile liquid-liquid extraction followed by the addition of NaCl to aide in partitioning. The recovered acetonitrile extract was analyzed by ultra-high-performance liquid chromatography (UHPLC) following previous methods (Martínez-

Carballo et al. 2007)) representing benzyldimethyldodecylammonium (BAC r=12) and benzyldimethyltetradecylammonium (BAC r= 14) degradation. Concentrations were calculated using the average concentration of the products. 3,4-DCA was analyzed by ultra-high performance liquid chromatography (UHPLC) using a solid phase extraction (Sep-Pak® C18 cartridge, Waters Corporation) and methanol elution following previous methods (Primel et al. 2007). Total ammonia was measured before and after exposure using ultraviolet-visible spectrophotometry (UV/Vis; Hach DR/2500, 115 Vac Hach) analysis at an excitation/emission value of 694 and 515 nm, respectively. Chemical concentrations were measured before and at test take down to confirm toxicant stability in solution.

Wastewater samples

Osmolality was measured with a Vapro® Vapor Pressure Osmometer (Model5600, ELItech group) pH, conductivity, dissolved oxygen and temperature were measured with an Advanced Electrochemistry Benchtop Meter 68X00650 and probes (Thermo Scientific). Total residual chlorine was measured using ultraviolet-visible spectrophotometry (UV/Vis; Hach DR/2500, 115 Vac Hach). Alkalinity and hardness were measured using colorimetric titration methods previously described (Standard Methods 2011).

SUPPLEMENTARY REFERENCES

Standard Methods for the Examination of Water and Wastewater, 2011.[1]

Standard Methods for the Examination of Water and Wastewater, Section 4020 Quality Assurance Control.[2]

CHAPTER V

COMPARING SENSITIVITY OF THREE FISH CELL LINES, RTGILL-W1, RTGUTGC, AND RTL-W1 THROUGH CYTOTOXICITY AND BIOMARKER ENDPOINTS FOR AQUATIC TOXICITY TESTING OF LEGACY AND EMERGING CHEMICALS OF CONCERN.

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Scott, Justin[a]#, Koons, Brice[a], and Minghetti, Matteo[a].

[a] Oklahoma State University, Stillwater, OK, 74078, USA

Corresponding author

ABSTRACT

Three cell lines derived from rainbow trout (*Oncorhynchus mykiss*), gill (RTgill-W1), intestine (RTgutGC,) and liver (RTL-W1) were evaluated through enzymatic cytotoxicity and gene expression for aquatic toxicity testing. Sensitivity of the cell lines was compared through exposure to the legacy chemicals copper (Cu) and cadmium (Cd) and chemicals of emerging concern benzalkonium chloride, sodium dodecylbenzene sulfonate, perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS) and, 3,4-dichloraniline, (3, 4-DCA). Cytotoxicity results indicated that, excluding LAS, RTgill-W1 cells were more sensitive to all other chemicals tested. Half maximal effective concentrations (EC50) were measured for all chemicals excluding PFOA and PFOS. While PFOA and PFOS exposures did not induce an inhibition of the cellular markers measured (i.e., cell metabolic activity, cell membrane and lysosome integrity) a stimulation effect was measured for lysosomal integrity. Gene expression results indicated a significant increase of 46- and 48- fold change for metallothionein in RTgill-W1 cells exposed to a no observed effect concentration (NOEC) and EC10 of Cd. PFOA and PFOS exposures in RTL-W1 cells did not affect the expression of the Peroxisome Proliferator Activated Receptor gamma (PPARγ). RTgill-W1 cells have shown to be overall more sensitive than RTL-W1 and RTgutGC cells for cytotoxicity assays and are warranted for their use in acute toxicity testing. Moreover, the use of MT gene expression measurements has shown to be a more sensitive endpoint in identifying metal toxicity in comparison to cytotoxicity assays.

KEYWORDS: Cell lines, cytotoxicity, biomarkers, aquatic toxicity, animal alternatives

INTRODUCTION

In both, regulatory whole effluent toxicity (WET) testing and chemical registration, *in vivo* fish assays utilize a large volume of specimens tested to measure and identify harmful environmental impacts. While fish toxicity testing methods have been vital in predicting toxicity through a set of standardized methods, advancements with *in vitro* ecotoxicological techniques have gained momentum. Moreover, fish *in vivo* assays used for chemical and effluent testing do not typically provide information on the mechanism of toxic action which may aid in toxicant identification. Cytotoxicity assays allow the identification of a toxicant's mode of action, decreased test duration and replacement of live animals used for aquatic toxicity testing. Recently, the International Organization for Standardization (ISO) and the OECD has accepted an *in vitro* alternative method using the rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1 (Bols et al. 1994) for chemical and effluent toxicity testing (ISO 21115:2019 OECD 249; 2021).

While this method has shown strong predictability for acute toxicity in fish (Tanneberger et al. 2013; Fischer et al. 2019; Scott et al. 2021), RTgill-W1 cells represent only one tissue type which does not fully characterize the organism holistically. Therefore, incorporation of the RTgill-W1 cell assay with additional fish cell lines derived from various tissue types could be useful to identify possible tissue specific mechanisms of toxic action related to a particular chemical of concern. Importantly, it should be investigated whether the gill is truly a preferred model for sensitivity or if other tissue types warrant incorporation into current ISO and OECD cytotoxicity methods.

Several studies have shown specific mode of toxic action in specific cell lines (i.e., gill, intestine, and liver) to chemicals in relation to their physio-chemical properties. For instance, it was shown that RTgill-W1 cells were shown to be more sensitive in terms of cytotoxicity to organic chemicals such as β-naphthoflavone and benzo(a)pyrene (BAP) compared to the rainbow trout and eel liver cell lines RTH-149 and Hepa E1. However, the Hepa E1 and RTH-149 expressed higher cytochrome p450 1A (CYP1A) activity, emphasizing the link between increased phase I biotransformation and decreased sensitivity of the liver cells (Franco et al. 2018). Another rainbow trout liver cell line, RTL-W1 (Lee et al. 1993), has also been shown biotransformation of BAP (Stadnicka-Michalak et al. 2018) and to be sensitive to metals common to mining effluents (Dayeh et al. 2005). RTgill-W1 cells have been shown to predict acute toxicity of several common whole effluent toxicity (WET) chemicals of interest including the legacy chemicals, copper (Cu) and cadmium (Cd) and chemicals of emerging concern (CECs), such as the quaternary ammonium compound (QAC), benzalkonium chloride (BAC) and the linear alkyl sulfonate (LAS), sodium dodecylbenzene sulfonate (Scott et al. 2021). The rainbow trout intestine cell line RTgutGC (Kawano et al. 2011) has shown to measure toxicity for heavy metals such as Cu, Cd, silver (Ag), and zinc (Zn) (Ibrahim et al. 2020), silver nanoparticles (Minghetti and Schirmer, 2016) and the organic chemical benzo(a)pyrene (BAP) (Stadnicka-Michalak et al. 2018).

Overall, the comparison of cytotoxicity of chemicals of varying origin such as legacy chemicals and CECs in fish cell lines derived from different tissues is lacking especially in view of incorporating novel cell lines into routine ecotoxicology testing procedures. Cytotoxicity assays have served as a strong representation for the

transitioning of novel *in vitro* approaches as *in vivo* replacements for both legacy and CECs acute exposures (Scott et al. 2021). Additionally, biomarkers have been useful in measuring exposure to common legacy contaminants in aquatic environments such as Cd (Kroon et al. 2017) and could potentially be used to further measure exposure to CECs. Moreover, the addition of multiple cell lines utilizing cytotoxicity and biomarkers could aid in identifying and reducing toxic impacts to aquatic populations from chemical and wastewater exposures. Therefore, our studies are warranted to better examine the sensitivity of different cell lines through routine cytotoxicity assays and incorporate biomarker endpoints aimed at increasing the identification of different classes of chemicals.

While legacy chemicals are well known for their toxicological impacts, CECs are problematic due to the lack of knowledge of their general toxicity and specific mechanism of toxic action (Petrie et al. 2014) particularly in fish where little is known about biological effects (Arukwe and Mortensen 2011). Building on this concern is the large production and volume of these chemicals, and the continued release in the environment regardless of fully understanding their risk and fate (Bolong et al. 2009). A good example is the rising concern with poly- and perfluorinated compounds (PFAS) and their lack of toxicological understanding (Podder et al. 2021). With the need for improved water quality conditions, CECs should be properly monitored to better understand their toxicity and impact on the environment with the same stringency that exists for legacy contaminants of concern. Specifically, there is a lack of knowledge on exposure and fate of CECs as well as the molecular and cellular mechanism of toxic

action. Therefore, using cell lines may allow the ability to better understand exposure scenarios and how it relates to toxicity in aquatic organisms.

Gene expression in specific cell types (e.g., hepatocytes) could be used to identify the toxic mechanism of action in a specific tissue type and aid in transitioning the *in vitro* assay's ability to measure acute toxicity of legacy and CECs. Common biomarkers such as metallothionein (MT), cytochrome p450 1A (CYP1A), and vitellogenin have been used to better understand toxic insults (Fent 2001). For instance, the protein metallothionein (MT), has been shown to be induced in response to excess metal accumulation (Minghetti et al. 2008). Moreover, studies have shown that MT has been expressed in several tissues of rainbow trout and have highlighted their use at detecting metal toxicity in aquatic systems (Chowdhury et al. 2005). The peroxisome proliferator-activated receptor (PPAR) is an important family of genes involved in cellular differentiation, adipogenesis and hormone and energy homeostasis (Fang et al. 2012). Previous studies showed perfluorooctanoic acid (PFOA) toxicity through suppression of PPAR genes which is involved in hepatic fatty acid metabolism in chicken embryos (Jacobsen et al. 2018), and in marine medaka (*Oryzias melastigma*) embryos (Fang et al. 2012). Inversely, studies have shown that PFAS increased expression of PPARα, PPARβ, PPARγ in proliferating and differentiating human mesenchymal stem cells (MSCs) (Qin et al. 2010). Therefore, more research is needed to determine the role PPAR genes on the molecular response to PFAS in fish cells.

Furthermore, PFAS has been observed as obesogenic, which, means that is capable of compromising lipid homeostasis promoting conditions such as obesity and non-alcoholic fatty liver disease (NAFLD) (Franco et al. 2020). Therefore, considering

that lipids are present in fetal bovine serum (FBS), a component of cell culture medium, one aspect of this study was to examine the role of FBS on the PFAS toxicity in RTL-W1.

The overall goal of this research was to compare the sensitivity of gill, liver and intestinal cell lines to various chemical exposures and determine if using multiple tissue types in combination with cytotoxicity and gene expressions assays allow a more sensitive and specific toxicity forensic tool. This was accomplished by comparing the sensitivity of three cell lines, RTgill-W1, RTgutGC and RTL-W1 to a selected chemicals containing legacy and CECs. Selected classes of chemicals consisted of two legacy metals, Cu and Cd and several CECs such as BAC the LAS, sodium dodecylbenzene sulfonate, two PFAS chemicals, PFOA and PFOS and the ISO and OECD reference chemical, 3,4-dichloroaniline. For gene expression assays, the cellular biomarkers MTb, and PPARγ were selected based on the specific biomarkers of exposure to a metal, Cd (i.e., legacy chemical) and the organics PFOA and PFOS (i.e. CEC).

MATERIALS AND METHODS

Cell culture

RTgill-W1 culturing and seeding techniques for cytotoxicity assays followed ISO and OECD guidelines (ISO 2019; OECD 2021). Briefly, cells were cultured in 75 cm^2 cell culture flasks (Greiner Bio-One, Kremsmunster, Austria) at 19 °C in normal atmosphere with Leibovitz's L-15 (Invitrogen, Thermofisher, Waltham, MA, USA) supplemented with 5% Fetal Bovine Serum (FBS; F6178, Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin for RTgill-W1 and RTL-W1 cells and 1%

gentamicin for RTgutGC. For the multiple viability assays a seeding density of 150,000 cells/mL (78,947 cells/cm^2), 140, 000 cells/ml (73,684 cells/cm^2), and 100,000 cells/ml (52,631 cells/cm^2) was used into 24 multiwell plates (Greiner Bio-One, Kremsmunster) for RTgill-W1, RTgutGC, and RTL-W1 cells, respectively. After seeding, all plates were incubated at 19 °C in the darkness for 48 hours (h) to allow development of a confluent monolayer. RTgill-W1 and RTL-W1 cells were seeded into 6 well plates (Greiner Bio-One, Kremsmunster) for the gene expression assay at the same seeding density of cells/cm^2 for each well (9.6 cm^2).

Chemical exposure preparation and analysis

Metals and soluble organics were dissolved in ultrapure water (16–18 M Ω cm^{-1} Barnstead GenPure, Thermofisher, Waltham, MA, USA) and insoluble organics into dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to create master stock solutions following previous literature methods (Scott et al. 2021). All dilution concentrations of toxicants are made in L-15/ex, a medium identical to Leibovitz's L-15 commercial medium but without amino acids and vitamins. Master stock solutions were made by mixing aliquot volumes into exposure medium to achieve the desired chemical concentrations. For organics the final solvent concentration of DMSO in solution was 0.5% v/v, in L-15/ex. PFAS chemicals were spiked into L-15/ex media without FBS for all cell lines or in L-15 supplemented with an increasing FBS concentration of 1, 5, and 10% for exposures with RTL-W1 cells.

Chemicals are listed in Table S1, and include category numbers, purity, and source. The concentration ranges (in ng/ml) for each chemical tested to the cells was: Cd

(as ion), 50 – 150,000; Cu, 65 - 3200; BAC, 5 – 4,000; LAS, 100 – 10,000; PFOA, 14 – 10,350; PFOS, 25 – 27,000; and 3, 4-DCA 3200 – 500,000. Cytotoxicity assays required chemical exposures higher than environmentally relevant concentrations in order to generate dose response curves for NOEC, EC10 and EC50 endpoints.

For metals, an aliquot of exposure solution in L-15/ex was acidified immediately after preparation to 5% nitric acid final concentration and measured using inductively coupled plasma mass spectrometry (ICP-MS; ICAP RQ, Thermofisher) analysis. The LAS was measured using a modified methylene blue standard method (Chitikela et al. 1995) in L-15/ex media through chloroform extraction and measured with an absorbance value of 652 nm (Cytation 5 multiwell plate reader; BioTek, USA). BAC is extracted from cell media using a 3:2 L-15/ex: acetonitrile liquid-liquid extraction followed by the addition of NaCl to aide in partitioning. The recovered acetonitrile extract was analyzed by ultra-high-performance liquid chromatography (UHPLC) following previous methods (Martínez-Carballo et al. 2007)) representing benzyldimethyldodecylammonium (BAC r=12) and benzyldimethyltetradecylammonium (BAC r= 14) degradation. Concentrations were calculated using the average concentration of the products. 3,4-DCA was analyzed by ultra-high performance liquid chromatography (UHPLC) using following a solid phase extraction (Sep-Pak® C18 cartridge, Waters Corporation) and methanol elution following previous methods (Primel et al. 2007). PFAs chemicals were not measured in solutions and are repored as nominal concentrations. Chemical concentrations were measured before (time zero; T0) and after exposure (24 h; T24) to confirm toxicant stability in solution. Quality control criteria for chemical analysis of measured exposure sample concentrations was set at ± 10% recovery at T0 and ± 20% at T24. If chemical

stability of the exposure concentrations did not meet quality control criteria the nominal concentrations were adjusted to the measured values. All chemicals were measured at least in triplicate (n = 3).

Multiple viability assay and cytotoxicity

Before exposure, cells were washed with phosphate buffer solution (PBS; Sigma-Aldrich, St. Louis, MO, USA) and then exposed to chemical concentrations for 24-h in the dark at 19 °C. After 24-h of exposure, cells were washed again, and the multiple endpoint viability assay was performed as previously described (ISO 2019; OECD 2021). The multiple endpoint viability measures cell metabolic activity, membrane integrity, and lysosomal integrity through the application of three commercially available dyes which are alamarBlue (AB; Invitrogen, Thermofisher, Waltham, MA, USA), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Thermofisher, Waltham, MA, USA), and 3-amino-7-dimethylamino-2-methylphenazine hydrochloride or Neutral Red (NR; Sigma-Aldrich, St. Louis, MO, USA), respectively (Lee et al. 2008; Minghetti and Schirmer 2016). Negative controls (L-15/ex) and solvent controls (L-15/ex with 0.5 v/v DMSO) were included in each plate.

RNA extraction, cDNA synthesis and quantitative PCR (qPCR)

For Cd the no observed effect concentration (NOEC) and effective concentration 10% (EC10) were 50 and 750 ng/ml. PFOA and PFOS cells were exposed to concentration 21 and 414 and 25 and 500 ng/ml, respectively to test a low and high environmentally relevant concentration of each chemical. MTb and PPARγ mRNA levels were analyzed by qPCR based on methods previously descried (Minghetti et al. 2008).

Briefly, total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, USA) following manufacturer's instructions on cells grown to confluency (*See section cell culture and seeding techniques)* in 6 well plates (Greiner Bio-One, Kremsmunster, Austria). The extracted RNA was DNAse treated using the TURBO DNAse kit (Thermo Fisher Scientific, Waltham, USA). Following DNase treatment, final total RNA concentrations were measured spectrophotometrically (Cytation 5 multiwell plate reader; BioTek, USA). RNA integrity was checked by electrophoretic separation and visualization using one μg of total RNA. Complimentary DNA (cDNA) synthesis was performed using the Maxima H minus cDNA kit (Thermofisher, Waltham, MA, USA) following the manufacture's protocol using 500 ng of total RNA. The MTb and PPARγ primer sequences used for qPCR are reported in Table 1.

Quantitative PCR was performed using the SYBR Premix Taq II (Takara Bio, USA) and thermocycler CFX Connect Real-Time PCR Detection System (BioRad, Hercules, CA, USA) following the manufacture's protocols. MTb mRNA levels were measured by absolute quantification, PPARγ by relative quantification. Absolute quantification is achieved by running the unknown cDNA samples in parallel to a set of reactions containing standards consisting of a serial dilution of linearized plasmid (TOPO® TA Cloning® Kits, Invitrogen, USA) containing the target gene cDNA sequences (Minghetti et al., 2008). Relative quantification is achieved by fold change and was calculated with ΔΔCt method with elongation factor 1 (ELF1) used as reference gene for normalization (Pfaffl 2001).

Statistical analysis

Statistical analysis was performed using GraphPad Software (Version 9.3.1). All data sets were measured for normality distribution. Excluding the gene expression, all other experiments were repeated at least 3 times in different days with cells of different passages. EC50 values were determined by the non-linear regression sigmoidal dose-response curve fitting module using the hill slope equation (GraphPad Software). Individual viability endpoints comparing toxicity of chemical to different cell types and chemical ranking of geometric EC50s was analyzed using one-way ANOVA and post hoc Tukey's test. Gene expression of exposed cells was compared to control and analyzed using one-way ANOVA and post hoc Dunnett's test. All statistical analysis tests were performed with alpha equal to 0.05.

RESULTS

Chemical confirmation

Confirmation of chemical concentrations is shown in Table 2. All reported chemical concentration analysis met their respective quality control criteria at T0. The initial and final exposure concentrations were very similar (within 20% of the initial nominal concentration) for all chemicals at T24 excluding 3, 4-DCA, based on the initial nominal concentrations. The concentrations of 3, 4-DCA at T24 have been adjusted based on their deviation of the two measured concentrations with T0 compared to the nominal.

Cytotoxicity to cell lines

While all chemical induced an inhibitory effect on cell metabolic activity, cell membrane integrity and lysosome integrity, PFOS induced a peculiar stimulation effect of lysosomal integrity when measured for all three cell lines (Figure 1). Such stimulation effect increased by increasing percentages of FBS (Figure 2). The ranking of EC50s for increasing toxicity of the chemicals was Cu > QAC > LAS > CD > 3, 4-DCA for each of the three cell lines (Table 3; one-way ANOVA post hoc Tukey's test; $p = 0.05$; $n = 3$). Specifically, RTgill-W1 cells were more sensitive to 4 out of 5 toxic chemicals (i.e. Cd, Cu, QAC, and 3, 4-DCA) compared to RTL-W1 cells. RTgutGC cells were more sensitive to only 2 of the 5 toxic chemicals (i.e. Cd and Cu) to RTL-W1 cells. Overall, RTL-W1 cells indicated to be the least sensitive to all chemicals excluding the LAS, which had similar cytotoxicity to all three cell lines.

Gene expression to cell lines

Gene expression of MTb was tested only in RTgill-W1 cells and it was found to significantly increase in cells exposed to Cd (one-way ANOVA, post hoc Tukey's test; $p = 0.0006$; $n = 3$). MTb expression in RTgill-W1 cells was found to be induced by a fold of 46 ± 9 and 48 ± 22 for the NOEC and EC10 Cd exposures, respectively compared to controls (Figure 4). No significant difference was found for PPARγ gene expression in RTL-W1 from PFOA and PFOS exposures for both the NOECs and high concentrations compared to controls (Figure 5).

DISCUSSION

Cytotoxicity and chemical sensitivity comparisons

The analysis of cytotoxicity of chemicals with diverse physicochemical properties in cell lines derived from the trout gill, gut and liver was envisioned to evaluate if chemicals showed tissue specific responses and to evaluate which cell type was the most sensitive. For the chemicals that demonstrated cytotoxicity (i.e., Cd, Cu, LAS, QAC, and 3, 4-DCA) EC50 values could be calculated for all three cell lines and showed varying sensitivity to chemicals (one-way ANOVA; post hoc Dunnett's multiple comparison test; alpha = 0.5) (Figure 3). Based on the cytotoxicity data, RTgill-W1 cells were the most sensitive for all chemicals excluding LAS, whereas RTL-W1 cells were the most tolerant and RTgutGC cells showed an intermediate response (Figure 3; Table 3). The ranking of the five chemicals that exhibited EC50 values was found to be identical to all three cell types indicating chemical toxicity is holistically similar through the three tissue types.

For metal sensitivity comparisons, the increase tolerance of RTL-W1 cells could be possibly explained by the fact the liver is a tissue that normally bioaccumulates and detoxifies heavy metals and other chemicals such as organics, (Arukwe and Mortensen 2011; Fazio et al. 2020) whereas the gill and gut are more involved in transporting of chemicals in the blood stream and their metabolic capacity is limited (El-Moselhy et al. 2014). For the organics, RTgill-W1 cells were most sensitive for the QAC and 3, 4-DCA compared to RTgutGC and RTL-W1 cells. For the LAS, results showed similar toxicity to all three cell lines, which could be explained by a general baseline toxic mechanism of action (MOA) due to LAS acting as a membrane toxicant. Previous studies have shown

that LAS has similar toxicity to enzymes in liver and gills of the teleost fish, Channa punctatus (Gupta et al. 1989) suggesting that different tissue types have similar responses to the anionic surfactant.

PFOA showed inhibition response well above environmentally relevant scenarios and also resulted in an increase of neutral red uptake which warrants further explanation. Due to the lack of knowledge of PFAs toxicity to aquatic vertebrates such as fish, it is difficult to understand this result. However, a previous study investigated PFOS exposures to the adult easter oyster *Crassostrea virginica* and found that significant cellular lysosomal damage occurred (Aquilina-Beck et al. 2020). Therefore, as lysosomes perform basic breakdown of toxic substances, PFOS may be having an increased toxic effect for lysosomal integrity in both invertebrate and vertebrate aquatic species. Moreover, by increasing the amount of FBS in the media, our results have shown that the addition of proteins and lipids may be an important mediator of cytotoxicity to the liver specific to lysosomal integrity (Figure 2) which agrees with previous studies using human liver cells (Franco et al. 2018)

Metallothionein and PPAR gamma gene expression

It has previously been shown that fish cell lines are a useful model to determine toxic insults at the molecular level as seen with increased MTb expression in response to metal toxicity (Ibrahim et al. 2020). In the present study, MTb gene expression in RTgill-W1 cells indicated that this biomarker is more sensitive than any of the cytotoxicity assays. Indeed, Cd exposure resulted in an induction of ~ 40-fold even in cells exposed to the NOEC (Figure 4). Previous studies have shown that MT expression in rainbow trout

increased for the following tissues with waterborne Cd exposures: kidney > gill > liver (Chowdhury et al. 2005). Interestingly, they also found that dietary cadmium exposure showed an increase in MT expression for the following tissues: kidney > cecae and posterior intestine > liver and stomach > midintestine > gills. Therefore, a rainbow trout kidney cell line such as RTK (Suryakodi et al. 2021), may prove useful for future studies to measure cytotoxicity of heavy metals with more sensitivity. Further research is needed to compare sensitivities of the other cell lines, but based on our results, RTgill-W1 cells and MTb have shown to be useful as a more sensitive model of metal toxicity through gene expression than the cytotoxicity assay.

PPARγ gene expression results for PFOA exposures indicated a slight inhibition in RTL-W1 cells in the highest concentration of 414 ng/ml (Figure 5), but was not significant (one-way ANOVA, post hoc Tukey's test, alpha = 0.05, n = 3). PFOS exposures resulted in no significant difference from control as well for both low (25 ng/ml) and high (500 ng/ml) concentrations. This could be due to the specific exposure (dose and time) used in this study. Studies have investigated exposure to marine medaka embryos at PFOS concentrations of 1, 4, and 16 mg/L which produced different effects of the PPAR isomers (Fang et al. 2012). While in the current study we did not get a significant response from our concentrations, future studies may investigate increased concentrations for cell line exposures. Previous studies have shown that the liver is a target organ for PFAs substances and toxicological effects include fatty acid metabolism, lipid transport and overt homeostasis, and cholesterol synthesis and PPAR isoforms have been shown to be involved in these effects (Arukwe and Mortensen 2011). Specifically, changes on PPAR expression levels indicated significant effects on the lipid β-oxidation

system. Furthermore, PFAS has been observed as obesogenic, which is capable of compromising lipid homeostasis promoting conditions such as obesity and non-alcoholic fatty liver disease (NAFLD) (Franco et al. 2020). The study by Franco et al., (2020) utilized a lipid deposition droplet method to determine if lipids would accumulate in human liver cells by potential obesogenic exposure. By using a quantitative metabolomic approach, dysregulation of lipid metabolism increase could be measured in the cells exposed to perfluorooctanoic acid (PFOA) at environmentally relevant concentrations. Additionally, an increase in overall liver weight was also measured in mice pups exposed to perfluorooctane sulfonic acid (PFOS) (Abbott et al. 2009), Therefore, future studies could utilize this method in the fish liver cell line, RTL-W1, to evaluate if PFOS induces a similar effect (i.e. deposition of lipid droplet), by increasing the about of protein content in the exposure medium.

CONCLUSION

Cytotoxicity using all three cell lines has highlighted a more holistic approach for aquatic toxicity testing for both legacy and CECs. Moreover, advanced biomarkers have been measured as a more sensitive endpoint for specific toxicants of concern (i.e., Cd and RTgill-W1 cells). While the three cell lines used in the present study offer more insight into various tissue responses to toxicants, future studies should aim to incorporate additional cell lines of other various tissue types (i.e., brain, heart, kidney, spleen, gonad, etc.). This inclusion may allow more sensitive endpoints to be measured, especially where the cell line fail to show the modes of actions (i.e. neurotoxicity, cardiotoxicity, renal toxicity, etc.) (Lin and Will 2012; Scott et al. 2021). Therefore the use of multiple cell lines may allow a more holistic understanding of the insult to fish at the organismal

and population level. Regardless, our results have highlighted that RTgill-W1 cells are more suited at predicting acute toxicity to fish populations and reinforce their value to the field of ecotoxicology which represent a valid replacement for acute fish toxicity testing.

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TABLES AND FIGURES

Table 1. Rainbow trout (Oncorhynchus mykiss primers used for qPCR**.**

Primer	Forward Primer (5' - 3')	Reverse Primer (3' - 5')	Repository ID
PPARγ	CCACAGCCAGGTTCAGGAG	TGTTGAGTAGGGAAGCGGTG	XM_036984365
MTb	GCTCTAAAACTGGCTCTTGC	GTCTAGGCTCAAGATGGTAC	M18104[a]
EF1a	ATATCCGTCGTGGCAACGTGGC	TGAGCTCGCTGAACTTGCAGGC	NM 001124339[a]

[a]GenBank (http://www.ncbi.nim.nih.gov/)

Table 2. Chemical confirmation of reference toxicants.

Chemical	Initial chemical stability % Mean ± sd	Final chemical stability % Mean ± sd
Cadmium as ion	99 ± 2	92 ± 4
Copper as ion	98 ± 10	102 ± 5
Benzalkonium chloride[a]	95 ± 2	89 ± 5
Sodium dodecylbenzene sulfonate	95 ± 3	91 ± 4
3,4-dichloroaniline	97 ± 9	78 ± 3
Perfluorooctanoic acid	NA	NA
Perfluorooctanoic sulfonic acid	NA	NA

[a]Benzalkonium chloride is as 74.5% benzyldimethyldodecylammonium chloride, 22.3% benzyldimethyltetradecylammonium chloride.

NA – Not applicable. Results are reported as nominal

Table 3. Geometric mean EC50 values and chemical toxicity ranking of RTgill-W1, RTGutGC, and RTL-W1.

Chemical	RTgill-W1	RTgutGC	RTL-W1
	EC50 Geometric mean ± sd (ng/ml)		
Cu	331 ± 115^{aA}	426 ± 39^{aA}	695 ± 119^{aB}
QAC	1067 ± 33^{bA}	1630 ± 282^{bB}	1956 ± 34^{bB}
LAS	3955 ± 417^{cA}	4607 ± 1127^{cA}	4664 ± 706^{cA}
Cd	8154 ± 540^{dA}	10 405 ± 1576^{dA}	26 365 ± 1964^{dB}
3,4 DCA	71 732 ± 1030^{eA}	100 817 ± 7102^{eB}	105 844 ± 4766^{eB}

Lowercase letters indicate significant difference of EC50 between chemicals for each individual cell types and uppercase letters indicate significant difference between cell types for each individual chemical (one-way ANOVA; post hoc Tukey's test; alpha = 0.05; n = 3).

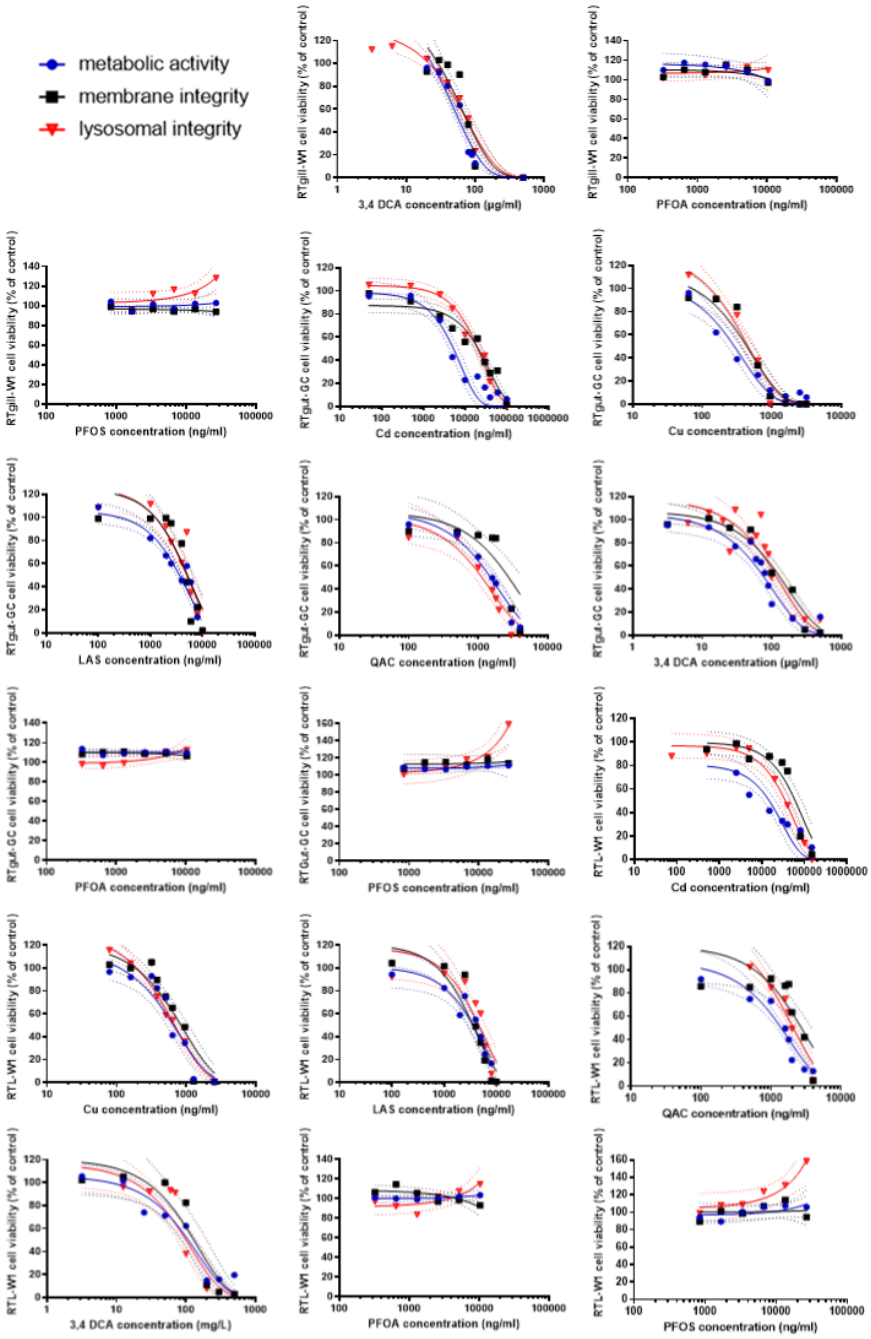

Figure 1. Dose response curves of selected toxicant exposures to RTgill-W1, RTgutGC, RTL-W1 cells. Results from viability assays are reported as percent viability based on the Fluorescent Units (FU) of the L-15/ex controls. Cytotoxicity assays of metabolic activity (alamarBlue) is indicated in blue (circles), membrane integrity (CFDA-AM) in black (squares), and lysosomal integrity (Neutral Red) in red (triangle). Values reported as mean (marker) and confidence intervals (dashed lines) of at least three independent experiments (n = 3).

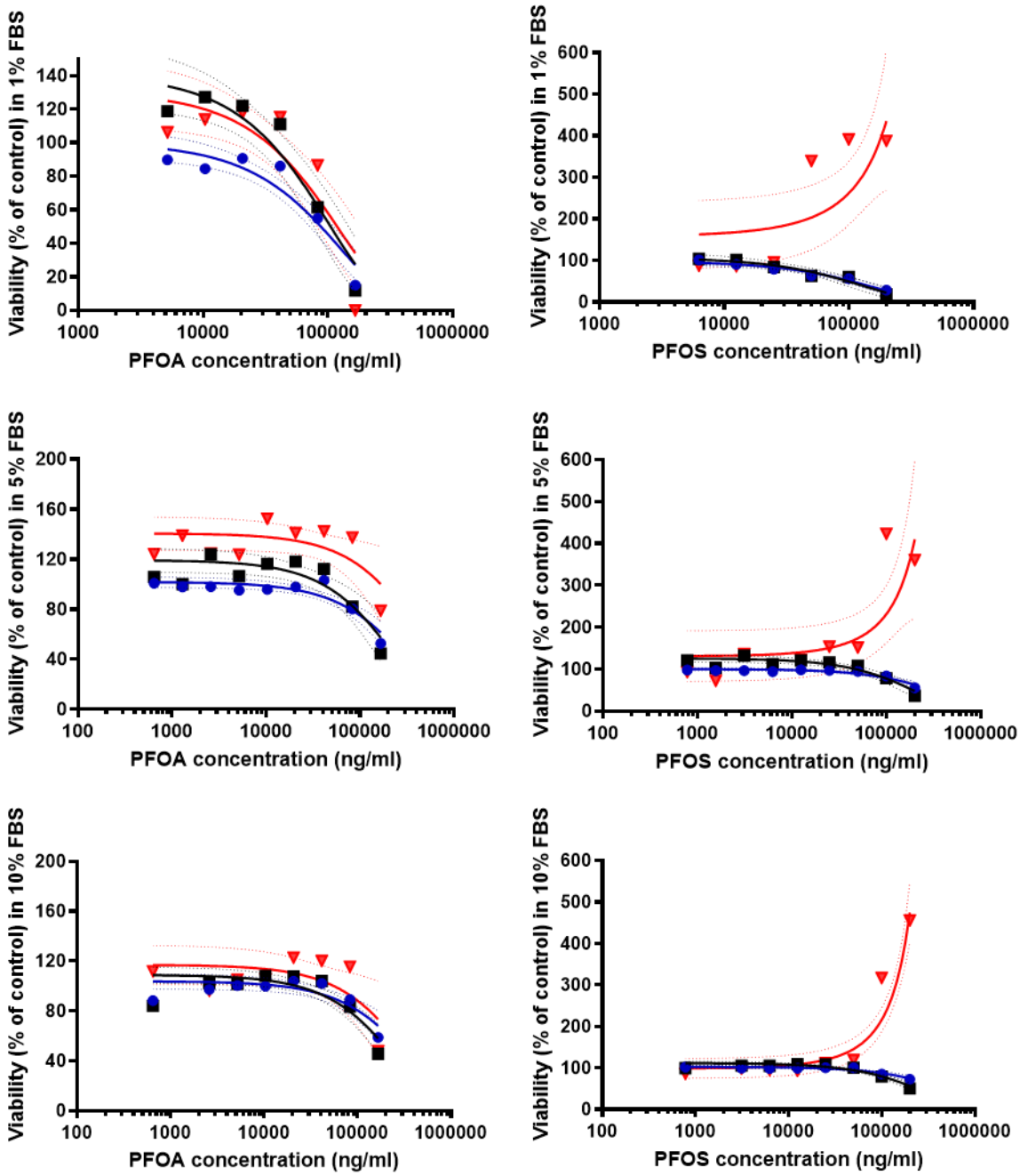

Figure 2. Dose response curves of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) exposures to RTL-W1 cells in L-15/FBS with increasing FBS of 1, 5 and 10%. Results from viability assays are reported as percent viability based on the Fluorescent Units (FU) of the L-15/FBS controls. Cytotoxicity assays of metabolic activity (alamarBlue) is indicated in blue (circles), membrane integrity (CFDA-AM) in black (squares), and lysosomal integrity (Neutral Red) in red (triangle). Values reported as mean (marker) and confidence intervals (dashed lines) of at least three independent experiments (n = 3).

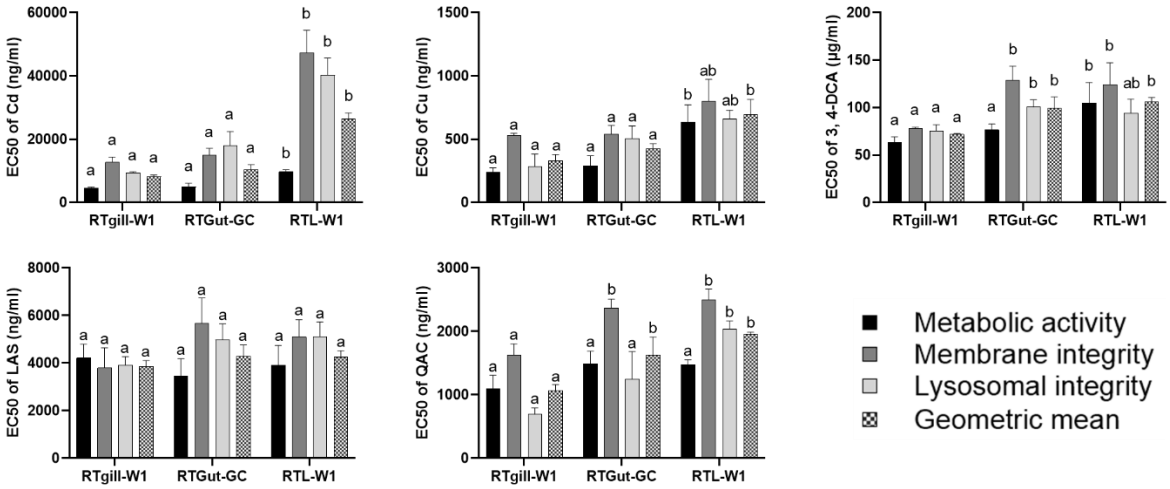

Figure 3. Effect concentrations of 50% population (EC50s) to three cell lines, RTgill-W1, RTGutGC, and RTL-W1 to cadmium (Cd), copper (Cu), 3, 4-dichloraniline, (3, 4-DCA), the linear alkyl sulfonate (LAS) sodium dodecylbenzene sulfonate, and the quaternary ammonium compound (QAC) benzalkonium chloride in L-15/ex. Cytotoxicity assays consisted of the multiple viability assay of metabolic activity (alamarBlue), membrane integrity (CFDA-AM), and lysosomal integrity (Neutral Red). Individual endpoints and geometric mean of all three values reported as mean and standard deviation. Lowercase letters indicate significant difference between endpoints in individual cell lines (one-way ANOVA, post hoc Tukey's test, alpha = 0.05, n = 3).

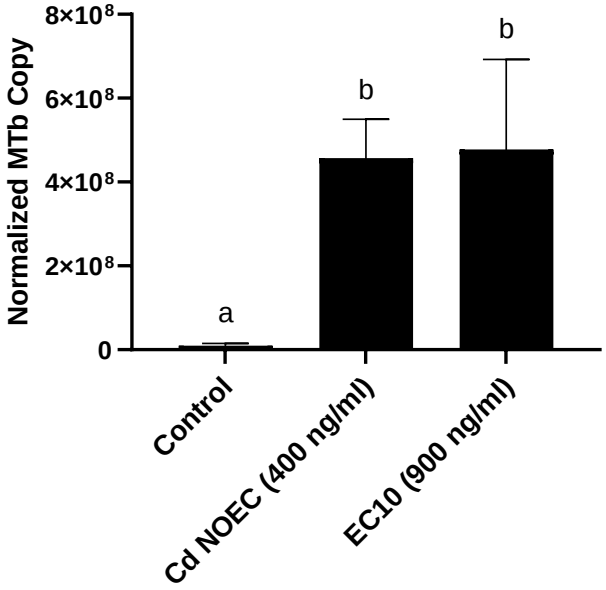

Figure 4. Gene expression of metallothionine (MTb) to RTgill-W1 cells for the no observed effect concentration (NOEC) and effect concentration of population (EC10) of cadmium (Cd) in L-15/ex. Lowercase letters indicate significant difference between control and Cd exposure concentrations (one-way ANOVA, post hoc Tukey's test, alpha = 0.05, n = 3).

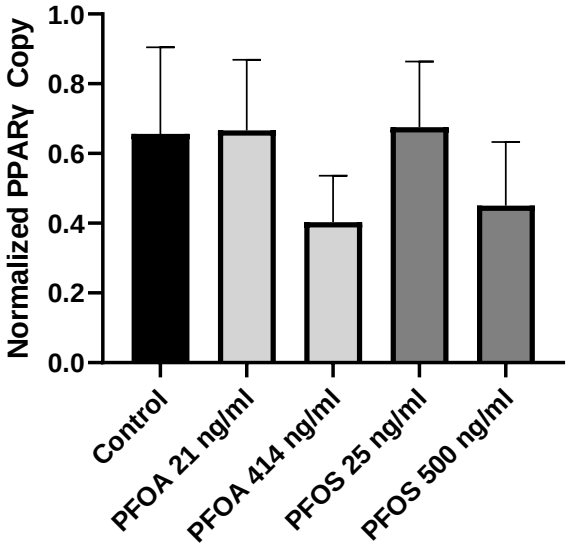

Figure 5. Gene expression of peroxisome proliferator activated receptor gamma (PPARγ) to RTL-W1 cells for concentrations of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). No significant difference was measured between control and exposure concentrations (one-way ANOVA, post hoc Tukey's test, alpha = 0.05, n = 3).

CHAPTER VI

GENERAL CONCLUSION

The overall findings of these studies have shown that fish cell line cytotoxicity and fish embryo assays are continuing to be highlighted for their applicability and incorporation into ecotoxicity testing based on their success as a more ethical and informative approach. Advanced toxicological screening tools such as these have been validated in helping to reduce and replace the number of fish used for aquatic toxicity testing. The acute toxicity predictability of both models has been highlighted and warrants further incorporation and advocacy towards current chemical registration and effluent testing.

Overall, *in vitro* fish cell lines and embryo assays have been reinforced as promising tools from our study for regulatory ecotoxicology, but still require some considerations for applications. Both cell lines and embryo models have shown to highlight their novelty to move away from solely relying on the use of live fish for aquatic toxicity testing. Specifically, both models were able to predict toxicity of chemicals of interest and actual environmental samples with RTgill-W1 cells being more sensitive to toxicants than fish larvae for acute toxicity. The FET was less sensitive than RTgill-W1 cells but showed a good correlation to larvae and allowed measurement of developmental and cardio toxicity that are lacking in current acute WET methods.

RTgill-W1 cells grown on transwell inserts were shown to tolerate freshwater exposures and a modified mannitol exposure medium. Although, this method might not be practical for current OECD and ISO method for routine toxicity testing, we could envision the use of transwell inserts for more mechanistic studies focusing on apical versus basolateral route of exposures and specialized exposure media. This reinforces the use of cytotoxicity assays cultured in traditional flat bottom wells and facilitates their application as a high throughput alternative. Moreover, the use of a hypoosmotic exposure medium in flat bottom wells was shown to increase the sensitivity to toxicants (i.e., Cd, Cu, ammonia, and 3, 4-DCA) which demonstrates that this novel exposure medium should be considered and incorporated in the ISO and OECD methods. Our results suggest that FETs and cytotoxicity assays serve as a better surrogate than larvae to detect toxicity and evaluate chemical mechanism of toxicity (i.e., cardio toxicity and subcellular endpoints, respectively). The ability to identify and confirm toxicity of specific chemicals is vital for instances where complex matrix exposures exist, and both models have shown to increase this capability.

Ultimately, incorporation of these models into current aquatic toxicity testing guidelines would need to be reinforced by intralaboratory and interlaboratory studies, such as round robin testing to determine how well the approach fits into current WET testing applications. If incorporated appropriately, cytotoxicity and FET tests may have the ability to reduce the large-scale use of live fish testing for acute WET testing as well as introduce advanced toxicological screening and identification capabilities. Cytotoxicity using all three cell lines has shown to offer a more holistic approach and could be useful in predicting lethal and sublethal chemical and effluent toxicity to the

organism as a whole. Additionally, biomarkers such as gene expression, allow a more sensitive endpoint to be used to aid in identifying specific classes of chemicals (e.g., metal toxicity for MTb expression).

Considerations for the *in vitro* model's incorporation may need a best fit approach to WET testing and chemical registration. Our approach has shown to be effective in correlating toxicity to the larvae and using the models in a tandem based approach. In looking at a tier-based approach between all three models' cytotoxicity assays could serve as the first step to acute toxicity testing and negate the need of fish embryos and larvae. Subsequently the embryos would be used when a negative cytotoxicity assay occurs and ultimately use larvae to ensure a false negative is not reported.

The *in vitro* models ultimately would need standardized test parameters for regulatory testing. In the case of WET testing, the number of samples for test renewal, technical and biological replicates, sample manipulation and preparation, endpoint verification and statistical analysis, and quality control measures will all need careful consideration. Ultimately state and federal agencies will need adoption or modification of the current ISO and OECD test guidelines for the regulatory methods and would be achieved through round robin testing and method validation and verification procedures.

Results from the present studies place emphasis on the ability of both *in vitro* models to predict acute toxicity to fish. Future studies are warranted to determine if the models can be shown to predict chronic toxicity. An example of this would be to increase exposure duration and a sample renewal component for 7-d and compare cytotoxicity and FET assays to correlate current chronic endpoints of fish larvae growth and biomass.

Furthermore, the use of more sensitive chronic endpoints such as cell proliferation, morphology, and apoptosis detection could be incorporated for sublethal effects. For the FETs, more sensitive endpoints could be used such as heart rate and pericardial development as seen with our study, as well as including eleuthero embryo stages, and other teratogenic endpoints, such as eye, snout, and tail morphologies. This would be important for the field of WET testing where dischargers have lower critical value exposure concentrations and are responsible for the makeup of a receiving body of water's volume requiring chronic testing methods to be used for reporting.

Biomonitoring components for ecotoxicology continue to be vital due to the difficulty in identifying and measuring chemicals in complex aquatic matrices such as effluents and determining toxicological impacts in chemical production and registration. This research has shown that advanced *in vitro* models can help facilitate these requirements using a more ethical, high throughput and informative technology.

VITA

Justin Scott

Candidate for the Degree of

Doctor of Philosophy

Dissertation: Advancing *In vitro* Fish Alternatives for Regulatory Ecotoxicity Testing

Major Field: Integrative Biology

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Integrative Biology at Oklahoma State University, Stillwater, Oklahoma in 2023

Completed the requirements for the Graduate Certificate in Interdisciplinary Toxicology at Oklahoma State University, Stillwater, Oklahoma in 2018

Completed the requirements for the Master of Science in Biology at Oklahoma State University, Stillwater, Oklahoma in 2018

Experience:
Graduate Teaching Assistant (2016-2023), Oklahoma State University.
Taught physiology labs for BIOL 3204. Served as lead TA Fall 2022 -2023.

Professional Memberships: Society of Environmental Toxicology and Chemistry (national and regional chapter).