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Copper Binding to Fish Gill Cell Lines: Towards Determination of Biotic Ligand Model Parameters

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Abstract

In aquatic environments, copper is a potentially toxic element that is also an essential nutrient, aiding in the growth and development of all aerobic organisms. In small quantities, copper acts as a micronutrient, playing a large role in many enzymatic reactions, however, at increased concentrations, copper is a potential aquatic pollutant that can cause metal toxicity. The biotic ligand model (BLM) is a useful tool that is often used for metals risk assessment and helps to establish protective water criteria guidelines. Previously, the BLM has been used to model metal complexation directly at the biotic ligand (*e.g.,* gill) for both whole organisms and primary gill cells but has not yet been used to model metal complexation directly for gill cells from a continuous cell culture. Cell viability curves, using two fluorescence indicator dyes, Alamar Blue and CFDA-AM, were generated using both the RTgill-W1 and LSgill-e cell lines to determine what concentrations of copper (II) sulfate induces cell toxicity. These tests confirmed the titratable range for both cell lines in order to maintain viability was 0-300 μM in L-15, a specific culture media. For this study, graphite furnace atomic absorption spectroscopy (GFAAS) was used to directly characterize copper binding to both a rainbow trout gill cell line (RTgill-W1) and a lake sturgeon gill cell line (LSgill-e) in a monolayer, in suspension, and in cell culture inserts where copper titrations were performed at non-toxic concentrations. Both cell lines showed weaker binding than primary rainbow trout gill pavement cells and up to an order of magnitude less binding than primary rainbow trout gill mitochondrial rich cells. Results suggest that the interaction of copper with cells derived from a continuous cell line is much weaker than interactions with cells from a primary culture. Based on research performed, these results suggest the use of RTgill-W1, or LSgill-e cell lines would most likely not suffice as an alternative to whole organism and primary cell testing for viability and toxicity assays.

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List of Abbreviations and Symbols

BLM	Biotic Ligand Model
GFAAS	Graphite Furnace Atomic Absorption Spectroscopy
RTgill-W1	Rainbow trout gill cell line
LSgill-e	Lake sturgeon gill cell line
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
FBS	Fetal Bovine Serum
Pen-Strep	Penicillin-Streptomycin
Cu	Copper
PBS	Phosphate-buffered saline
NOM	Natural Organic Matter
L-15	Leibovitz's L-15 Medium
LOD	Limit of detection
CCC	Criterion Continuous Concentration

Table of Chemical Sources

Chemical	Source
L-15 Media	Leibovitz L-15 supplemented with L-glutamine (Millipore Sigma)
Gibco TrypLE Express trypsinizing solution	TrypLE™ Express Enzyme (1X), no phenol red (ThermoFisher Scientific)
Copper (II) sulfate	Copper (II) sulfate pentahydrate (Millipore Sigma CAS Number: 7758-99-8)
NaCl	Sodium Chloride (BDH CAS Number: 7647-14-5)
$CaCl_2$	Calcium chloride dihydrate (Fisher Chemical CAS Number: 10035-04-8)
Tissue culture grade water (L-15/ex)	Gibco™ Distilled Water (ThermoFisher Scientific CAS Number: 15230170)
KCl (L-15/ex)	Potassium chloride (Millipore Sigma CAS Number: 7447-40-7)
$MgSO_4$-$7H_2O$ (L-15/ex)	Magnesium sulfate heptahydrate (Millipore Sigma CAS Number: 10034-99-8)
$MgCl_2$-$6H_2O$ (L-15/ex)	Magnesium chloride hexahydrate (Millipore Sigma CAS Number: 13446-18-9)
Na_2PO_4 (L-15/ex)	Sodium phosphate dibasic (Millipore Sigma CAS Number: 7558-79-4)
KH_2PO_4 (L-15/ex)	Potassium biphthalate (J. T. Baker CAS Number: 877-24-7)
Galactose (L-15/ex)	D-(+)-Galactose (Millipore Sigma CAS Number: 59-23-4)
Pyruvate (L-15/ex)	Sodium pyruvate (Millipore Sigma CAS Number: 113-24-6)
Aluminum oxide (ISE)	Aluminum oxide powder (Millipore Sigma CAS Number: 1344-28-1)
Pd + $Mg(NO_3)_2$ (Matrix modifier)	Palladium and magnesium matrix modifier (Perkin Elmer)

1. Introduction

1.1 Copper in Aquatic Environments

Copper (Cu) is a trace metal element that occurs naturally at very low levels in many environments including soils, sediments, and waters (Flemming and Trevors 1989). However, it is important to note that this Cu occurs in water as Cu^{2+} and not elemental Cu. Due to human activities including mining, construction, agriculture and pesticide use, an increase in soil and water contamination from Cu is of particular concern for aerobic organisms (Panagos *et al*. 2018). In small quantities, Cu is essential for all aerobic organisms due to its role as a micronutrient (Clearwater et al. 2002; Grosell and Wood 2002). As a micronutrient, it proves to be essential for normal cellular metabolism, production of red blood cells and many other enzymatic reactions as a co-factor including its major role in the reaction catalyzed by mitochondrial cytochrome c oxidase (Clearwater et al. 2002; Grosell and Wood 2002). However, at increased concentrations Cu can prove to be toxic to many organisms, including fish (Ransberry et al. 2015). Due to the anthropogenic sources of Cu in freshwater systems mentioned previously, increased amounts of Cu exposure in these environments can be considered a major aquatic pollutant due to the potential risk it has on these aquatic organisms; thus, maintaining optimal levels is essential (Bopp et al. 2008).

The concentration of Cu in unpolluted freshwater environments ranges from <1-6 μg/L, however, even with concentrations ranging from 10-1000 μg/L effects of Cu toxicity are potentially not detectable in fish (Kamunde and Wood 2004). In some polluted environments, concentrations of Cu can range from <1 μg Cu/L to as high as 9000 μg Cu/L where normal biochemical activities in fish are affected at these high copper concentrations (Kamunde and

Wood 2004). In aquatic environments, Cu can be found either complexed by inorganic and/or organic ligands or suspended with particulate matter and can also be found in free ionic form where the most toxic forms are typically $CuOH^+$ and Cu^{2+} (Allen and Hansen 1996; Kamunde and Wood 2004). These waterborne metals then bind to fish gills serving as a route of Cu uptake due to the volume of water that is passed through fish gills, accumulation can also occur (Wood and Jackson 1980). For fish, the gill acts as the first point of contact where free Cu^{2+} drastically reduces and often inhibits Na^+/K^+-ATPase activity at the Mg^{2+} binding site (Playle 1998; De Boeck et al. 2004). This large reduction of Na^+/K^+-ATPase activity causes a downstream effect in the reduction of sodium uptake which often results in ionoregulatory failure and sometimes proves to be lethal (Playle 1998; De Boeck et al. 2004).

As previously mentioned, in aquatic environments, Cu can be found in many forms: complexed by inorganic and/or organic complexes, suspended with particulate matter and it can be found in the free ionic form with an oxidation state of +2 (Kamunde and Wood 2004). To begin with organic complexes, natural organic matter (NOM) is a complex mixture of organic substances that is very important in aquatic environments (Sillanpää et al. 2015). NOM contains a large amount of metal-binding groups that are considered weak such as phenolic and carboxyl groups, and a small amount of strong metal binding groups including nitrogen and sulfur binding sites (Richards et al. 2001). As was previously mentioned, free ionic metals are often considered to be the most toxic in aquatic environments and in the presence of NOM, the amount of free metal is drastically lowered (Richards et al. 2001). This decrease is caused by chelation of the free ionic metals to the NOM and thus, NOM reduces metal toxicity to aquatic organisms (Richards et al. 2001). Next, looking at inorganic complexes, the biggest difference between organic and inorganic substances is that generally, inorganic substances usually do not contain a

carbon atom, except bicarbonate, where organic substances contain many (Shallenberger 1997). Compared to organic complexes, inorganic complexes have a much higher penetrating capacity as NOM that has complexed with metal ions are unavailable for uptake by aquatic organisms (Moiseenko 2019).

However, along with metal complexation, Cu toxicity depends strongly on many factors including temperature, water chemistry, size, species and condition of the organism, and the season (De Boeck et al. 2004). Cu toxicity is directly related to the concentration of the free Cu^{2+} ion, and not the total Cu concentration (Allen and Hansen 1996). In order to predict this toxicity and accumulation on the gills of freshwater fish, the biotic ligand model (BLM) is often used. The BLM takes into account the environmental parameters previously mentioned including temperature, water chemistry, size, etcetera, to predict toxicity based on water chemistry (Grosell et al. 2007). Using the BLM, the amount of Cu accumulated in/on the gill of the fish can be predicted which is proportional to the toxic effects of Cu in freshwater environments, assessing the risk that Cu has in these environments (Grosell et al. 2007).

1.2 Biotic Ligand Model

The biotic ligand model is an equilibrium model based on logK values that is used to establish appropriate water quality criteria by taking into account many environmental parameters that affect metal toxicity in fish (Grosell et al. 2007). The basis of the BLM combines two models: the gill surface interaction model (Pagenkopf et al. 1974; Pagenkopf 1983) and the free ion activity model of toxicity (Campbell 1995) and is then combined into one widely used model which is widely known as the BLM (Di Toro et al. 2001). With these two models taken into account, the BLM is based on the idea that toxicity is not simply dependent on the total concentration of metal(s) in the aqueous environments but is also dependent on metal-ligand

complexation and the interaction of the metal with competing cations at the site of toxicity (Di Toro et al. 2001). A schematic illustration of the BLM can be seen in Figure 1.

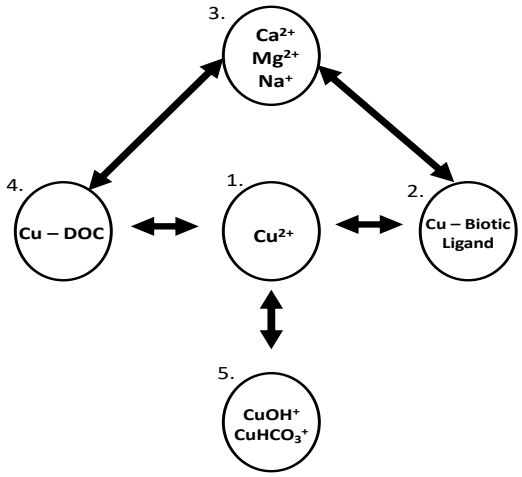


Figure 1. Schematic illustration of the biotic ligand model. **(**1) Free metal ion – copper for this research. (2) Site of action – the biotic ligand or the gill for this research. (3) Competing cations. (4) Complexation of copper with organic matter. (5) Complexation of copper with inorganic complexes. Figure adapted from (Di Toro et al. 2001).

The basis behind the BLM is that the accumulation of metal at the "biotic ligand" is directly proportional to toxicity (Smith et al. 2017). To predict this toxicity, the biotic ligand is most often thought of as the gill as it is both on the surface of the organism and it can interact with dissolved ions in the exposure water (Janes and Playle 1995; Smith et al. 2015). For fish gill research, the biotic ligands are often thought to be important uptake pathways for active ions which for Cu^{2+}, is an Na^{+} transporter (Niyogi and Wood 2004). The BLM is used to predict the amount, if any, of bound metal at the biotic ligand (2. in Fig. 1) where the amount of accumulation is related to the toxicological response (Paquin et al. 2002). For predicting this acute toxicity in freshwater organisms, the BLM takes into account the complexation of the metal to both organic and inorganic ligands (4. And 5. in Fig. 1), metal binding to the biotic ligand, and cations that compete with the metal for binding at the biotic ligand (Villavicencio et

al. 2005). The BLM predicts this acute metal toxicity relative to the free ion, Cu^{2+} based on the affinity of this metal to the biotic ligand, the gill (Antunes et al. 2006). How the free ion is measured and its influence on the BLM will be discussed further in sections 1.4 Ion Selective Electrode and 1.5 Graphite Furnace.

As the BLM has many input factors, water composition and characteristics have an impact on the toxic effects that metals may pose in aquatic environments. Water characteristics including pH, dissolved organic carbon, conductivity, and hardness all affect Cu toxicity on aquatic organisms (Park et al. 2018). Water chemistry affects the bioavailability of metals in aquatic environments, which is how BLM generates site-specific criteria (Paquin et al. 2000). For example, as mentioned previously, copper potentially exists in many complexes. In aquatic environments with high concentrations of natural organic matter (NOM), copper complexes, both organic and inorganic, will form which will account for the majority of copper in these waters (Paquin et al. 2000). This means that there will only be a small amount of free copper (Cu^{2+}), which is directly related to copper toxicity potentially resulting in less toxic effects on organisms in environments with more NOM (Paquin et al. 2000). Compared to aquatic environments with less NOM, more dissolved Cu may be existing as the free ion, Cu^{2+}, which could have more toxic effects on organisms in these environments.

Typically, to perform metal analysis in an experimental setting, living fish are exposed to the metal(s) of interest, generally 3 or 24 hours, and then the fish are sacrificed where the gill is used to perform metal analysis (Smith et al. 2017). Performing metal analysis on live fish comes with some disadvantages including having to very carefully control the water chemistry, having to work with a very diverse (size, and weight) group of fish having the reproducibility between animals very low, as well as having to estimate the concentration of free metal through

calculation and not direct measurements whereas metal complexation can be measured directly for gill cells from a continuous cell culture (Janes and Playle 1995; Smith *et al.* 2017). There are also ethical disadvantages as the fish must be sacrificed for analysis, as well as the high costs associated with animal testing which should all be considered. To overcome these challenges, *in vitro* models are available for fish gill cell lines in culture (Wood *et al.* 2002) which will be discussed further in section 1.3 Continuous Cell Lines. Previously, the BLM has been used to model metal complexation directly at the biotic ligand (*e.g.,* gill) for both whole organisms and primary gill cells but has not yet been used to model metal complexation directly for gill cells from a continuous cell culture.

1.3 Continuous Cell Lines

In previous research by Smith *et al.* (2017), an *in vitro* method involving reconstructed primary epithelia on cell culture inserts was performed, the media composition was controlled on both the apical (top) and basolateral (bottom) of the 'artificial gill' allowing the artificial gill to closely resemble an intact living fish (Smith *et al.* 2017). However, the primary gill cells used in the research by Smith *et al.* to characterize copper binding still requires fish to be sacrificed for the research. A potential alternative is continuous cell lines, eliminating the need to repeatedly sacrifice fish.

In vitro studies, specifically using immortal cell lines, has gained research interest in place of whole organism testing and more specifically, in place of primary gill cells (Kaur and Dufour 2012). Primary cell lines come directly from the tissue of interest on the organism (*e.g.* the cells from the gill of the fish), and can be maintained *in vitro* for a limited period of time, often maintaining the characteristics of the cells *in vivo* (Cruz-Tapias et al. 2013). On the other hand, cell lines, continuous cell lines or immortalized cell lines, are often used to replace the use

of primary gill cells. Continuous cell lines can theoretically live forever, having an indefinite lifespan making these cell lines immortal (Geraghty et al. 2014). Although cell lines offer many advantages which will be discussed later, there are some limitations that must be considered when working with these cell lines instead of primary cell cultures. An optimal cell line should function and maintain the same cellular features as that of primary cell cultures which can sometimes prove to be difficult, as primary cell lines are not always well characterized (Kaur and Dufour 2012). Also, continuous cell lines change over time as they are passaged and thus the responsiveness to stimuli, genotypic and phenotypic variation may have also occurred which needs to be taken into consideration (Kaur and Dufour 2012). However, using cell lines in place of whole organisms and primary gill cells does offer many advantages including the ease of use, cost effectiveness, providing a virtually unlimited supply of material and working with cell lines bypasses ethical concerns and controversies that may be associated with using primary human and/or animal tissues (Kaur and Dufour 2012). One major advantage that working with cell lines has, is that they provide a single sample with consistent, reproducible results (Kaur and Dufour 2012).

One specific cell line that is widely used in the study of fish health and toxicity is the RTgill-W1 cell line (Lee et al. 2009). The RTgill-W1 cell line was derived from the gill of the rainbow trout, *Oncorhynchus mykiss* and was established in Dr. Niels Bols' lab at the University of Waterloo (RTgill-W1 where the 'W1' is representing its establishment in Waterloo). This cell line is widely available making it ideal for many research projects. The RTgill-W1 cells have an epithelioid morphology and form tight monolayer sheets which can be grown in both tissue culture flasks or in cell culture inserts (transwell membranes) (Lee et al. 2009). This cell line is ideal for many toxicology studies due to its ability to withstand both hypo- and hyper-osmotic

environments and it is grown at room temperature making it a flexible model system for studying fish gill function, toxicity at the fish gill, and for further understanding the mechanism of toxicity at the gill of the fish (Lee et al. 2009). The RTgill-W1 cell line was one of two cell lines used in this research with the other cell line being LSgill-e.

Unlike the RTgill-W1 cell line, the LSgill-e cell line is novel and has not yet been used to model or study metal toxicity at the gill. The LSgill-e cell line was derived from the gill of the lake sturgeon, *Acipenser fulvescens* and was establish in Dr. DeWitte-Orr's lab at Wilfrid Laurier University. The LSgill-e cell line has an epithelial (-e) morphology where visually, the accumulation and secretion of substances can be observed (Vo et al. 2018). The secretion of these mucosal materials makes the media very viscous, a characteristic not shared with the RTgill-W1 cell line, making these great cell lines to compare and contrast.

As previously mentioned, the LSgill-e cell line is novel and therefore, there is a lot to learn about this particular cell line. Due to this, there is no literature evidence as to whether the LSgill-e mucosal materials bind metals. However, it was found in other fish species such as the cap (*Cyprinus carpio*), mucus copper complexes were dominant in the gill environment with a pH range of 6-9 (Tao et al. 2001), evidence that mucus secreted by fish can bind copper. For this specific fish species, it was determined that bioavailable copper species were much lower due to mucus complexation and pH change to acidic conditions (Tao et al. 2001). It was also found that in rainbow trout, acid exposure with a pH below 3.5, mucous production is significantly enhanced however in these acidic conditions, the ability of this mucus to bind copper was abolished (Evans 1987).

In this study, RTgill-W1 and LSgill-e were cultured using the three methods: (1) monolayer, (2) suspension, and (3) cell culture insert. Cells in a monolayer are grown on the

bottom of cell culture flasks in a two-dimensional layer where exposure to media and potential toxin occurs from the top. Cells in suspension are collected from cell monolayers with the use of trypsin to produce a single-cell suspension in media, where it is this cell suspension that is treated with the potential toxin. This is the method that least resembles the gill of an intact living fish. Lastly, most closely resembling the gill of an intact fish are cell culture inserts. For this method, cells are grown in a trans-well insert to form an epithelial layer with both apical and basolateral orientation. Access to both the apical (top) and basolateral (bottom) of the cells is controlled, where the toxin can be added to the top of the cells which represents the outside of the gill and then the bottom chamber is analogous to the blood of the fish. In this study, after the cell lines were in culture (monolayer, suspension, or insert) two main experiments were performed: assessing cell viability when exposed to a potential toxin (*i.e.,* copper) and/or measuring free copper when exposed to a potential toxin (*ie.* copper) for application in the BLM.

1.3.1 Viability Indicator Dyes

One method used to determine cellular viability for both the RTgill-W1 and LSgill-e cell lines after exposure to potential toxins in culture (monolayer, suspension, and insert) is using two fluorescent indicator dyes: alamar blue and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM). Alamar blue and CFDA-AM are noninvasive, nontoxic, simple to use, and directly applicable to the cell lines being used throughout this study, RTgill-W1 and LSgill-e (Schreer et al. 2005). Alamar blue provides a measure of cellular metabolic activity when the nonfluorescent alamar blue dye is reduced to a pink colour that is fluorescent which occurs in the media due to cellular activity (O'Brien et al. 2000; Schreer et al. 2005). CFDA-AM provides a measure of cell membrane integrity where it can be converted from a nonfluorescent substance to a fluorescent dye by the cells indicating the integrity of the membrane as only an intact membrane can support

the esterase activity required for the conversion (Bopp and Lettieri 2008). The use of both of these fluorescent dyes together on the same set of cells, effectively measures cell line viability assays for fish with similar incubation times, and they can be detected at different wavelengths with no interferences (Schreer et al. 2005; Bopp and Lettieri 2008).

Once cell viability curves were established for copper in RTgill-W1 and LSgill-e, measurement of free and bound copper could be determined. As mentioned previously, the BLM can be used to characterize copper binding directly to the gill cells. This has previously been done for whole fish and primary gill cells but has yet to be done for fish gill cells from a continuous cell line. If continuous cell lines were to show the same or similar binding to that of primary gill cells, there is huge potential for animal free exposure testing.

1.4 Ion Selective Electrode

As mentioned previously in section 1.2 Biotic Ligand Model, the BLM helps to establish appropriate, and protective water quality criteria (Park et al. 2018). In aquatic environments, metal complexation is essential because the formation of inorganic and organic complexes makes a large fraction of the total metal in these aquatic environments nonbioavailable (Di Toro et al. 2001). However, as was depicted in Figure 1, in solution some of the metal exists as the free metal ion which in this case, as Cu^{2+} where this is most often thought of to be the most bioavailable species (Di Toro et al. 2001). The bioavailable species is important to determine as the amount of bioavailable metal in the aquatic environment is the amount of metal that is free or available to reach the toxic site of action (the gill of the fish) (Adams et al. 2020). One analytical method often used to determine the concentration of bioavailable metal is the ion selective electrode (ISE) (Smith et al. 2017).

An ion selective electrode is a type of electrode that is often used in titrimetry for the determination of logK values, binding affinities, for different surfaces including fish gill cell lines (Smith et al. 2017). Working with ISE's has many advantages including the relative simplicity and ease of use when working with the ISE in which the activity of a single metal ion, in this case Cu^{2+}, is determined from the measured potential, in mV (Luider et al. 2004). An ISE is immersed into an aqueous solution containing the free ion that is to be measured, along with an external reference electrode with the circuit being completed with the potential being measured on a potentiometer (Basics 2016). A potential difference occurs across the ISE membrane (Figure 2) when the free ions in the aqueous sample solution diffuse from the area of high ion concentration to the side with the lower ion concentration, where this potential is then read on the potentiometer as the output potential (Basics 2016). When the sample solution is at equilibrium, the potential is dependent upon the concentration of the free ion outside the membrane, where the electrode potential is linearly related to the logarithm of the activity of the free metal ion according to the Nernst equation (Smith et al. 2017).

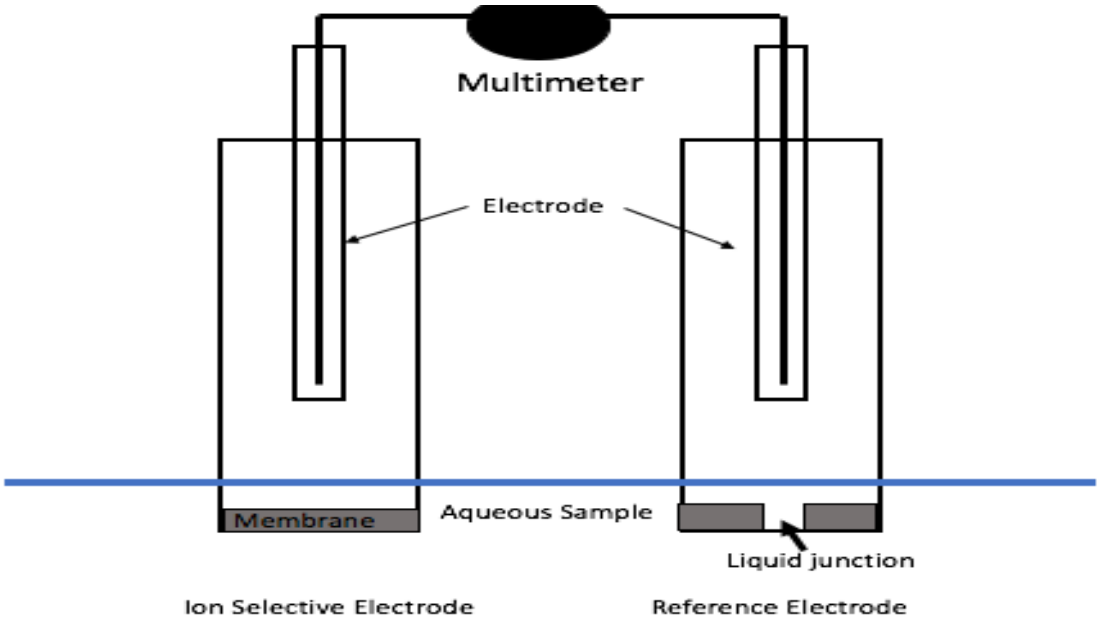


Figure 2. Schematic illustration of an ion selective electrode and reference electrode in an aqueous sample solution. Figure adapted from Dr. Wojciech Wroblewski at CSRG, University of Warsaw, Poland (Wroblewski 2005).

The output response from the ISE is a direct measure of the free ion concentration which is thought to be directly proportional to bioavailability (Luider et al. 2004; Smith et al. 2017). In order to model the amount of free ion in the sample solution using the Nernst equation, a one point internal calibration procedure is completed in order to determine the slope of the Nernst equation, $E=E^{o}+m\log[Cu^{2+}]$ where a Nernstian slope of 29.6 is assumed (Smith et al. 2017). From here, the concentration of the free metal ion can be calculated, as well as the complexed copper can also be determined indirectly by subtracting the calculated free ionic copper concentration from the total dissolved copper concentration (Luider et al. 2004). Although ISE's are a great method for determining the concentration of the free metal ion in solution, there are a few limitations. Some of these limitations include a possible lack of sensitivity in systems with very low free ion concentrations, and the possibility of electrode interferences (Amacher 1984). This insufficient buffering is the main limitation as strong binding is required for the electrode to work well. Due to these possible limitations when using an ISE, there is another analytical method that can be used, and was used in this research, to measure the concentration of free metal ions in solution: the graphite furnace.

1.5 Graphite Furnace

Graphite furnace atomic absorption spectroscopy (GFAAS) is a type of spectrometry that atomizes the sample of interest, giving an output absorbance signal that is characteristic to the metal being analyzed in which the quantitative amount of the element can be determined (Holcombe and Borges 2010). Using the graphite furnace for determining free metals concentrations in samples offers many advantages including that only small sample sizes are

required (~25 μL), it is more sensitive and has a much lower limit of detection than flame-based methods which makes it a great tool for determining trace elements in samples with reproducible results (Sperling 2006).

A graphite furnace usually consists of three main steps: a drying stage, an ashing stage and an atomization stage (Larue and Tyson 1993). In the first step, the drying stage, after the sample has been acidified, loaded and is ready for analysis, the sample is drawn up by the graphite furnace automatically where it is then evaporated at a low temperature (between 80-200 ºC) (Larue and Tyson 1993). The temperature of this stage is chosen specifically for each sample so that the solvent is evaporated as fast as possible from the sample without causing any loss of sample through spattering in the graphite tube (Larue and Tyson 1993). In the second step, ashing, temperatures are much higher than the first step (between 350-1600 ºC) in order to remove both organic and inorganic material from the sample (Larue and Tyson 1993). In the third step, atomization, the graphite furnace reaches the highest temperatures (between 1800-3000 ºC) where the sample is atomized and free analyte atoms are generated in the light path (Larue and Tyson 1993). After the atomization of the sample of interest in the graphite furnace, the atoms absorb the light and then output absorbance values to the detector. With the output absorbance values and the Beer-Lambert law (A=εcl) concentration values can be determined from a calibration curve created with standards of known metal concentrations.

When using a graphite furnace for analyzing samples with metals, it is important to take into account the stability of the analyte and the volatility of the sample matrix. A matrix modifier can be added in excess to the sample, as it converts the analyte element to a higher phase so that it is much more thermostable and to increase the volatility of the sample (Schlemmer and Welz 1986). With the addition of a matrix modifier to the sample, very high temperatures are able to

be reached to remove the bulk of the contaminants without losing any amount of sample analyte before it reaches the atomization stage (Schlemmer and Welz 1986). Another benefit of adding a matrix modifier to a sample before analysis in a graphite furnace is its ability to greatly reduce sample inferences and scattering (Schlemmer and Welz 1986). Throughout the graphite furnace portion of this research, a matrix modifier, specifically for copper, was used for the above-mentioned reasons. The matrix modifier used, as recommend by Perkin Elmer for graphite furnace atomic absorption with copper was Pd + $Mg(NO_3)_2$.

1.6 Research Objectives and Goals

The overall objective of my research is to test the feasibility of continuous cell lines for Cu binding instead of primary gill cell testing and/or whole fish testing.

The objectives and goals of this research are broken down into two main components:

1. To determine the effects of copper exposure, cell density, and media composition on the viability of both RTgill-W1 and LSgill-e continuous cell lines. This was done in order to investigate the range of copper in which the cells from the cell lines remain viable.
2. To measure copper binding to RTgill-W1 and LSgill-e cells in monolayer, in suspension, or in insert using graphite furnace analysis.

1.7 Hypothesis

1. Continuous cell lines will be more resistant to copper toxicity than primary fish gill cells.
    a. Cell lines are immortal and can be grown indefinitely. Due to their immortal nature, cell lines are often different from their primary counterparts (Kaur and Dufour 2012). As a result of these genetic differences, downstream affects may be observed such as altered cellular functions, responsiveness to stimuli, and

phenotype (Kaur and Dufour 2012). Also, as a result of continual passaging for the upkeep and maintenance of cell lines, variation in genotype and phenotype can occur (Kaur and Dufour 2012). As serial passaging can alter a cell line and the responsiveness to stimuli, it is believed that the RTgill-W1 cell line will be more resistant to copper toxicity than primary rainbow trout gill cells.

2. LSgill-e will be more resistant to copper toxicity than RTgill-W1 cells.
    a. Unlike the RTgill-W1 cell line, the LSgill-e cell line accumulates and secretes substances that can visually be observed (Vo et al. 2018). The secretion of mucosal materials makes the media very viscous, unlike the very fluid media observed with the RTgill-W1 cell line. It is hypothesized that the presence of this mucus-like substance secreted by LSgill-e cell line will act as a barrier, providing some protection to the cells when exposed to toxic concentrations of copper.
3. With both RTgill-W1 and LSgill-e cells, a higher cell density will result in lower cellular toxicity than with a lower cell density.
    a. It has been shown that with lower cell densities, cell toxicity has been said to increase and in contrast, it was also determined that copper toxicity decreased as the number of cells in culture increased (Franklin et al. 2002). It is hypothesized that with a higher cell density, decreased toxicity will be observed as the concentration of toxicant per cell would be lower as well as more cells can metabolize more toxicant than less cells.
4. Copper binding will be greater for LSgill-e cells in a monolayer, suspension, and insert than RTgill-W1 cells.

a. As previously mentioned in hypothesis (2) above, unlike the RTgill-W1 cell line, the LSgill-e cell line accumulates and secretes mucosal materials that makes the resulting media very viscous (Vo et al. 2018). It is hypothesized that due to the secretion of mucous from the LSgill-e cell line, it will aid in binding copper due to the consistency. It is thought that this mucous-like substance will in addition to the larger cell size in LSgill-e than RTgill-W1, will be able to bind more copper in the vicinity of the cell in comparison to the RTgill-W1 cells.

5. The greatest amount of copper binding for both cell lines will be observed in the cell culture insert.
    a. In this study, three different methods for researching copper binding to two different cell lines was examined: suspension, monolayer, and insert. From suspension, to monolayer, to insert, the cells in culture become increasingly more 'gill-like' in the insert than compared to that of cells in suspension and in monolayer. Thus, it is hypothesized that when cells are plated in a way making them more 'gill-like' more copper binding will be observed than when cells are in suspension and in monolayer (Smith et al. 2017).

1.8 Significance of Research

The Canadian Council on Animal Care is a Canadian organization that is responsible for maintaining, setting, developing and overseeing standards for animal care and ethics in Science in Canada (Brunt 2017). In 2016, more than 4.3 million animals were used for scientific research purposes where 37.2%, over 1.6 million of these animals were fish, making them the most utilized animal for research (Brunt 2017). It is important to note that not all procedures are harmful to the animal with more than 38% consisting of animal breeding and roughly about 6%

that were euthanized for regulatory tests and animal testing (Brunt 2017). However, in today's society, the use of animals for research purposes is becoming much more controversial and finding alternatives to this testing, especially for fish, is very important.

Commonly, the BLM has been used to model metal complexation directly at the biotic ligand (*e.g.,* gill) for whole organisms by exposing living fish to various metals for a designated length of time and then the fish is harvested for the gill to perform metal analysis, sacrificing the fish (Playle et al. 1993; Macrae et al. 1999). The BLM has also been used to characterize copper binding to primary rainbow trout gill cells, as demonstrated in Smith *et al.* (2017). However, to obtain these primary gill cells from the fish, fish still need to be sacrificed. To date, the biotic ligand model has not yet been used to model metal complexation directly for gill cells from a continuous cell culture. The use of continuous cell lines could provide a powerful approach, combatting the use of intact living fish and primary gill cells for regulatory animal testing and ultimately, providing an alternative to this testing.

2. Materials and Methods

2.1 Gill Cell Line Growth and Maintenance

The rainbow trout gill cell line developed from the rainbow trout (*Oncorhynchus mykiss)* was obtained from Dr. Niels Bols (University of Waterloo) as a very generous gift. The lake sturgeon gill cell line developed from the lake sturgeon (*Acipenser fulvescens)* was obtained from the Dr. Stephanie DeWitte-Orr lab at Wilfrid Laurier University where it was developed (Vo et al. 2018). Both the rainbow trout and lake sturgeon gill cell lines were grown in 75cm^2 Falcon cell culture flasks at room temperature in L-15 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep). L-15 media is a type of cell

culture media that is specifically designed to support cell growth in environments without CO_2 (Bols et al. 1994). FBS is added to the culture media as it contains components that are essential for cell proliferation and maintenance as well as stimulates cellular growth in cell cultures (van der Valk et al. 2018) and pen-strep is added to prevent cell contamination (Ryu et al. 2017).

Throughout this study, both gill cell lines were prepared in three different ways depending on the experiment: monolayer, suspension, and insert. For all three methods, the procedures started with either the RTgill-W1 cell line or the LSgill-e cell line grown to confluency (~7 days) in 75 cm^2 Falcon Cell Culture Flasks, at room temperature, 20 °C, before being used for each respective experiment.

2.2 Cell Viability Assays

Cell viability assays were performed using both cell lines, RTgill-W1 and LSgill-e using different media compositions as well as varying seedings densities. Below in Table 1, a list of all experiments conducted examining cell viability and toxicity can be found.

Table 1. Cell viability experiments performed for both the RTgill-W1 and LSgill-e cell lines.

Figure Number	Cell Line	Experimental Parameters
Figure 3.	RTgill-W1	Media: L-15 Cell Density: 20,000 cells/well $CuSO_4$ Concentration Range: 10 μM – 975 μM
Figure 7.	LSgill-e	Media: L-15 Cell Density: 12,500 cells/well $CuSO_4$ Concentration Range: 5 μM – 500 μM

Figure 8 & 9.	RTgill-W1	Media: L-15 Cell Density: 10,000 cells/well – 25,000 cells/well (four densities examined) $CuSO_4$ Concentration Range: 1 μM – 200 μM
Figure 10.	RTgill-W1	Media: Titration Media Cell Density: 20,000 cells/well $CuSO_4$ Concentration Range: None
Figure 11.	RTgill-W1	Media: Titration Media Cell Density: 20,000 cells/well $CuSO_4$ Concentration Range: 1 μM – 200 μM
Figure 11.	LSgill-e	Media: Titration Media Cell Density: 12,500 cells/well $CuSO_4$ Concentration Range: 1 μM – 200 μM

2.2.1 RTgill-W1 – Copper Toxicity in L-15 Media

Once confluent (typically 5-7 days), the RTgill-W1 cells from the 75cm^2 Falcon cell culture flasks were detached from the cell culture flask with a trypsinizing solution (Gibco TrypLE Express trypsinizing solution), typically 3-7 minutes depending on cell confluency at the time of detachment. Cells were then centrifuged, and the pellet of cells was resuspended in ~1 mL of L-15 cell culture media supplement with 10% FBS and 1% penstrep and then counted using the Countess II FL Automated Cell Counter using trypan blue. Once counted, the RTgill-W1 cells were resuspended in L-15 media to a final cell density of 20,000 cells/well with a final well volume of 100 μL/well in a 96-well plate. Once plated, the cells were left to attach to the cell culture plate overnight at 20°C. The following day, copper (II) sulfate was added ranging in

concentrations from 10 μM - 975 μM in L-15 media with a no copper control and left to incubate for 24 hours at room temperature. After the incubation with copper, the copper and media were then removed, cells were then rinsed with phosphate-buffered saline (PBS) and cell viability was then determined using two fluorescent indicator dyes, alamar blue and CFDA-AM. A solution of 10 mL PBS, 10 μL CFDA-AM (from a 4 mM stock solution) and 500 μL alamar blue (AlamarBlue™ Cell Viability Reagent from Invitrogen by Thermo Fisher Scientific) was made. Each culture well in the 96-well plate received 100 μL of this solution. After a one-hour incubation period, the wells were read on a fluorometric multiwell plate reader and cell viability was analyzed as a percentage of the control. The fluorescence spectrophotometer was set up and the plate was read using 560/590 nm (excitation/emission) settings. The way that alamar blue works is it quantitatively measures metabolic activity of cells in any given sample by measuring cellular proliferation. When alamar blue is used, healthy, growing cells in culture cause a chemical reduction of the dye from a non-fluorescent blue to a fluorescent red (Rampersad 2012). When cells are continuously growing, the viable cells maintain the fluorescent red, however when cell growth is inhibited an oxidized environment is maintained keeping the alamar blue its non-fluorescent blue colour (Thermo Scientific 2012).

2.2.2 LSgill-e – Copper Toxicity in L-15 Media

Performing the viability toxicity assay for the LSgill-e cell line followed the same procedure in which was used for the RTgill-W1 cell line outlined above in section 2.2.1. However, the cells were plated at a density of 12,500 cells/well. The cell seeding density was based off of previous work (Vo et al. 2018). For the LSgill-e cell line, copper (II) sulfate was added ranging in concentrations from 5 μM – 500 μM with L-15 media with a no copper control.

Incubation time and alamar blue and CFDA-AM procedure was completed the exact same as with the RTgill-W1 cell line in section 2.2.1.

2.2.3 RTgill-W1 – Copper Toxicity in L-15 Media with Varying Cell Density

Next, the effect of cell seeding density on copper toxicity was examined for the RTgill-W1 cell line when exposed to various concentrations of copper (II) sulfate. For this experiment the following cell densities were examined: 10,000 cells/well, 15,000 cells/well, 20,000 cells/well and 25,000 cells/well with a final well volume of 100 µL/well in L-15 growth media. Once plated, the cells were left to attach to the cell culture plate overnight at 20°C. The following day, copper (II) sulfate was added ranging in concentration from 1 µM – 200 µM with L-15 media with a no copper control and left to incubate for 24 hours at room temperature. After the incubation with copper, cell viability was determined using alamar blue and CFDA-AM, as described above.

2.2.4 RTgill-W1 – Toxicity in Titration Media without Copper

Titration media, a closer resemblance to that of freshwater in comparison to L-15 media, was made using the same concentrations as outlined in Smith *et al.* (2017). The solution composition was 150 mM NaCl and 2 mM $CaCl_2$ in ultrapure water (18.2 MΩ resistance, Milli-Q A-10 Synthesis Water System). For this experiment, once confluent, (typically 5-7 days) the RTgill-W1 cells from the 75cm^2 Falcon cell culture flasks were detached from the cell culture flask with a trypsinizing solution (Gibco TrypLE Express trypsinizing solution), typically 3-7 minutes depending on cell confluency at the time of detachment. Cells were then centrifuged, and the pellet of cells was then resuspended in ~1 mL of L-15 media and then counted using the Countess II FL Automated Cell Counter using trypan blue. Once counted, the RTgill-W1 cells

were resuspended in L-15 media for a final cell density of 20,000 cells/well and left to attach overnight at 20°C. The following day, cells were rinsed with the titration media and 100 μL of titration media was added to each well. Viability was then measured using two fluorescent indicator dyes, alamar blue and CFDA-AM, as described above, at six different time intervals: 2h, 4h, 6h, 11h, and 24hrs.

2.2.5 RTgill-W1 – Copper Toxicity in Titration Media

The RTgill-W1 cells were prepared for experiment as described above. Once counted, the RTgill-W1 cells were resuspended in L-15 media for a final cell density of 20,000 cells/well in L-15 media with a final volume of 100 μL/well. Once plated, cells were left to attached overnight at 20°C. The following day, the cells were rinsed with titration media (150 mM NaCl and 2 mM $CaCl_2$) and copper (II) sulfate was added ranging in concentrations from 1.0 μM – 200 μM in titration media with a no copper control and left to incubate for 24 hours at room temperature. After the incubation with copper, the copper and media were then removed, cells were then rinsed with PBS and cell viability was then determined using two fluorescent indicator dyes, alamar blue and CFDA-AM, as described above.

2.2.6 LSgill-e – Copper Toxicity in Titration Media

Performing the toxicity assay with titration media for the LSgill-e cell line followed the same procedure in which was used for the RTgill-W1 cell line outlined above in section 2.2.5. However, the cells were plated at a density of 12,500 cells/well. For the LSgill-e cell line, copper (II) sulfate was added ranging in concentrations from 1.0 μM – 200 μM with titration media and a no copper control. Incubation time and the alamar blue and CFDA-AM procedure was completed the exact same as with the RTgill-W1 cell line in section 2.2.5.

2.2.7 Statistical Analysis and Modelling

After performing toxicity assays, statistical analysis was performed to determine what concentrations of copper (II) sulfate were significantly different from the control. For this analysis, GraphPad Prism™ Version 9.0.0 was used. After the data was inputted in GraphPad, the data for each viability experiment was analyzed to check for Normal (Gaussian) distribution using the Shapiro-Wilk normality test. If the data was not normally distributed, the data was transformed until it was normally distributed after checking once again with the Shapiro-Wilk normality test. Once the data was normally distributed, a one-way ANOVA with a Dunnett's post-test was used to determine the significance of each concentration of copper (II) sulfate compared to the control.

As well as performing statistical analysis for the cell viability assays, Winward BLM was used to determine effect concentration for the conditions used throughout the experiments. When using Winward BLM, toxicity prediction mode was used with Cu as the metal in Freshwater. The ion ratios used were the default North American median values for freshwater with Ca:Mg 1.98, Ca:Na 1.57, Ca:K 11.47, SO_4:Cl 1.95 and $pCO_2 = 10^{-3.2}$. The temperature was set at 20.00℃, pH at 7.7, hardness at 90.00mg/L $CaCO_3$ as is present in Lake Ontario (Dobson 1984) and DOC at 3.88 mg C/L as per the predicted average of the DOC concentration globally in lake water (Toming et al. 2020). For each viability experiment that was performed, the criterion continuous concentration (CCC) was determined. This is an estimate of the highest concentration of copper that could be present under these specific conditions in which the organism could be exposed to forever resulting in no toxic effects.

2.3 Ion Selective Electrode Titrations

For all titrations, acid-washed glass and plastic was used. For acid-washing, glassware and plastic was soaked for 24 hours in 10% trace metal grade nitric acid and then washed extensively with ultrapure water (18.2 MΩ resistance, Milli-Q A-10 Synthesis Water System). All titrations were completed at room temperature. Both cell lines, RTgill-W1 and LSgill-e were grown to confluency in 75cm^2 Falcon cell culture flasks prior to titration and then were detached from the cell culture flask with a trypsinizing solution (Gibco TrypLE Express trypsinizing solution), typically 3-7 minutes depending on cell confluency at the time of detachment. This was completed on the day of the titration. For cell seeding densities, media composition and volumes see section 3.2 where it is discussed for each specific experiment performed as they varied from experiment to experiment.

Before titrations were started, the cupric ISE (Orion model 94-29, Boston, MA) was polished to a mirror-like shine finish using aluminum oxide powder. A reference electrode (double junction Ag/AgCl Model 900200) was used as a reference and the potential was observed on a Tanager potentiometer (model 9501). It is said that the detection limit is restricted by electrode dissolution and the associated saturated of the diffusion layers on the electrode prevents the electrode to measure samples with total Cu levels below 10^{-6}mol dm^{-3} (Zirino et al. 2002). However, in the presence of strong binding ligands, cupric concentrations can get to total Cu levels as low as 10^{-19}mol dm^{-3} (Avdeef et al. 1983; Marcinek et al. 2021).

For all titrations completed, the same principle method was used. Titrant (copper (II) sulfate) was added and the electrode potential was monitored closely until stabilization. The measurement was said to be stable of there was no random drift of more than 0.1mV/min for at least 5 minutes (Smith et al. 2017). The time it took to equilibrate varied from 15 minutes – 2

hours after each single addition of titrant. Throughout the titration from start to finish, the pH was monitored to maintain a pH at 7.70 $\pm$0.03 (Combination pH Electrode, Epoxy Body, Thermo Fisher Scientific) and adjusted using nitric acid and sodium hydroxide and finally, following the final addition of titrant, the pH of the sample was acidified below a pH of 4.5 and once stable, the mV value was recorded and the titration was complete. The final acidified pH value was used as a calibration for each individual titration for the electrode being used. For the titrations, two copper stock solutions were prepared using copper (II) sulfate: 100 mM and 10 mM and stock solution used was dependent upon the total copper concentration that needed to be achieved for each individual titration. The concentration of copper increased throughout the titration with copper being added in steps throughout the duration of the titration.

Following the titration, an internal calibration procedure was used in order to determine the intercept of the Nernst equation as outlined in Smith *et al.* (2017). For this calibration method, for Cu^{2+}, a Nernstian slope of 29.6 was assumed (Smith et al. 2017). This method of internal standard calibration was developed by Tait *et al.* and has showed great reproducibility and corrects for any sample-specific bias (Tait et al. 2016; Smith et al. 2017). The final acidified sample, with the pH below 4.5, the total copper concentration is assumed to be equal to the free copper concentration (Smith et al. 2017). The free copper concentration in all samples was calculated following the equation as outlined below in the equations. Nernst equation for calculating the free Cu^{2+} calculation using the data from an ISE. Where E_{acid} represents the potential of the acidified sample, E° represents the standard potential, and $[Cu^{2+}]$ is the total concentration of copper added to the sample at the time of acidification (Equation 2) and $[Cu^{2+}]$ is the total amount of free copper present in the sample at each copper addition, correlated to its potential.

$$E_{acid} = E° + mlog[Cu^{2+}] \quad (1)$$

$$E° = E_{acid} - 29.6\log\,[Cu^{2+}] \quad (2)$$

$$logCu^{2+} = \frac{E-E^{°}}{29.6} \quad (3)$$

2.4 Graphite Furnace

For all experiments performed using the graphite furnace, the same principle method was used, however the cells were prepared in one of three ways depending on the experiment: in a monolayer, in suspension, or using a cell culture insert. All graphite furnace experiments were completed at room temperature. For all the methods of cell preparation mentioned below, both cell lines, RTgill-W1 and LSgill-e were grown to confluency 75cm^2 Falcon cell culture flasks in L-15 media supplemented with 10% FBS and 1% pen-strep prior to analysis and then were detached from the cell culture flask with a trypsinizing solution (Gibco TrypLE Express trypsinizing solution), typically 3-7 minutes depending on cell confluency at the time of detachment. After detachment, cells were then centrifuged and the pellet of cells was resuspended in ~ 1 mL of media (titration media or L-15 depending on the experiment, this is discussed in further detail for each experiment in section 3.3) and then counted using the Countess II FL Automated Cell Counter using trypan blue. From here, the cells were prepared for experiment either in a monolayer (section 2.4.1), in suspension (section 2.4.2) or in cell culture inserts (section 2.4.3). For all experiments analyzed using the graphite furnace, prior to analysis of samples, samples of known copper (II) sulfate concentrations were run in order to produce calibration curves to be able to determine free copper concentrations after sample analysis. The graphite furnace that was used for this research was the PinAAcle 900 Atomic

Absorption Spectrometer in Graphite Furnace Mode with a detection limit of 0.014 $\mu g/L$ determined using elemental standards with very dilute aqueous solutions with a 98% confidence interval (PerkinElmer 2011).

2.4.1 Monolayer

For cells in a monolayer, the day prior to analysis, the cells were plated in individual T-25 cell culture flasks at the appropriate cell density, depending on the experiment which will be discussed below, in L-15 media supplemented with 10% FBS and 1% penstrep. The cells (either RTgill-W1 or LSgill-e) were left to attach overnight at room temperature. The following day, the L-15 media was removed from the flask and the cells were washed thoroughly with titration media. To each individual cell culture flask, different concentrations of copper (II) sulfate were added and then left to incubate at room temperature for three hours. Specific volumes, cell seeding densities and copper concentrations can be seen below (Table 2) as it is unique for each individual monolayer experiment and cell line that was performed. Following the three-hour incubation period, the titration media was removed from the cell culture flasks and placed into 1.5 mL microcentrifuge tubes where each sample was acidified to 0.2% using nitric acid. For the RTgill-W1 cell line, cells were plated at a density of 340,000 cells/well with 88% live cells and 12% dead, in L-15 media the day prior to analysis using the graphite furnace. The following day, the L-15 media was removed, and the cells were thoroughly rinsed with titration media, prior to being exposed to copper (II) sulfate concentrations of 2 μM, 4 μM, and 6 μM in titration media for an incubation period of three hours. Following incubation, the media was taken from the RTgill-W1 cells and acidified to 0.2% using nitric acid and the samples were then ready for analysis. Once samples were acidified, they were loaded into the graphite furnace and were then ready for analysis. For analysis of each individual sample, a Pd + $Mg(NO_3)_2$ matrix

modifier was used. For the matrix modifier, 5 µL of the working solution (0.1% Pd + 0.06% $Mg(NO_3)_2$) (PerkinElmer) was automatically added to each 10 µL sample before being injected into the graphite furnace. After standard and sample analysis was completed, the output absorbance values of the standards were used to create a Beer's Law Plot and this line of best fit was then used to calculate the free copper concentrations of the samples from the absorbance value that was determined from the graphite furnace.

Table 2. Graphite furnace monolayer experiments performed for both the RTgill-W1 and LSgill-e cell lines.

Figure Number	Cell Line	Experimental Parameters
Figure 17.	Calibration Curve (No Cells)	Media: Titration Media Cell Density: N/A $CuSO_4$ Concentration Range: 1 µM – 8 µM
Figure 18.	RTgill-W1	Media: Titration Media Cell Density: 340,000 cells/well $CuSO_4$ Concentration Range: 2 µM, 4 µM, 6 µM
Figure 19.	Calibration Curve (No Cells)	Media: Titration Media Cell Density: N/A $CuSO_4$ Concentration Range: 1 µM – 6 µM
Figure 20.	LSgill-e	Media: Titration Media Cell Density: 340,000 cells/well $CuSO_4$ Concentration Range: 2 µM, 4 µM
Figure 21.	Calibration Curve (No Cells)	Media: Titration Media Cell Density: N/A $CuSO_4$ Concentration Range: 1 µM – 15 µM

Figure 22.	RTgill-W1	Media: Titration Media Cell Density: 493,000 cells/well $CuSO_4$ Concentration Range: 1 μM – 17.5 μM
Figure 23.	LSgill-e	Media: Titration Media Cell Density: 317,000 cells/well $CuSO_4$ Concentration Range: 1 μM – 17.5 μM

2.4.2 Suspension

For cells in suspension, the day of analysis, after the cells had been detached from the 75cm^2 Falcon cell culture flasks, the cells were suspended at the appropriate cell density in titration media depending on the experiment and the cell line being used (Table 3). The cells were each resuspended in titration media in 1.00 mL volumes in Falcon tubes. To each individual Falcon tube, different concentrations of copper (II) sulfate were added and then left to incubate at room temperature on an orbital rocker for the cells to remain in suspension, for three hours. For the RTgill-W1 cell line, cells were detached from the cell culture plate the day of the experiment, counted and were then seeded at a density of 500,000 cells/mL with 91% live and 10% dead, suspended in titration media. The cells were kept in suspension by remaining on an orbital rocker throughout the duration of the incubation period. The cells in suspension were exposed to copper (II) sulfate concentrations from 1 μM – 17.5 μM in titration media for an incubation of three hours on an orbital rocker. Following incubation, the cells were filtered out using a 0.45 μm syringe filter and the sample with no cells was acidified to 0.2% nitric acid and were then ready for analysis. Following the three-hour incubation period, the cells were filtered out using a 0.45 μm syringe filter and the titration media, now with no cells, was acidified to

0.2% using nitric acid. Once samples were acidified, they were loaded into the graphite furnace sample tray and were then ready for analysis. For analysis of each individual sample, a Pd + $Mg(NO_3)_2$ matrix modifier was used, as described above (section 2.4.1). For the LSgill-e cell line, cells were detached from the cell culture plate the day of the experiment, counted and were then seeded at a density of 400,000 cells/mL with 88% live and 12% dead, suspended in titration media. The cells were kept in suspension by remaining on an orbital rocker throughout the duration of the incubation period. The cells in suspension were exposed to copper (II) sulfate concentrations from 1 µM – 15 µM in titration media for an incubation period of three hours on an orbital rocker. Following the incubation, the cells were filtered out using a 0.45 µm syringe filter and the sample with no cells was acidified to 0.2% with nitric acid and the samples were then ready for analysis.

Table 3. Graphite furnace suspension experiments performed for both the RTgill-W1 and LSgill-e cell lines.

Figure Number	Cell Line	Experimental Parameters
Figure 25.	Calibration Curve (No Cells)	Media: Titration Media Cell Density: N/A $CuSO_4$ Concentration Range: 1 µM – 15 µM
Figure 26.	RTgill-W1	Media: Titration Media Cell Density: 500,000 cells/well $CuSO_4$ Concentration Range: 1 µM – 17.5 µM
Figure 27.	LSgill-e	Media: Titration Media Cell Density: 400,000 cells/well $CuSO_4$ Concentration Range: 1 µM – 15 µM

2.4.3 Cell Culture Insert

For cells in cell culture inserts, the media composition, cell densities, and timelines can be found outlined below in Table 4 as these variables changed as the experiment progressed. On the first day when plating the cells in the cell culture inserts, the cells were detached from the 75cm^2 Falcon cell culture flasks, the cells were suspended at the appropriate cell density in L-15 media at the appropriate cell density depending on the cell line and experiment. The cells were then plated in the cell culture inserts and this was considered day 0. On day 0, the RTgill-W1 cell line was plated in 44cm^2 cell culture inserts at a density of 3.77×10^6 cells/mL and the LSgill-e cells were plated at a density of 1.47×10^6 cells/mL. Throughout the duration of this insert experiment, the cells in the cell culture insert had both the apical and basolateral sides exposed to L-15 media where TEER values were measured and media was changed consistently throughout which will be outlined below in section 3.3.3. After appropriate TEER values were established, the L-15 media on the apical side of the insert was removed, and the cells were rinsed three times with titration media. Copper (as $CuSO_4$ solution) was then added in titration media to the apical side of the insert prior to a three-hour incubation period. The basolateral side remained L-15 media. After the three-hour incubation period with copper (II) sulfate, samples from both the apical and basolateral sides were taken and acidified to 0.2% nitric acid. Once samples were acidified, they were loaded into the graphite furnace sample tray and were then ready for analysis.

Table 4. Graphite furnace cell culture insert experiments performed for both the RTgill-W1 and LSgill-e cell lines.

Figure Number	Cell Line	Experimental Parameters
Figure 28. TEER Values	RTgill-W1	Media: L-15 Media Cell Density: Day 0: 3.77×10^6 cells/mL

		$CuSO_4$ Concentration Range: N/A
Figure 29. TEER Values	LSgill-e	Media: L-15 Media Cell Density: Day 0: 1.47×10^6 cells/mL $CuSO_4$ Concentration Range: N/A
Figure 30. Figure 31.	Calibration Curve (No Cells)	Media: Titration Media Cell Density: N/A $CuSO_4$ Concentration Range: 1 μM – 225 μM
Figure 32.	RTgill-W1 Sorption Data	Media: Titration Media Cell Density: $CuSO_4$ Concentration Range: 1 μM – 200 μM
Figure 33.	LSgill-e Sorption Data	Media: Titration Media Cell Density: $CuSO_4$ Concentration Range: 1 μM – 200 μM

3. Results and Discussion

3.1 Cell Viability Assays – Cell Monolayer Exposures

For this research, cell viability assays were performed to determine the cytotoxic concentrations of copper (II) sulfate for both the RTgill-W1 and LSgill-e cell lines. This was done in order to determine what concentration(s) of copper (II) sulfate induced cell toxicity to ensure that throughout future experiments, the cells remained alive and viable. This was important to ensure that the cells from both cell lines resemble the gill of an intact, living fish as close as possible. Cell viability was measured as a percentage of the control using two fluorescent indicator dyes: alamar blue and CFDA-AM after 24-hour copper (II) sulfate exposure. When examining cell viability, it is said that cells with viability percentages at or above 80% are considered non-cytotoxic and thus have not been exposed to detrimental concentrations of the pollutant (López-García et al. 2014). For this research, when cells were at or above 80% cell viability after exposure to copper, they were considered to be viable and that concentration of copper was considered to be non-cytotoxic. To further prove these findings, statistical analysis was also performed to determine what concentrations of copper (II) sulfate were significant or not.

For the first experiment, RTgill-W1 cells in a monolayer were exposed to copper (II) sulfate concentrations ranging from 10 μM – 975 μM. When looking at metabolic activity as indicated by alamar blue, the RTgill-W1 cells were still more than 80% viable even when exposed to $CuSO_4$ concentrations as high as 500 μM in L-15 media (Figure 3). This indicated that the safe titratable range for copper (II) sulfate in future RTgill-W1 experiments in L-15 media was up to 500 μM. This was a much greater copper concentration than what would be observed in natural aquatic environments. As mentioned earlier in section 1.1, the concentration

of Cu in unpolluted freshwater environments ranges from <1-6 μg/L however even with concentrations ranging from 10-1000 μg/L, effects of Cu toxicity are potentially not detectable in fish (Kamunde and Wood 2004). When looking at cell membrane integrity which was indicated by CFDA-AM, the RTgill-W1 cells were less than 70% viable when exposed to $CuSO_4$ concentrations up to 500 μM and were 80% viable when exposed to a total copper concentration of 300 μM (Figure 3). Once the threshold where the cells still remained 80% viable was established, it was important to determine the EC_{50} for copper (II) sulfate with RTgill-W1 cells in L-15 media. In order to calculate the EC_{50} values when looking at both alamar blue and CFDA-AM, higher, potentially more toxic, concentrations of $CuSO_4$ were needed, up to a final concentration of 975 μM (Figure 3**)**. When the total concentration of copper was increased to almost 1 mM, nearly 100% cell death was observed (Figure 3). The EC_{50} value, the concentration of copper than would prove lethal to 50% of the RTgill-W1 cells in L-15 media, when examined by metabolic activity was calculated to be 636 ± 0.2 μM and when examined by cell membrane integrity was calculated to be 566 ± 0.5 μM. Further statistics involving this data can be seen below (Table 6). Both of the models for calculating the EC_{50} values can be seen below in Figure 4 (does-response curve for alamar blue) and Figure 5 (dose-response curve for CFDA-AM). Dose-response curves were modelled using GraphPad Prism™ (Version 9.0.0) nonlinear regression model [Inhibitor] vs. normalized response was used to determine the EC_{50} values with 95% confidence intervals.

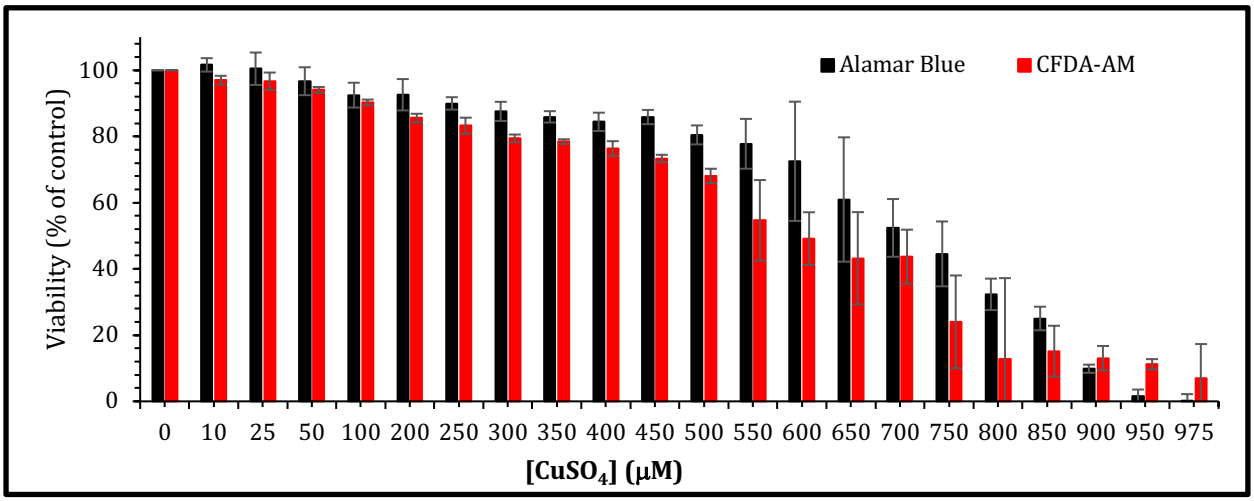


Figure 3. Viability of RTgill-W1 cell cultures in a monolayer in 96-well plates after 24h incubation with copper (II) sulfate concentrations ranging from 10 µM – 975 µM in L-15 media. Cell viability was assessed using two indicator dyes, alamar blue (black) and CFDA-AM (red). Each concentration was performed in triplicate with a cell density of 20,000 cells/well.

To further examine the effect that copper had on the viability of the RTgill-W1 cell line, stats were run using GraphPad Prism™ (Version 9.0.0) to determine what concentrations of copper (II) sulfate were significantly different than the control, RTgill-W1 cells with no copper exposure, indicating nearly 100% cell viability. A one-way ANOVA was performed with a Dunnett's post-test, which can be found in Table 5 below. Prior to the one-way ANOVA the alamar-blue data was transformed by squaring all of the data to ensure normal (Gaussian) distribution. The results obtained using CFDA-AM already had normal (Gaussian) distribution and no transformation of data was needed prior to the one-way ANOVA analysis. For all statistical analysis performed throughout this research, non-significant data (NS) indicated $P>0.05$, $P\leq 0.05$ was indicated by '*', $P\leq 0.01$ was indicated by '**', $P\leq 0.001$ was indicated by '***' and lastly, $P\leq 0.0001$ was indicated by '****'. It was determined that for both alamar blue and CFDA-AM, when total copper concentrations were higher than 600 µM, the viability became significantly different from the control, as indicated by the smaller P values. This is indicative that the cells were becoming less viable with higher concentrations of copper. This

was expected as a significant amount of cell death was observed at high copper concentrations for the RTgill-W1 cell line. The concentrations of copper that were lower than 600 µM, the P values were much lower than 0.05, therefore, they are considered to be not statistically significant. This further proved that at total copper concentrations below 600 µM , cell viability is much greater as evidenced by the low statistical variability between the samples and the control. It was also determined that under these conditions for the RTgill-W1 cell line the CC was 12.11 $\mu g/L$ to observe no toxic effects for an indefinite amount of time.

Table 5. One-way ANOVA with a Dunnett's posttest stats for the viability of RTgill-W1 cells in a monolayer.

[$CuSO_4$] Added (μM)	**Alamar Blue**		**CFDA-AM**	
	Summary	**P Value**	**Summary**	**P Value**
10	NS	0.9998	NS	0.9998
25	NS	>0.9999	NS	0.9996
50	NS	0.9991	NS	0.9994
100	NS	0.9893	NS	0.9989
200	NS	0.9130	NS	0.9947
250	NS	0.8110	NS	0.9749
300	NS	0.4661	NS	0.9183
350	NS	0.4092	NS	0.9192
400	NS	0.3140	NS	0.7392
450	NS	0.2874	NS	0.6909
500	NS	0.0727	NS	0.4558
550	NS	0.1209	NS	0.2249
600	NS	0.0584	*	0.0125
650	**	0.0058	**	0.0038
700	***	0.0010	**	0.0042
750	***	0.0002	****	<0.0001
800	****	<0.0001	***	0.0002
850	****	<0.0001	****	<0.0001
900	****	<0.0001	****	<0.0001
950	****	<0.0001	****	<0.0001
975	****	<0.0001	****	<0.0001

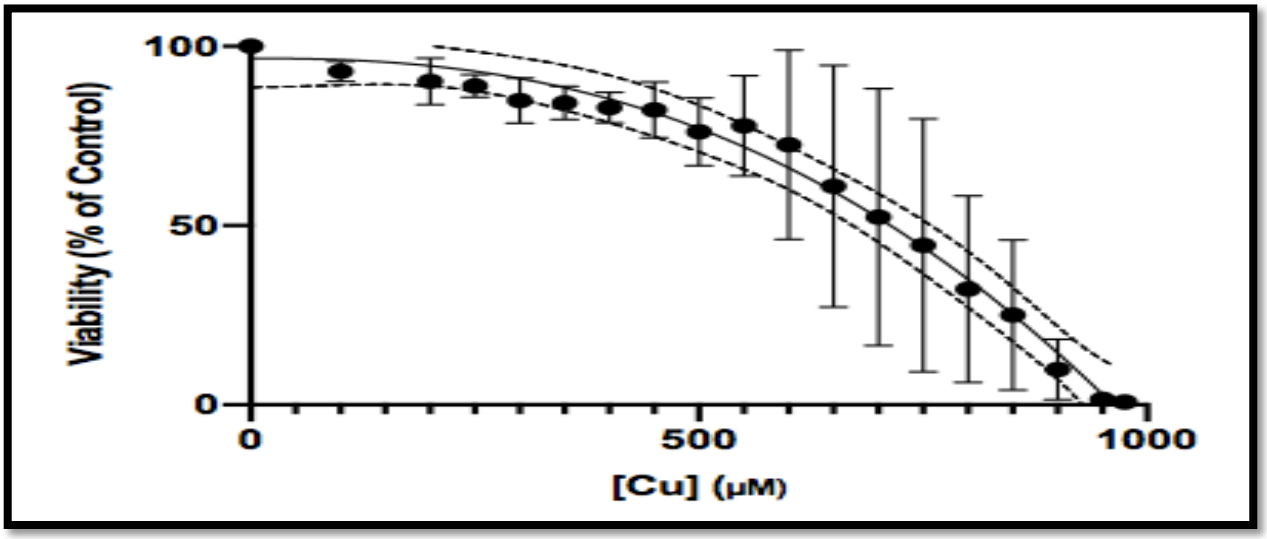


Figure 4. Dose-response curve for RTgill-W1 cells in a monolayer exposed to CuSO4 ranging from 10 µM – 975 µM with cell viability analyzed using alamar blue. The line of best fit (solid black line) can be seen as well as the 95% confidence intervals (black dashed line). This data (circles) was taken from Figure 3 above.

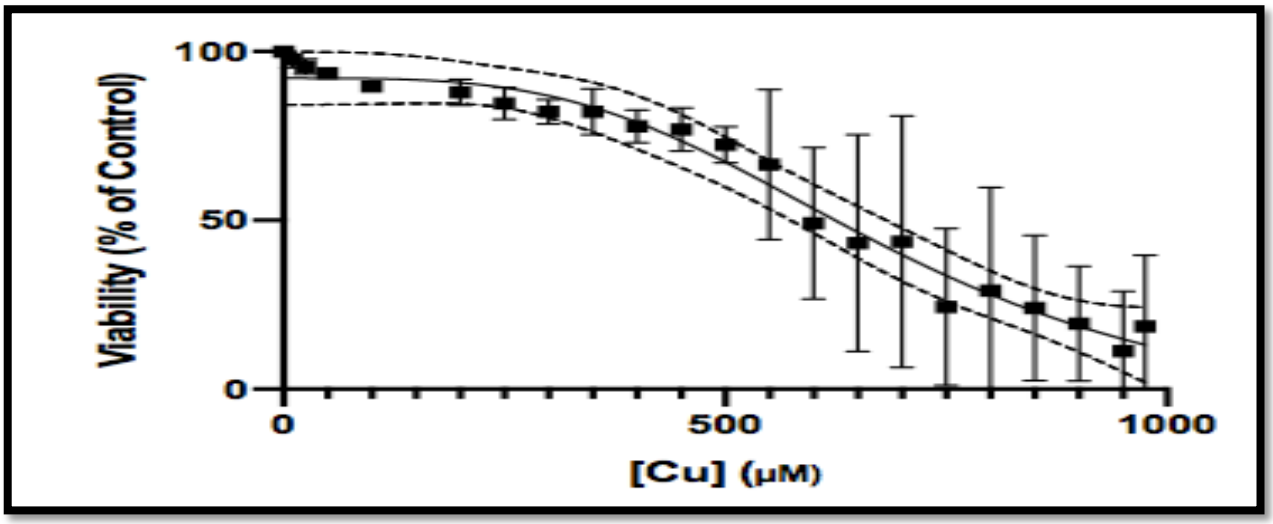


Figure 5. Does-response curve for RTgill-W1 cells in a monolayer exposed to CuSO4 ranging from 10 µM – 975 µM with cell viability analyzed using CFDA-AM. The line of best fit (solid black line) can be seen as well as the 95% confidence intervals (black dashed line). This data (circles) was taken from Figure 3 above.

Table 6. EC_{50} and statistical data for the RTgill-W1 monolayer viability experiment. Calculated using the GraphPad Prism™ 9 (Version 9.0.0) nonlinear regression model [Inhibitor] vs. normalized response to determine the EC_{50}, R^2 and SEM.

	Alamar Blue	**CFDA-AM**
EC_{50}	636 µM	567 µM
R^2	0.968	0.955
SEM	7.38	6.96

In Figure 3, it could be seen quantitatively that at high total Cu concentrations of 975 μM, cell toxicity was observed in the RTgill-W1 cell line. To further examine the toxicity that Cu had on these cells, qualitative results (*i.e.,* microscopy) were observed after the cell were exposed to copper for 24-hours. In Figure 6 below, it can be seen that on the left when cells were in L-15 media alone, the cell shape was generally epithelial-like with no cell shrinkage or toxicity observed. In the middle, the RTgill-W1 cells were exposed to 300 μM of copper (II) sulfate in L-15 media and as seen in Figure 3 this is consistent with approximately 80% cell viability. This was visually observed as the majority of the cells remained epithelial-like but some were beginning to shrink in size – an indication that the cells were beginning to die. Lastly, on the right, the cells were exposed to 975 μM of copper (II) sulfate in L-15 media and quantitatively, at this copper concentration, nearly 100% cell death was previously observed. At this concentration, some of the cells can be seen detached and floating in the media indicating that they have died. The majority of the RTgill-W1 were no longer epithelial-like and had shrunk drastically in size due to the Cu toxicity.

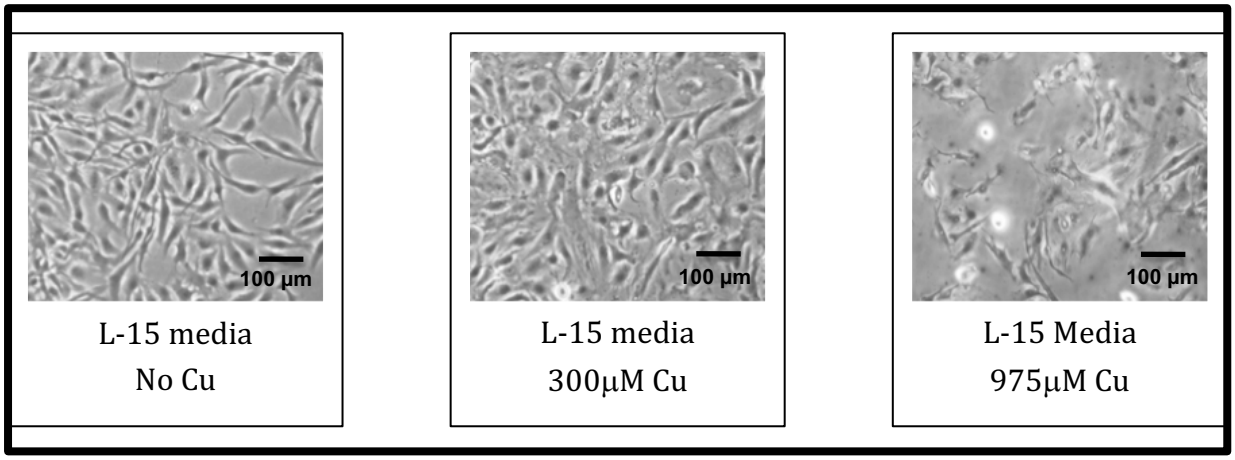


Figure 6. RTgill-W1 cells under a microscope at 100X magnification. Cells were grown in L-15 media with either no Cu exposure (left), 300 μM Cu exposure for 24hours (middle), or 975 μM Cu exposure for 24hours (right). With no Cu, cells are nearly 100% viable with no Cu toxicity observed. At 300μM, some Cu toxicity can be observed

with some cell shrinkage. At 975μM, cell toxicity is very prominent with nearly 100% cell death and visual cell shrinkage.

In addition to the RTgill-W1 cell line, viability assays in L-15 media were also performed using the LSgill-e cell line. Cell viability was analyzed using alamar blue and CFDA-AM as mentioned previously. When the cell viability analysis was performed with the RTgill-W1 cell line, it was determined that with copper concentrations as high as 500 μM, cell viability remained greater than 80% and therefore, when performing the cell viability assays for LSgill-e, Cu was only added to a maximum concentration of 500 μM as this concentration would not be exceeded in later experiments and no cytotoxic effects were observed as seen in Figure 7 below. In future experiments it was known that copper concentrations up to 500 μM could be used, and cells would remain viable in L-15 media. However, as copper concentrations of 975 μM had to be reached in order to see cell death in the RTgill-W1 cell line, it is likely that ligands in L-15 culture media complex Cu resulting in less bioavailable copper for cellular uptake. This was examined further later in the research. For the LSgill-e cell line, when cell viability was examined using metabolic activity as indicated by alamar blue, viability remained greater than 80% with all copper concentrations from 5 μM - 500 μM. When cell viability was examined using cell membrane integrity as indicated by CFDA-AM, viability also did not drop below the 80% threshold indicating no significant cytotoxic effects were observed up to a final copper concentration of 500μM. From this, a safe titration range was established, from 5 μM – 500 μM for the LSgill-e cells in L-15 media which could then be used in future experiments. Statistical analysis was performed, specifically, a one-way ANOVA was completed with a Dunnett's posttest which can be found below in Table 7. The data was checked for normalized (Gaussian) distribution prior to analysis using the Shapiro-Wilk test and no transformation of data was

required. It was observed that at lower concentrations of Cu when examined using alamar blue, (5 – 50 μM) there was no significant difference between the control (100% cell viability), with the exception of one concentration (25 µM), and lower concentrations indicating there was still significant cell viability. However, as concentrations of copper increased, from 100 µM – 500 µM, slight differences between the control and the increasing concentrations of copper was observed. When looking at cell viability as measured using CFDA-AM, there was no significant difference between the control and any of the concentrations of copper added (5 – 500 μM). No dose-response curve model was provided for the LSgill-e cells in L-15 media as cell viability did not drop below 50% with the copper concentrations used. It was also determined that under these conditions for the LSgill-e cell line the CC was 12.11 $\mu g/L$ to observe no toxic effects for an indefinite amount of time.

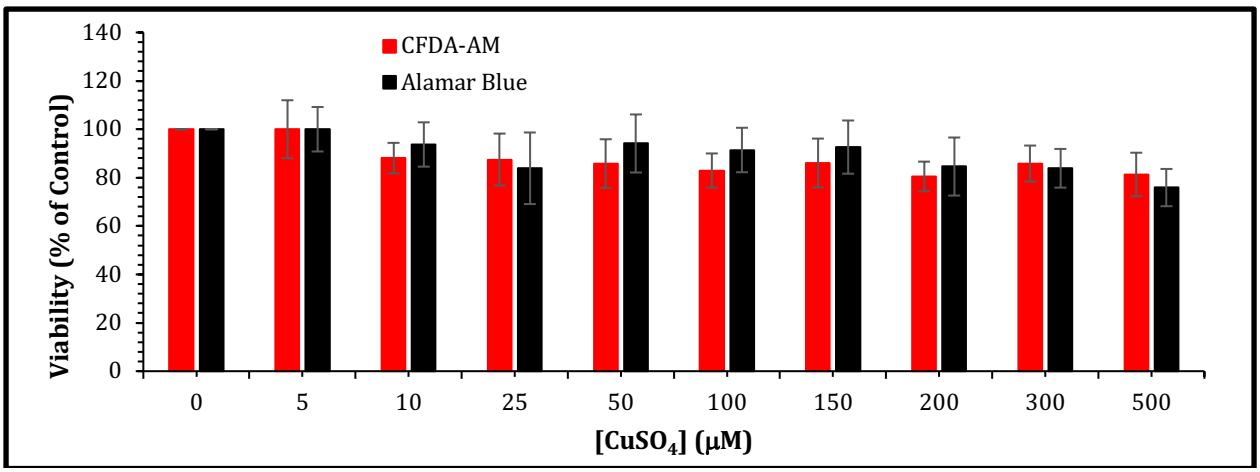


Figure 7. Viability of LSgill-e cell cultures in a monolayer in 96-well plates after 24h incubation with copper (II) sulfate concentrations ranging from 5µM-500µM in L-15 media. Cell viability was assessed using two indicator dyes, alamar blue (black) and CFDA-AM (red). Each concentration was performed in triplicate with a cell density of 12,500 cells/well.

Table 7. One-way ANOVA with a Dunnett's posttest stats for the viability of LSgill-e cells in a monolayer.

[$CuSO_4$] Added (μM)	Alamar Blue		CFDA-AM	
	Summary	P Value	Summary	P Value
5	NS	>0.999	NS	>0.9999
10	NS	0.0979	NS	>0.9999

25	***	0.0002	NS	>0.9999
50	NS	0.1307	NS	0.9999
100	*	0.0198	NS	0.9999
150	*	0.0459	NS	0.2635
200	***	0.0003	NS	0.9999
300	**	0.0036	NS	0.9999
500	****	<0.0001	NS	0.9999

After the cell viability toxicity assays were completed for both the RTgill-W1 and LSgill-e cell lines to investigate what concentrations of copper (II) sulfate induced cell toxicity in L-15 media, it was important to investigate whether or not cell seeding density had any influence on cell toxicity and viability. When viability tests are performed, it is important to take into account cell seeding density as the cellular response to toxins, in this case Cu, are said to be affected by the cell density number (Cristina Da Silva Gasque et al. 2014). At higher cell densities, Cu may become bound to the gill cell and unavailable providing less toxic effects than when at lower cell densities, the cells are more affected by the toxin (Cristina Da Silva Gasque et al. 2014). Also, at lower cell densities, the cells may undergo more stress and thus toxicity could be increased. To examine further examine this, the RTgill-W1 cell line was exposed to copper (II) sulfate, with cell seeding densities of 10,000 cells/well, 15,000 cells/well, 20,000 cells/well and 25,000 cells/well in L-15 culture media. Cell viability was assessed using both alamar blue and CFDA-AM, as seen in Figure 8 and Figure 9, respectively.

When examining cell viability as cellular metabolic activity for RTgill-W1 cells exposed to copper (II) sulfate (Figure 8) it was determined that with Cu concentrations from 1 μM to as high as 200 μM, the cells in monolayer remained almost 100% viable after a 24-hour exposure time. This was true for the cell densities of 10,000 cells/well, 20,000 cells/well and 25,000 cells/well and with a cell density of 15,000 cells/well, viability decreased to about 80%. These results were not as expected as even with a very low cell density of 10,000 cells/well viability

remained similar when compared to the cell seeding density of 25,000 cells/well which was expected to have a higher cell viability due to less exposure to the toxin, Cu. For this experiment, it can be said that cell seeding density in the range tested (10,000 cells/well – 25,000 cells/well) did not have a drastic effect on cell toxicity as measured by alamar blue for RTgill-W1 cells in a monolayer. Also, for this data, statistical analysis was also performed using a one-way ANOVA with a Dunnett's posttest which can be found below in Table 8. All data was normally distributed as per the Shapiro-Wilk test and no data transformations were required. It was determined that for all cell densities, 25,000 cells/well – 10,000 cells/well and all concentrations of copper, cell viability was not significantly different than the no copper control (100% cell viability).

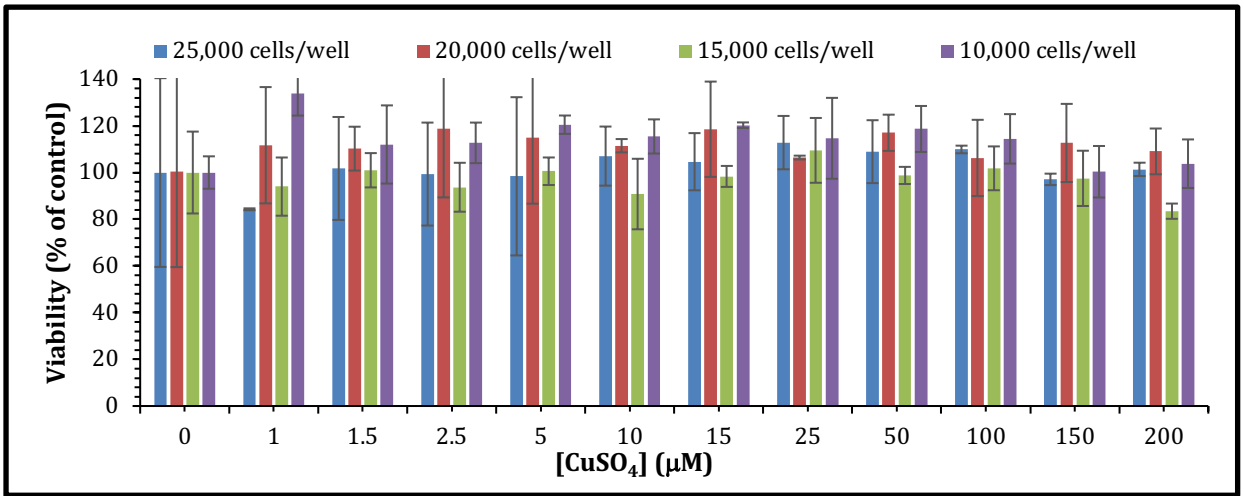


Figure 8. Viability of RTgill-W1 cell cultures in a monolayer in 96-well plates after 24h incubation with copper (II) sulfate concentrations ranging from 1µM-200 µM in L-15 media. Cell viability was assessed using a fluorescent indicator dye, alamar blue – a measure of cellular metabolic activity. Each concentration was performed in triplicate for each cell plating density; 10,000 cells/well, 15,000 cells/well, 20,000 cells/well and 25,000 cells/well.

Table 8. One-way ANOVA with a Dunnett's posttest stats for the viability of RTgill-W1 cells in a monolayer at various cell densities with Alamar Blue.

$[CuSO_4]$ Added (μM)	Alamar Blue							
	25,000 cells/well		20,000 cells/well		15,000 cells/well		10,000 cells/well	
		P Value		P Value		P Value		P Value
1	NS	0.9997	NS	0.7954	NS	0.9910	NS	0.9316
1.5	NS	0.9999	NS	0.9652	NS	0.9999	NS	0.9993
2.5	NS	0.9994	NS	0.7966	NS	0.9891	NS	0.9960
5	NS	0.9997	NS	0.7195	NS	0.9999	NS	0.9840

10	NS	>0.9999	NS	0.6527	NS	0.8192	NS	0.9927
15	NS	0.9999	NS	0.8354	NS	0.9998	NS	0.9842
25	NS	0.9999	NS	0.8478	NS	0.8420	NS	0.9961
50	NS	0.9998	NS	0.3863	NS	0.9999	NS	0.9897
100	NS	>0.9999	NS	0.9075	NS	0.9997	NS	0.9933
150	NS	0.9995	NS	0.3865	NS	0.9997	NS	0.9996
200	NS	0.998	NS	0.9483	NS	0.3289	NS	0.9994

Cell viability was also examined as cell membrane integrity for RTgill-W1 cells exposed to copper (II) sulfate (Figure 9) with multiple seeding densities from 10,000 cells/well – 25,000 cells/well. It was determined that with all four cell densities examined with Cu concentrations ranging from 1 μM to as high as 200 μM, cell viability did not drop below 80%. Viability remained very consistent with all four densities examined which once again, was not as expected as lower cell densities are expected to have more toxic effects than with higher cell densities (Cristina Da Silva Gasque et al. 2014). It can be said that for this experiment, cell seeding density did not have a drastic effect of cell toxicity as measured by CFDA-AM for RTgill-W1 cells in a monolayer. Seen below in Table 9, a one-way ANOVA was run with a Dunnett's posttest can be found. All data was normally distributed as per the Shapiro-Wilk test and no data transformations were required. Just like with the alamar blue above, it was determined that for all cell densities, 25,000 cells/well – 10,000 cells/well and all concentrations of copper, cell viability was not significantly different than the no copper control.

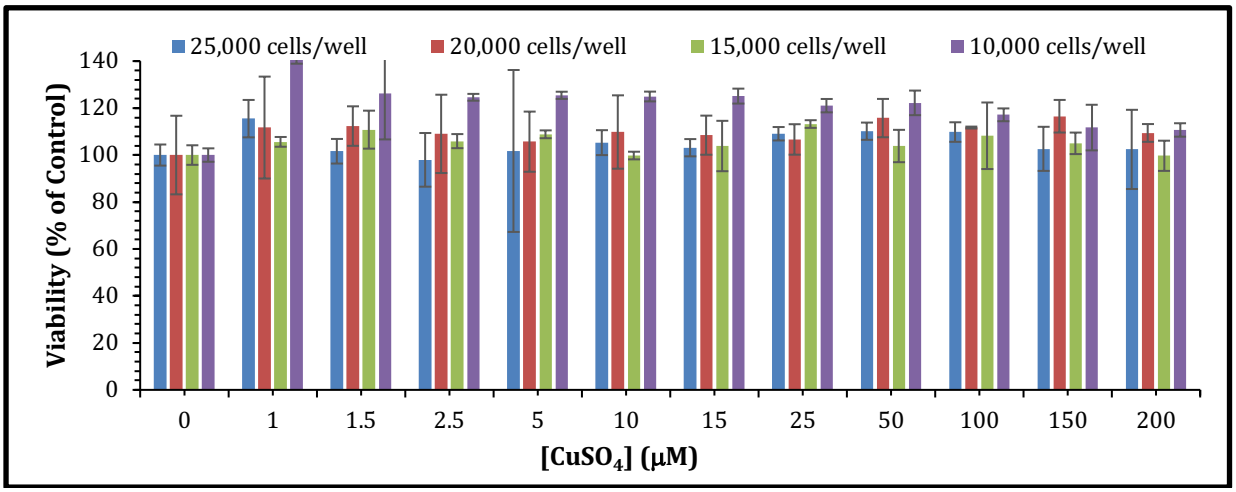


Figure 9. Viability of RTgill-W1 cell cultures in a monolayer in 96-well plates after 24h incubation with copper (II) sulfate concentrations ranging from 1µM-200 µM in L-15 media. Cell viability was assessed using a fluorescent indicator dye, CFDA-AM – a measure of cell membrane integrity. Each concentration was performed in triplicate for each cell plating density; 10,000 cells/well, 15,000 cells/well, 20,000 cells/well and 25,000 cells/well.

Table 9. One-way ANOVA with a Dunnett's posttest stats for the viability of RTgill-W1 cells in a monolayer at various cell densities with CFDA-AM.

[$CuSO_4$] Added (μM)	CFDA-AM							
	25,000 cells/well		20,000 cells/well		15,000 cells/well		10,000 cells/well	
		P Value		P Value		P Value		P Value
1	NS	0.6865	NS	0.9028	NS	0.9447	NS	0.4683
1.5	NS	0.7890	NS	0.8586	NS	0.3994	NS	0.8836
2.5	NS	0.3255	NS	0.9736	NS	0.9258	NS	0.8560
5	NS	0.8039	NS	0.9969	NS	0.6261	NS	0.8480
10	NS	0.9969	NS	0.9585	NS	>0.9999	NS	0.8475
15	NS	0.9375	NS	0.9780	NS	0.9929	NS	0.8451
25	NS	0.9997	NS	0.9930	NS	0.2162	NS	0.9302
50	NS	0.9994	NS	0.6224	NS	0.9931	NS	0.9204
100	NS	0.9996	NS	0.8676	NS	0.7085	NS	0.9670
150	NS	0.8997	NS	0.5310	NS	0.9715	NS	0.9962
200	NS	0.8770	NS	0.9584	NS	>0.9999	NS	0.9992

As the effects of cell density on the toxicity of RTgill-W1 cells was not as expected, why this was happening needed to be investigated. One major factor that could have an effect on cell toxicity is media composition. L-15 culture media contains many components including phenol red, sodium pyruvate, L-glutamine and many others that are essential for the growth, development and maintenance of cell cultures (Cad). While this complex basal media is great for

maintaining cell cultures in preparation for experiment, it may contain many more potentially protective binding ligands as discussed in section 1.2 – Biotic Ligand Model, than other medias (Dayeh et al. 2004). These protective compounds in L-15 media could be the reason why in earlier experiments in this research, cell toxicity was observed at much higher copper concentrations than what would be expected with simpler media (Dayeh et al. 2004). Two types of simpler media that were examined for this research were L-15/ex (Appendix A) and titration media. Titration media was used for the majority of experiments in this research due to its composition of only NaCl and $CaCl_2$ providing a much closer composition to typical freshwater than basal L-15 culture media. It was also important to examine cell toxicity and viability in media other than the L-15 basal media as this media does not closely resemble freshwater whereas titration media more closely resembles the freshwater in natural aquatic environments.

L-15/ex media is a modification of Leibovitz' L-15 media containing only salts, sodium pyruvate and galactose and was originally developed for short term exposure using the RTgill-W1 cell line (Yue et al. 2015). Due to the longer exposure time of experiments done in this research and the composition of the L-15/ex media, experiments completed using this media can be found in Appendix A. Prior to exposing the RTgill-W1 cell line to titration media and copper at the same time, it was important to examine the effect of titration media on the cells alone with no addition of the potential toxicant, Cu. If the titration media was found to be toxic to the cells than it would have been difficult to examine the effects of Cu on the cells, so the toxicity of the media alone was examined first (Figure 10). If it was found that titration media was toxic to the cells, it would be unknown whether or not if the copper was causing the toxicity, if the titration media was causing the toxicity, or if there was a combined effect with both the copper and titration media causing cell toxicity. The RTgill-W1 cells were exposed to the titration media for

two hours, four hours, six hours, eleven hours, and twenty-four hours where at each time interval viability was examined. As can be seen in Figure 10 below, cell viability that was analyzed using both alamar blue and CFDA-AM remained greater than 80% when exposed to only the titration media for 24 hours. As mentioned previously, viability percentages at or above 80% are indicative of non-cytotoxic effects so further experiments using titration media could then be performed. Even though the cells would not be exposed to titration media for this long in future experiments, it can be said that the cells would remain alive and viable. Thus, the toxic effects of copper were then able to be investigated without limited possibility of the media binding Cu and protecting the cells which was observed with the L-15 media. This is discussed in further detail in section 1.2 Biotic Ligand Model, applying to studies using cell monolayers only and not cell suspension or cells in cell culture inserts.

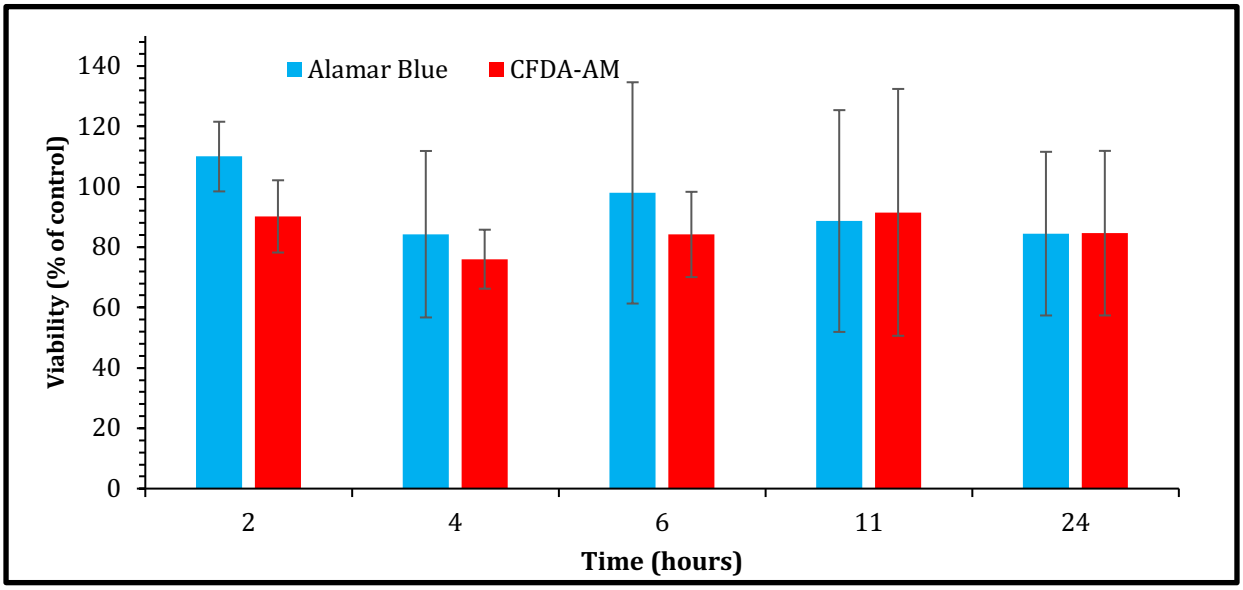


Figure 10. Viability of the RTgill-W1 cells in a monolayer in 96-well plates after 2h, 4h, 6h, 11h and 24h incubation with titration media. Cell viability was assessed using two fluorescent indicator dyes, alamar blue (blue) and CFDA-AM (red). Each time-point was performed in triplicate with a cell density of 20,000 cells/well.

As it was determined that RTgill-W1 cell viability remained at or greater than 80% when exposed to titration media for up to 24 hours, viability was then assessed for both the RTgill-W1

and LSgill-e cell lines in titration media, more closely resembling "real" freshwater, exposed to various concentrations of copper (II) sulfate. Due to time restraints and other conflicts discussed in Section 4, LSgill-e viability in titration media alone with no copper exposure was not examined. For the RTgill-W1 cell line, concentrations of Cu from 1.0 μM – 200 μM were used to examine cell viability (Figure 11). When looking at CFDA-AM for RTgill-W1, a measure of cell membrane integrity, cell viability remained at or greater than 80% until a concentration of 50 μM was reached where cell viability was at 78% and viability began to drop to 5% when exposed to a total copper concentration of 200 μM. When looking at alamar blue for the RTgill-W1 cell line, when exposed to a copper concentration of 50 μM, cell viability was only at 48% and at a copper concentration of 200 μM, cell viability was only at 3% indicating that the majority of the cells had died. When comparing cell viability for the RTgill-W1 cell line in titration media to cell viability in L-15 media it is evident that the titration media does not contain any Cu binding ligands (except weak Cl^- and OH^-), so cell toxicity occurred much faster at lower Cu concentrations in titration media compared to in L-15 media. Thus, for the RTgill-W1 cell line, media composition played a major role on copper induced cell toxicity. When looking at the LSgill-e cell line, it can be seen (Figure 11) that even with copper concentrations as high as 500 μM, cell viability still remained very high, at 97% viability for alamar blue and 100% for CFDA-AM. This may vary due to the reasons discussed in section 1.3 Continuous Cell Lines due to the mucus they produce or the relatively low passage number in comparison to the RTgill-W1 cell line, in which they more closely resemble primary cells. Thus, in titration media LSgill-e can be said to be more resilient to copper toxicity with concentrations up to 200 μM when compared to the RTgill-W1 cell line. In Table 10 below, results from a one-way ANOVA was run with a Dunnett's posttest can be found. The Shapiro-Wilk normality test could not be

performed as this experiment was only performed in duplicate. Normal (Gaussian) distribution was assumed and a one-way ANOVA was performed. It was determined that for both alamar blue and CFDA-AM analysis of cell viability for RTgill-W1 and LSgill-e, all concentrations of copper added were significantly different from the control that had 100% cell viability. This is indicative of cell viability drastically decreasing when Cu is added in systems with titration media. Although the LSgill-e cell line did not decrease in cell viability as drastically as the RTgill-W1 cell line when exposed to copper, statistical differences were still observed from the control as viability was inconsistent and as a percentage of the control, was more viable in some cases than the control. This could be due to a cell seeding density error and 100% cell viability could be assumed.

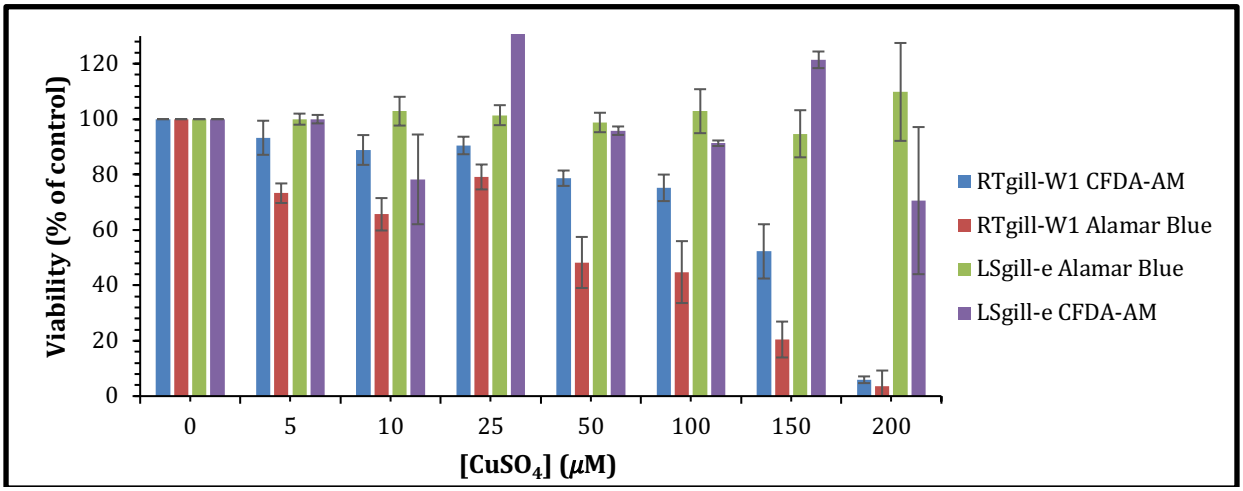


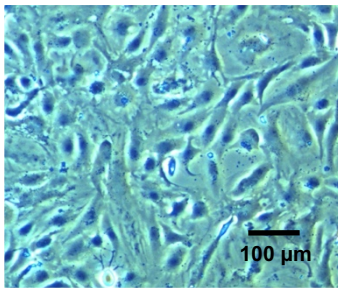
Figure 11. Viability of both the RTgill-W1 and LSgill-e cell lines in a monolayer in 96-well plates after 24h incubation with copper (II) sulfate in titration media. Cell viability was assessed using two fluorescent indicator dyes, alamar blue and CFDA-AM. Each concentration was performed in duplicate for each cell line with a cell density of 20,000 cells/well for RTgill-W1 and 12,500 cells/well for LSgill-e.

Table 10. One-way ANOVA with a Dunnett's posttest stats for the viability of RTgill-W1 and LSgill-e cells in a monolayer, in titration media. Grey boxes indicate no experiment run for that cell line at that specific copper concentration.

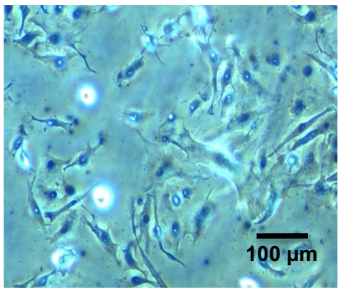
[$CuSO_4$] Added (μM)	RTgill-W1				LSgill-e			
	Alamar Blue		CFDA-AM		Alamar Blue		CFDA-AM	
		P Value	****	P Value		P Value		P Value
1	****	<0.0001	****	<0.0001				
1.5	****	<0.0001	****	<0.0001				

2.5	****	<0.0001	****	<0.0001				
5	****	<0.0001	****	<0.0001	NS	>0.9999	NS	>0.9999
10	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
15	****	<0.0001	****	<0.0001				
25	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
50	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
100	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
150	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
200	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001

When looking at the results above, it was determined that at high Cu concentrations of 200 µM, cell toxicity and nearly 100% cell death occurred in the RTgill-W1 cell line. To further examine the toxicity that Cu had on these cells, qualitative results were assessed after the 24-hour copper exposure. In Figure 12 below, it can be seen that on the left when cells had no copper exposure, it was titration media only, cell shape was epithelial-like with no toxicity observed. On the right, the RTgill-W1 cells had been exposed to 200 µM of copper (II) sulfate in titration media where it was previously determined that nearly 100% cell death occurred at this concentration which was further verified in the figure below. At this concentration, the cell density was visually much lower as the cells have died and detached from the cell culture flask. The RTgill-W1 cells in Figure 12 were also no longer epithelial-like and shrunk and shriveled drastically due to the Cu toxicity.



Titration Media No Cu



Titration Media 200µM Cu

Figure 12. RTgill-W1 cells under a microscope at 100X magnification. Cells were grown in L-15 media and then exposed to $CuSO_4$ in titration media for 24 hours (right) at a concentration of 200µM. With no Cu, cells were nearly 100% viable with no Cu toxicity visually observed (left). At 200µM, cell toxicity was very prominent with nearly 100% cell death with cell shrinkage and death visually observed (right).

To further examine the relative toxicity of the lake sturgeon and rainbow trout gill cell lines, other characteristics need to be taken into account as to why the lake sturgeon cell line seems to be more tolerant to copper toxicity than the rainbow trout gill cell line. One possibility is the passage number (number of times the culture has been subcultured) of the LSgill-e cells compared to the RTgill-W1 cells. The lower that the passage number is, the closer the cell line is to the primacy cell culture and thus, less distant from the primary organism. The LSgill-e cell line used had a maximum passage number of ~50 whereas the RTgill cell line reached as high as ~150, very distant from the primary organism. Overtime, it is possible that the RTgill-W1 cells have lost some essential functions that are required to protect the organism from toxicity and thus when exposed to copper, protection was decreased, and higher cell toxicity was observed. Another possibility, as discussed earlier, could be the production of mucus in the LSgill-e cell line and not the RTgill-W1 cell line. The mucus produced by the LSgill-e cells could be acting as an added barrier providing protection from the copper toxicity in comparison to the non-mucus producing cell line, RTgill-W1. Further details about the mucus production can be found in section 1.3 Continuous Cell Lines.

As optimal media composition, cell density and copper concentration were now determined, for both the RTgill-W1 and LSgill-e cell lines, copper binding could be examined. This was done using two different analytical methods: the ISE and the graphite furnace.

3.2 Ion Selective Electrode

After completing the practice ISE titrations with salicylic acid and copper (II) sulfate (Appendix C), the first titration with RTgill-W1 cells in suspension was performed, Figure 13

below. The RTgill-W1 cells were grown to confluence in L-15 media and then suspended at a density of 141,000 cells/mL in titration media to a final volume of 25 mL with 84% live and 16% dead. To keep consistent with Smith *et al.* (2017), who used 250,000 primary cells/mL, as close to this density as possible was attempted however due to number of cells available this was not the case for all of the experiments performed. The RTgill-W1 cells remained suspended throughout the duration of the experiment with the use of a stir bar. As can be seen below, Figure 13, the first copper addition, 2 μM was titrated in and left to stabilize for three hours. However, the electrode would not fully stabilize or even begin to stabilize throughout this titration, which could be a result of too low of a copper concentration or not a high enough cell density. To adjust for this, the total copper concentration was increased to 5 μM. Approximately 2.1 hours after the second addition of copper, the electrode did not stabilize and more copper was titrated in to a final concentration of 8 μM. After this final addition of copper and over six hours, the electrode would still not stabilize and no acidification step was completed (acidification step discussed in section 2.3). In order for an ISE to stabilize, sufficient buffering is required. A couple of possibilities as to why this was not occurring could be that the cell density was just too low and with the relatively low concentration of copper added, buffering was insufficient and the electrode would not stabilize. The second possibility was that the cell line may not have the same binding strength as either the primary gill cells or whole organism thus the total concentration of copper (II) sulfated needed to be increased. It was also observed throughout this titration that the RTgill-W1 cells were dying as at the beginning of the titration the cells were 84% live at the and only 43% live at the end of the titration. This could have been a result of the stir bar being used to keep the cells in suspension, killing the cells throughout the titration and that the dead cells were not binding copper causing the electrode not to stabilize. These are all factors that were

taken into account when performing future ISE titrations. It is important to note that ISE's work best in well-buffered systems such as systems with strong Cu^{2+} binding and/or high concentrations of Cu^{2+}.

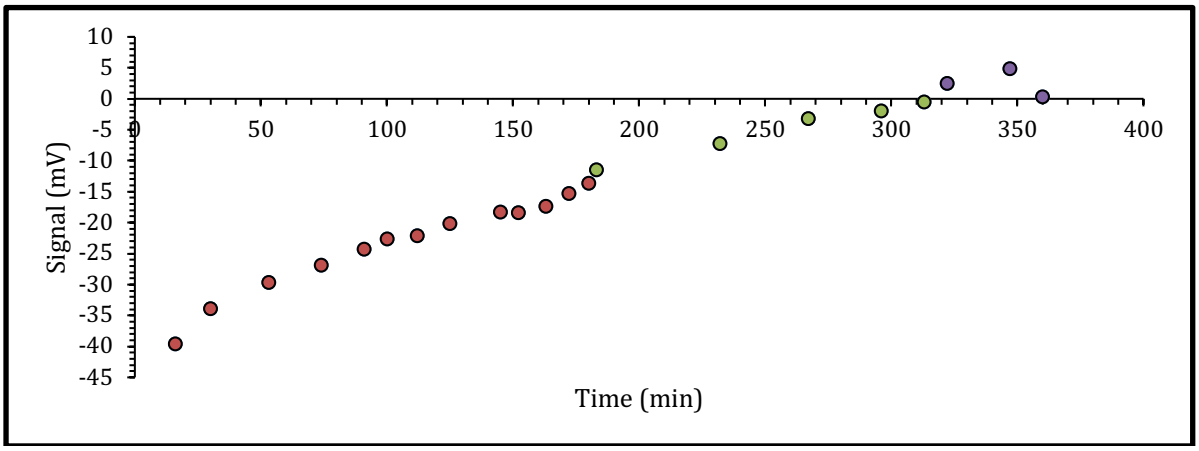


Figure 13. Cell suspension titration data for the RTgill-W1 cell line, as time after the first addition of copper with the signal (mV). Copper (II) sulfate was added in three increments, 2µM (red circles), 5µM (green circles), and 8µM (purple circles) to 25mL of rainbow trout gill cells with a cell density of ~141,000 cells/mL in titration media. pH was monitored and maintained at 7.7±0.05 throughout the titration and adjusted using nitric acid and sodium hydroxide when necessary.

Before and after the above copper ISE titration, an alamar blue assay was performed to assess metabolic activity of the rainbow trout gill cells (Figure 14). Cell free media averaged 181 relative fluorescent units (RFUs), while titration media and cells in suspension averaged 106.8 RFUs, indicating the live cells were successfully metabolizing the alamar blue. After the copper titration, the RFUs decreased to 29 indicating a loss of cell viability. The observed cell death could be due to the combination of toxic concentrations of copper in the sample, with consistent stirring, exposing the cells to abnormal culture conditions.

Suspension titrations were also completed using the LSgill-e cell line in titration media. For the titration, the cells were suspended at a density of 500,000 cells/mL to a final volume of 25 mL with 67% live cells and 33% dead. The cell density was increased in comparison to the RTgill-W1 titration in Figure 13 above to try to achieve more (or any) Cu binding than what was observed in the RTgill-W1 titration. As can be seen in Figure 14 below, the titration was

performed using copper (II) sulfate concentrations of 10 µM, 20 µM, and 30 µM. Higher copper concentrations were used for the LSgill-e cell line for the first titration as it was noted that with the previous titration in Figure 13 above, higher concentrations were likely needed to achieve a stable signal. Cell density was also increased for this titration in comparison to the RTgill-W1 suspension titration to aid in stabilizing the electrode throughout the titration. The first copper addition, 10 µM was added to the LSgill-e cells in suspension and left to stabilize for ~2 hours. However, throughout these two hours, the electrode would not stabilize, so the concentration of copper was increased to 20 µM. The electrode still would not stabilize so the concentration was increased to 30 µM where the electrode still would not stabilize. Throughout the titration with each addition of copper, the signal, in mV should be increasing and not decreasing as observed. The sample was then acidified in order to determine the intercept of the Nernst equation to get an idea for the trend of this titration. Following the titration, the cells were only 3% live and 97% dead, counted using trypan blue and Countess cell counter for a live/dead cell count, which may indicate why the electrode would not stabilize.

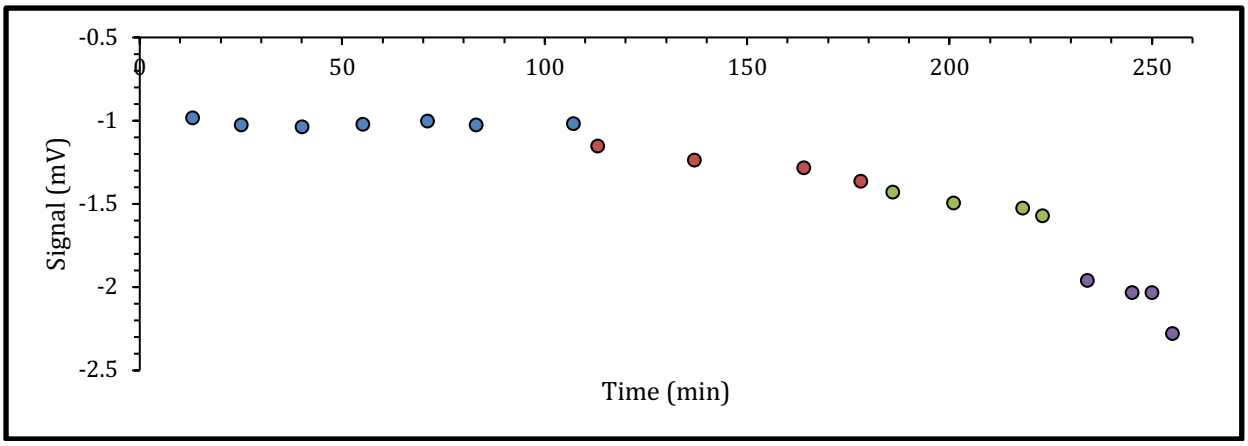


Figure 14. Cell suspension titration data for the LSgill-e cell line, as time after the first addition of copper versus the signal (mV). Copper (II) sulfate was added in three increments, 10µM (blue circles), 20µM (red circles), and 30µM (green circles) to a final volume of 25mL of LSgill-e cells at a cell density of ~500,000 cells/mL in titration media. pH was monitored and maintained at 7.7±0.05 throughout the titration and adjusted using nitric acid and sodium hydroxide. Following the last addition of copper, the sample was acidified to a pH of 3.9 (purple circles).

Cell viability was again measured using alamar blue before and after titration. For LSgill-e the RFUs in titration media alone was 168, and with cells the average RFUs were 893.3, indicating the LSgill-e cells were viable at the beginning of the titration. Following the titration, the RFUs were 30.5 indicating a loss of cell viability with 97% cell death over the duration of the titration period. Similar to RTgill-W1 this is most likely due to the stressful titration conditions.

Although the ISE would not stabilize, the amount of bound and free copper for the LSgill-e suspension could still be calculated as the sample was acidified. For each addition of copper, the average signal was used in the calculation as well as the average signal after acidification. This can be seen in Figure 15 below. In this figure, it can be seen that when the total copper concentration increased, the amount of free copper decreased. This is strongly indicative that this titration did not work. As the amount of total copper increased, the amount of Cu^{2+} decreased which should not happen as free copper should also be increasing (or remaining constant). However, when the total copper concentration added was 10 µM, the amount of free copper measured was 3.3×10^{-5} µM. When the total copper concentration added was 20 µM, the amount of free copper measured was 3.2×10^{-5} µM. When the total copper concentration added was 30 µM, the amount of free copper measured was 3.1×10^{-5} µM. However, for this titration the ISE would not stabilize and one main reason for this could be that the amount of total copper added was not enough. As mentioned previously in section 1.4, the signal from the ISE is dependent on the activities of the ion in solution (Štulík 2004). As the output signal measured is based on the measurement of the potential from the ion in solution, it is very likely that if higher concentrations of total copper were added, there would have been more success in stabilizing the electrode. This titration would need to be re-done in order to further understand

what was happening in the solution as the total copper concentration was increased. This would be great supplementary future work that would aid in further understanding of this research.

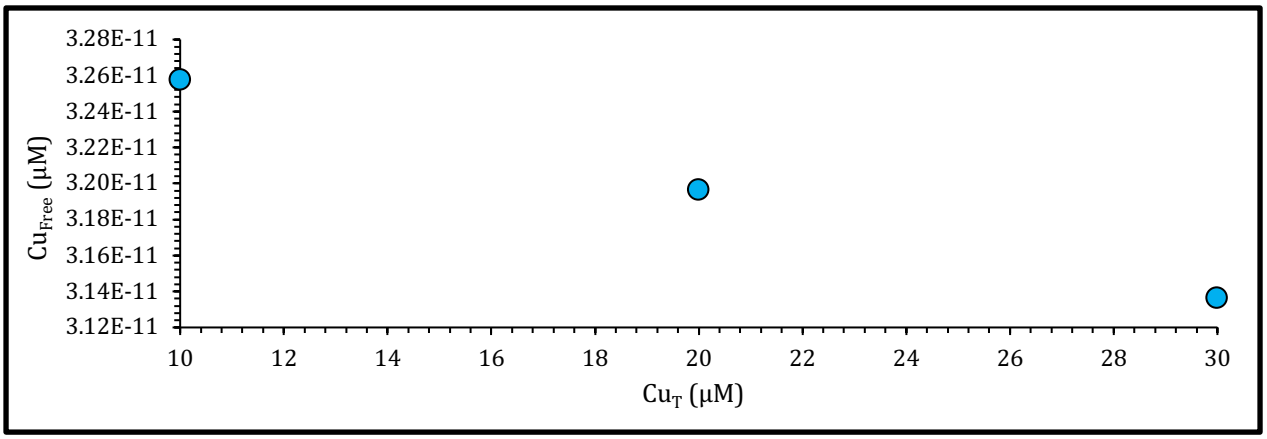


Figure 15. LSgill-e cell suspension titration data measured as the free ion, Cu^{2+} , plotted against the total copper in the sample. The blue circles represent the titrant addition of copper to titration media in concentrations of 10 µM, 20 µM and 30 µM.

In a previous paper by Smith *et al*. (2017), both primary rainbow trout gill cells in suspension and as reconstructed epithelia were successful with the ISE to examine free copper in all of the samples. However, in the present study, as mentioned earlier, the electrode would not stabilize for both the RTgill-W1 and LSgill-e cell lines in suspension so it was important to try alternate culture conditions to see if the electrode would stabilize and if reproducible results could be achieved. This can be seen below in Figure 16 for LSgill-e cells grown in a monolayer. It was determined that even after nearly 6 hours of copper additions to LSgill-e cells in a monolayer, the electrode still would not stabilize.

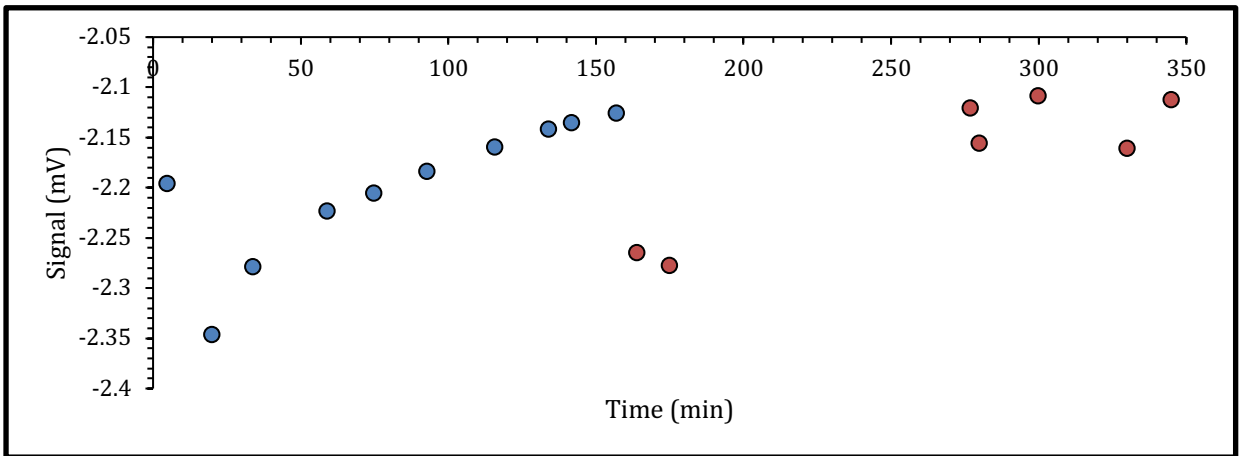


Figure 16. Cell monolayer titration data for the LSgill-e cell line, as time after the first addition of copper (II) sulfate versus the signal (mV). Copper (II) sulfate was added in two increments: 29 µM (blue circles), and 45 µM (red circles) to a final volume of 4 mL in a large petri dish in titration media with a cell density of ~ 500,000 cells/mL. pH was monitored and maintained at 7.7±0.05 throughout the titration and adjusted using nitric acid and sodium hydroxide.

After performing an ISE titration for cells in a monolayer and for cells in suspension, another method to determine the free concentrations of copper in samples had to be introduced, the graphite furnace. As can be seen in Appendix C, the ISE would stabilize for practice titrations consisting using just salicylic acid with various additions of copper (II) sulfate. This indicates that the ISE was functioning properly, and it was not stabilizing due to many possibilities mentioned previously including insufficient number of cells to stabilize the electrode, too low of copper concentrations, insufficient Cu^{2+} buffering, etcetera. Results and experiments for examining RTgill-W1 and LSgill-e samples using the graphite furnace can be seen below in section 3.3.

Once again, as the electrode consistently failed to stabilize, biotic ligand model parameters were unable to be determined. However, these were able to be determined in previous research by Smith *et al*. (2017) for primary rainbow trout gill cells in suspension. This can be indicative that for continuous cell lines, the binding capacity is not strong enough and therefore the electrode could not stabilize. This could indicate that cells from a continuous cell line are not

a good alternative to toxicity testing in place of primary gill cells or the whole organism. However, in order to make this conclusion certain, further experiments as discussed in section 4.5 below should be performed.

3.3 Graphite Furnace

A graphite furnace is a great tool for determining the concentration of metals in various samples. One important thing to note when analyzing samples using the graphite furnace is that the Beer-Lambert law needs to be applied and as such calibration curves were produced prior to the analysis of the samples. For the calibration curves, standards of known Cu concentrations, in titration media, were run through the graphite furnace and characteristic absorbance values to each individual standard were produced. From here, a standard calibration curve was produced as Cu concentration vs. absorbance. The concentration of free copper in the samples was then able to be calculated based on the Beer-Lambert law and the standard calibration curve (García and Báez 2012). For the monolayer samples, the sample solution was taken using a micropipette from the top of the monolayer, for the suspension samples, the cells were filtered and the filtered samples were measured using GFAAS and for the inserts, samples were taken from both the top and bottom of the insert using a micropipette. The concentration of free copper was calculated using the line of best fit from the standard curve and the absorbance value that was determined from the graphite furnace. This was completed for all samples analyzed using the graphite furnace. The solution that is measured in the graphite furnace is Cu_{Free} which can be found in the equation below.

$$Cu_T = Cu_{Bound} - Cu_{Free} \quad (4)$$

The concentration of measured free copper is indicative of Cu not bound to the gill cells in culture, and not ionic Cu^{2+} species specifically but rather all Cu species in solution.

3.3.1 Graphite Furnace Analysis for Cells in a Monolayer

To begin, the copper binding to both RTgill-W1 and LSgill-e cell lines was examined for cells in a monolayer. The calibration curve for the RTgill-W1 cells in a monolayer can be seen below in Figure 17. Working standards with copper concentrations of 0 µM, 2 µM, 4 µM, 6 µM, and 8 µM were analyzed and from here the calibration curve was produced.

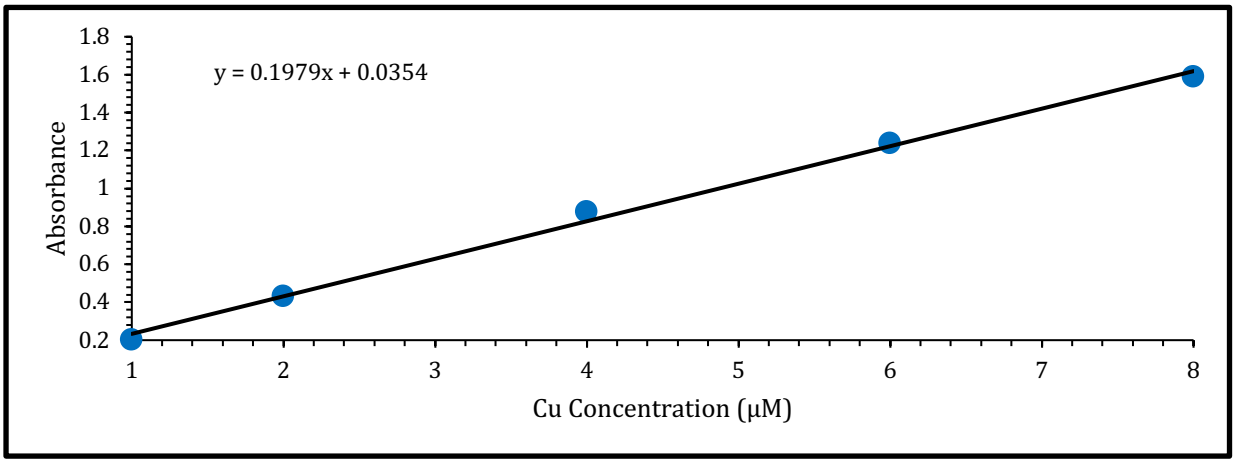


Figure 17. Calibration curve for RTgill-W1 cells in a monolayer with $CuSO_4$ concentrations of 1µM, 2 µM, 4 µM, 6 µM, and 8 µM analyzed using a graphite furnace. All samples were acidified using HNO_3 to 0.2% in titration media. The calibration curve had an R^2 value of 0.9965 and a line of best fit equation y=0.1979x+0.0354.

As can be seen below in Figure 18, copper binding to the RTgill-W1 cell line was observed for the cells in a monolayer. With the addition of all three concentrations of copper, binding was observed as all three samples had free copper concentrations below the 1:1 line indicating that the RTgill-W1 continuous cell line cells do offer some binding power. As can be seen in Table 11, all three samples had bound copper. The concentration of bound copper to the cells was determined by subtracting the measured free [Cu] from the total [Cu] added to the monolayer. However, the concentration of copper per cell was very low. With a cell density of 340,000 cells/well it was hypothesized that the amount of [Cu]/cell would be much higher which was not the case. In all three of these samples the majority of the copper added to the samples

remained free. This data indicates that the binding capacity of the RTgill-W1 cells in monolayer is much less than that of primary gill cells as will be discussed later on in this section.

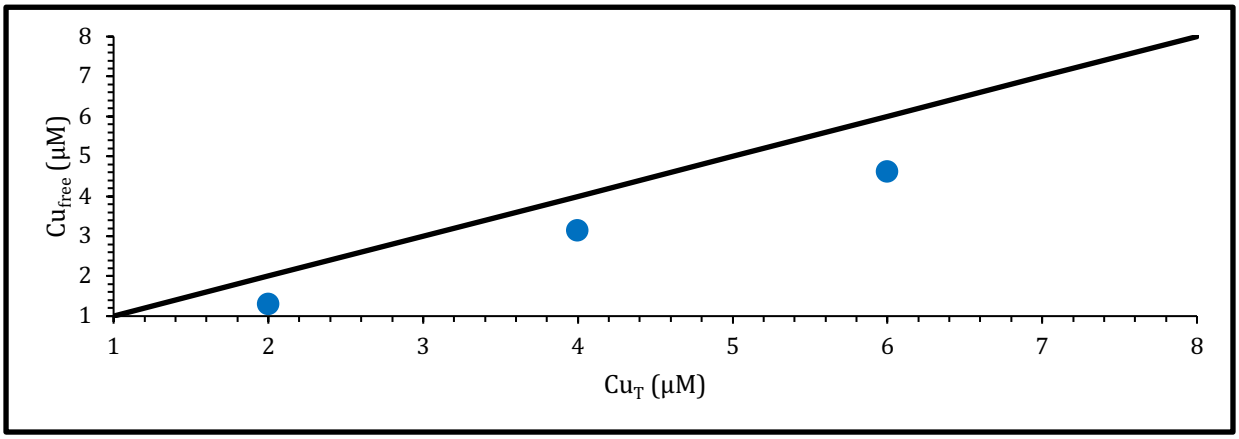


Figure 18. Graphite furnace analysis for copper binding to RTgill-W1 cells in a monolayer. Three different concentrations of $CuSO_4$ were added to RTgill-W1 cells (~340,000 cells/mL) in a monolayer and left to incubate for three hours before the free concentration of copper in solution was analyzed using the graphite furnace. The black line indicates the 1:1 line of free Cu to total Cu.

Table 11. GFAAS results for copper binding to RTgill-W1 cells in monolayer.

Total [Cu] Added (µM)	Measured Free [Cu] (µM)	Bound [Cu] (µM)	[Cu]/Cell (µM)
2	1.29	0.70	2.1×10^{-6}
4	3.14	0.86	2.5×10^{-6}
6	4.61	1.39	4.1×10^{-6}

After analyzing copper binding to the RTgill-W1 cells in monolayer, copper binding to the LSgill-e cell line was analyzed. The calibration curve for the LSgill-e cells in a monolayer can be seen below in Figure 19. Working standards with copper concentrations of 1 µM, 2 µM, 4 µM, and 6 µM were analyzed and from here the calibration curve was produced.

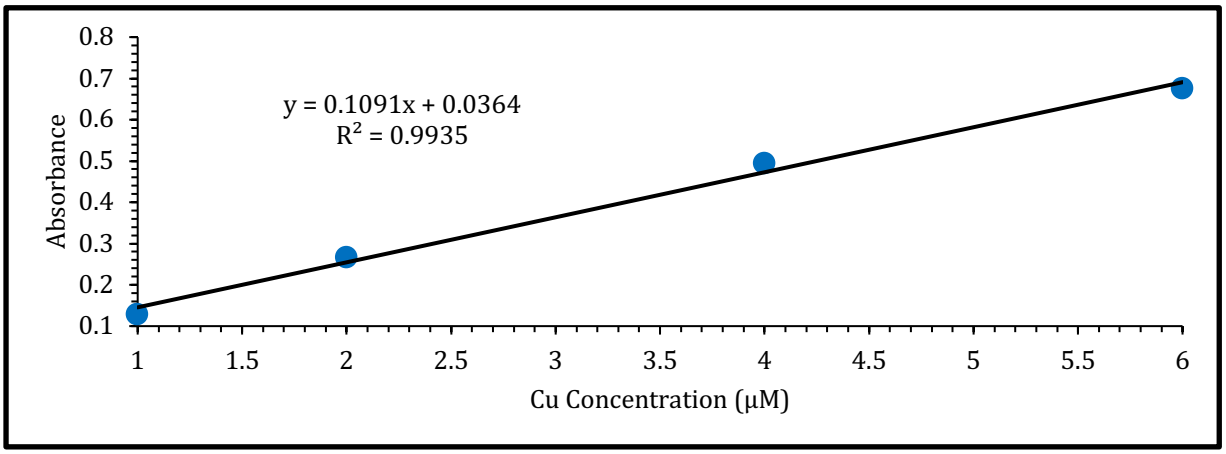


Figure 19. Calibration curve for LSgill-e cells in a monolayer with CuSO4 concentrations of 1µM, 2 µM, 4 µM, and 6 µM analyzed using a graphite furnace. All samples were acidified using HNO_3 to 0.2% in titration media. The calibration curve had an R^2 value of 0.9935 and a line of best fit equation y=0.1091x+0.0364.

For the LSgill-e cell line, cells were plated at a density of 340,000 live cells/well in L-15 media the day prior to analysis. The following day, the L-15 media was removed, the cells were thoroughly rinsed with titration media, prior to being exposed to copper (II) sulfate concentrations of 2 µM, and 4 µM in titration media for an incubation period of three hours. Following incubation, the media was taken from the LSgill-e cells and acidified to 0.2% using nitric acid and the samples were then ready for analysis.

As can be seen below in Figure 20, copper binding to the LSgill-e cell line was observed for the cells in a monolayer. With the addition of two different copper concentrations, binding was observed as both samples had free copper concentrations below the 1:1 line, which could be an indication that this continuous cell line has a copper binding site(s). As can be seen in Table 12, both samples had bound copper, once again suggesting some binding capacity of the LSgill-e cell line. Similar to the RTgill-W1 cell line, the majority of the copper added was measured as free, unbound copper possibility indicating weak, but some, binding capabilities. When higher concentrations of copper were added to the sample, more copper was determined to be bound to the cells which was expected.

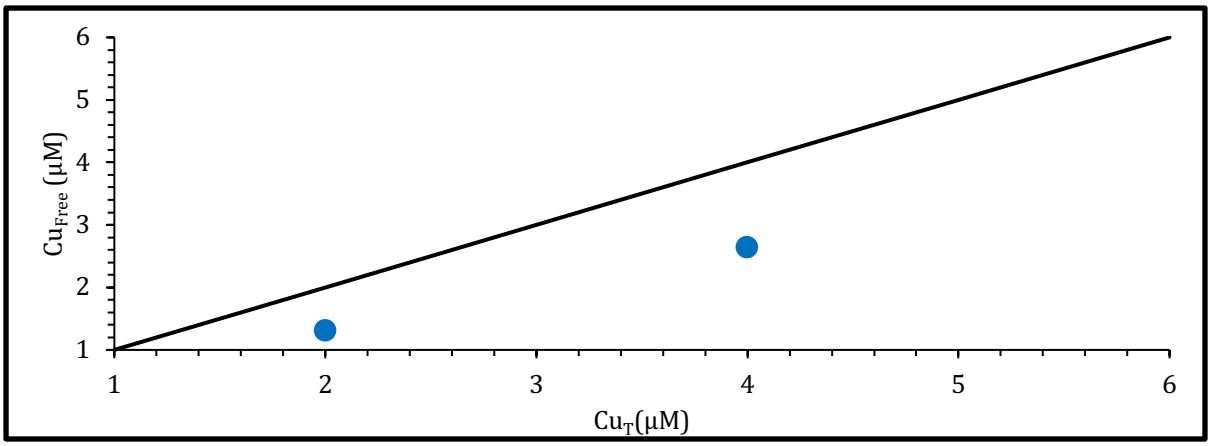


Figure 20. Graphite furnace analysis for copper binding to LSgill-e cells in a monolayer. Two different concentrations of $CuSO_4$ were added to LSgill-e cells (~340,000 cells/mL) in a monolayer and left to incubate for three hours before the free concentration of copper was analyzed using the graphite furnace. The black line indicates the 1:1 line of free Cu to total Cu.

Table 12. GFAAS results for copper binding to LSgill-e cells in monolayer.

Total [Cu] Added (µM)	Measured Free [Cu] (µM)	Bound [Cu] (µM)	[Cu]/Cell (µM)
2	1.30	0.70	2.1×10^{-6}
4	2.64	1.36	4.0×10^{-6}

When comparing copper binding for both the RTgill-W1 and LSgill-e cell line from Figures 18 and 20 above, when 2 µM of total copper was added, the amount of [Cu]/cell remained the same for both the RTgill-W1 and LSgill-e cell lines. However, when 4 µM of total copper was added, the LSgill-e cell line had a much greater concentration of copper bound per cell at 4.0×10^{-6} µM Cu/cell where the RTgill-W1 cell line had only 2.5×10^{-6} µM Cu/cell. This is an indication that the LSgill-e cells in monolayer had a higher binding capacity than that of the RTgill-W1 cell line for this experiment. The above experiments were only completed to examine whether or not the graphite furnace would work for these experiments. As there was great success, more experiments were performed with more samples and concentrations which can be found below.

The calibration curve for the next experiment, seen below in Figure 21, was used for analyzing the samples for both the RTgill-W1 and LSgill-e cells in monolayer. This sample data can be seen below in Figure 22, and Figure 23. The line of best fit equation y=0.0852x+0.0797 was used to determine the concentrations of free copper found in each sample and from here, the concentrations of bound copper and bound copper/cell was able to be determined.

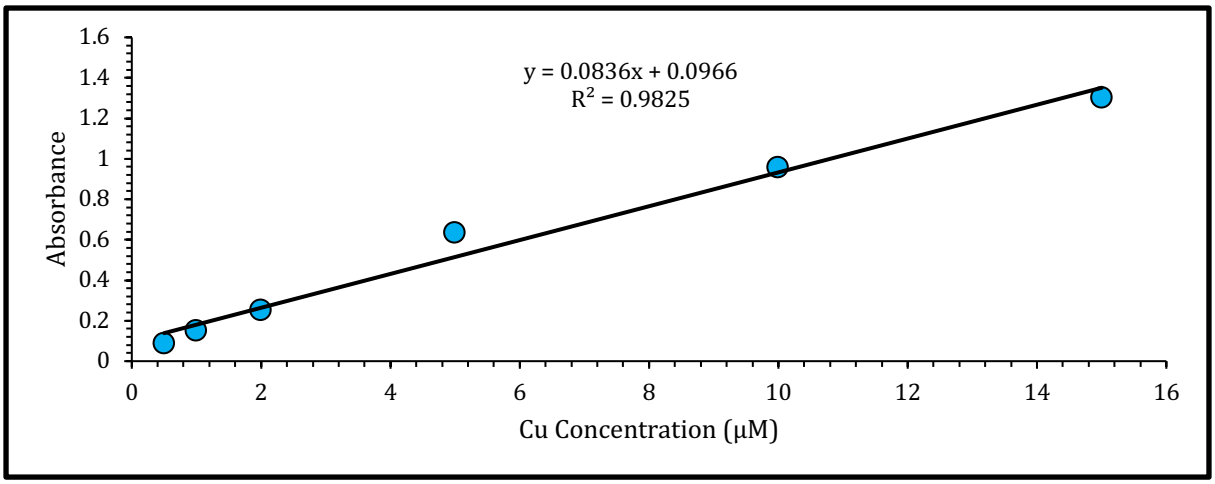


Figure 21. Calibration curve for determining free copper concentrations in RTgill-W1 and LSgill-e monolayer samples. All standards were acidified using HNO_3 to 0.2% in titration media. The calibration curve had an R^2 value of 0.9832 and a line of best fit equation y=0.0852x+0.0797.

For the RTgill-W1 cell line, cells were plated at a density of 493,000 cells/mL with 90% live and 10% dead, in L-15 media the day prior to analysis. The following day, the L-15 media was removed, the cells were thoroughly rinsed with titration media, prior to being exposed to copper (II) sulfate concentrations from 1 µM – 17.5 µM in titration media for an incubation period of three hours. Following incubation, the media was taken from the RTgill-W1 cells and acidified to 0.2% using nitric acid and the samples were then ready for analysis. This sample analysis can be seen in Figure 22 below. The black line indicates the 1:1 line for the total amount of copper added to the concentration of free copper in the sample. As all data is below this 1:1 line, this is an indication that there was bound copper to the RTgill-W1 cell line. The

concentrations of bound copper can be seen below in Table 13. However, one major problem to this experiment was that in addition to bound copper to the cell line(s), copper binding was also observed to the vessel. In future research, which will be discussed further in section 4.6 this experiment needs to be performed with a vessel that would not bind copper as it is uncertain as to what copper was bound to the vessel and what copper was bound to the RTgill-W1 cells.

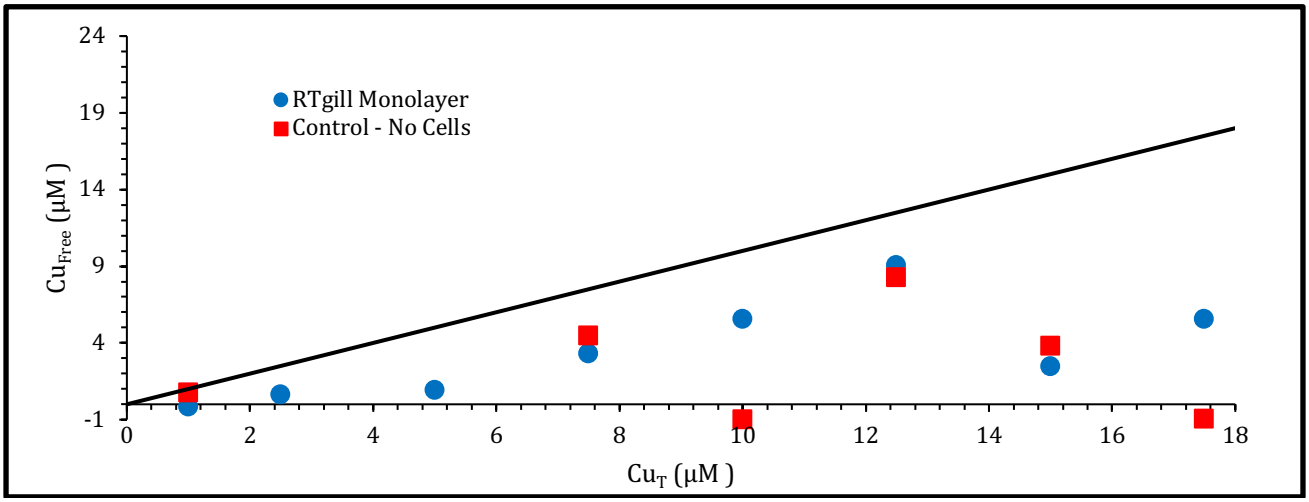


Figure 22. Graphite furnace analysis for copper binding to RTgill-W1 cells in a monolayer. $CuSO_4$ was added from 1 µM – 17.5 µM to the cells in the monolayer in titration media at a density of 493,000 cells/mL with an incubation period of three hours before analyzing the free copper concentration using the graphite furnace. The black line indicates the 1:1 line of free Cu to total Cu, the blue circles represent free Cu not bound to the RTgill-W1 cells.

Table 13. GFAAS results for copper binding to RTgill-W1 cells in monolayer.

Total [Cu] Added (µM)	Measured Free [Cu] (µM)	Bound [Cu] (cells) (µM)	Bound [Cu] (Vessel) (µM)	[Cu]/Cell (µM)
1	Below limit of detection (LOD)	1.17	0.266	2.38×10^{-6}
2.5	0.63	1.87	3.63	3.80×10^{-6}
5	0.89	4.11		8.34×10^{-6}
7.5	3.30	4.20	3.05	8.52×10^{-6}
10	5.53	4.47	10.99	9.06×10^{-6}
12.5	9.04	3.46	8.27	7.01×10^{-6}
15	2.46	12.54	3.81	2.54×10^{-5}
17.5	5.54	11.96	18.47	2.43×10^{-5}

For the LSgill-e cell line, cells were plated at a density of 317,000 cells/mL with 83% live and 17% dead, in L-15 media the day prior to analysis using the graphite furnace. The following day, the L-15 media was removed, the cells were thoroughly rinsed with titration

media, prior to being exposed to copper (II) sulfate concentrations from 1 µM – 17.5 µM in titration media for an incubation period of three hours. Following the incubation period, the media was taken from the LSgill-e cells and acidified to 0.2% using nitric acid and the samples were then ready for analysis. This can be seen in Figure 23 below. Similar to the RTgill-W1 cell line, all data can be observed below the 1:1 line, indicating copper binding to the LSgill-e cell line, all concentrations of total, bound, free and [Cu]/cell can be seen below in Table 14. Copper was still observed to be bound to the vessel, which would need to be corrected in future experiments.

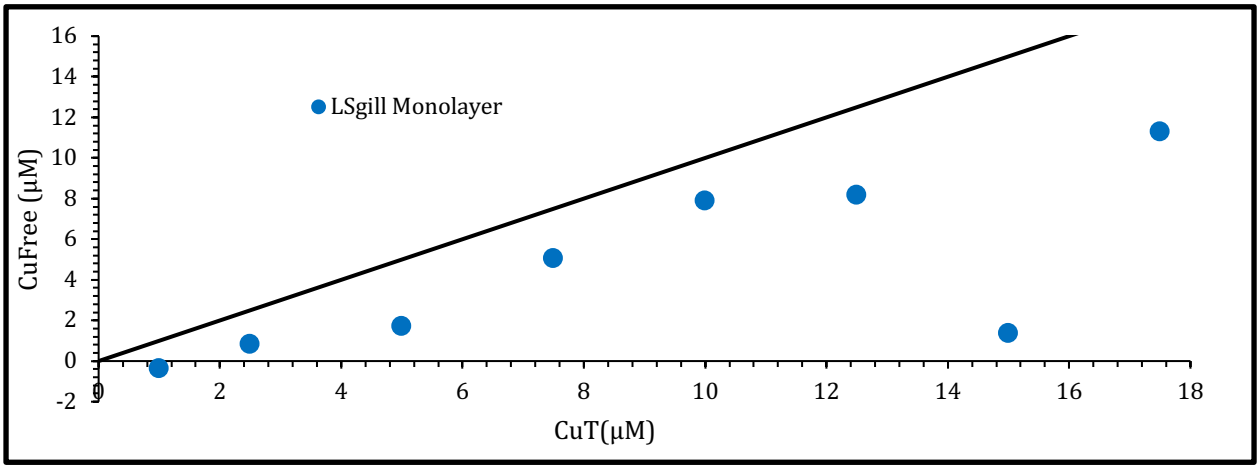


Figure 23. Graphite furnace analysis for copper binding to LSgill-e cells in a monolayer. $CuSO_4$ was added from 1 µM – 17.5 µM to the cells in the monolayer in titration media at a density of 317,000 cells/mL with an incubation period of three hours before analyzing the free copper concentration using the graphite furnace. The black line indicates the 1:1 line of free Cu to total Cu, the blue circles represent free Cu not bound to the LSgill-e cells.

Table 14. GFAAS results for copper binding to LSgill-e cells in monolayer.

Total [Cu] Added (µM)	Measured Free [Cu] (µM)	Bound [Cu] (µM)	Bound [Cu] (Vessel) (µM)	[Cu]/Cell (µM)
1	Below LOD	1.36	0.266	4.29×10^{-6}
2.5	0.84	1.66	3.63	5.25×10^{-6}
5	1.70	3.30		1.04×10^{-6}
7.5	5.05	2.45	3.05	7.72×10^{-6}
10	7.89	2.11	10.99	6.65×10^{-6}
12.5	8.18	4.32	8.27	1.36×10^{-6}
15	1.36	13.64	3.81	4.30×10^{-5}
17.5	11.28	6.22	18.47	1.96×10^{-5}

Binding to the vessel was observed for both the RTgill-W1 and LSgill-e cell lines, which would need to be taken into account in future experiments. Both the RTgill-W1 and LSgill-e cell lines did exhibit copper binding, however, the extent of this binding was unknown due to the binding of copper to the vessel as well as the cells. In Table 15 below, a comparison of the free copper and bound copper in each sample can be seen compared for both the RTgill-W1 and LSgill-e cell lines. For this study, the amount of bound copper was important to examine as this is what is thought to cause toxic effects to the organism. Higher concentrations of bound copper would indicate stronger binding capabilities to the cell line and the possibility to use continuous cell lines for toxicity testing in place of primary or whole organism testing. When comparing the binding capabilities for both cell lines, there is no distinct trend. The LSgill-e cell line bound more copper at copper concentrations of 1 µM, 12.5 µM, and 15 µM whereas the RTgill-W1 cell line had more bound copper at concentrations of 2.5 µM, 5 µM, 7.5 µM, 10, and 17.5 µM. Therefore, no strong conclusion could be drawn from this experiment as to what cell line should stronger copper binding capacities.

Table 15. Comparing free copper and bound copper from the RTgill-W1 and LSgill-e cells in a monolayer.

Total [Cu] Added (µM)	**Measured Free [Cu] (µM)**		**Bound [Cu] (µM)**	
	RTgill-W1	LSgill-e	RTgill-W1	LSgill-e
1	Below LOD	Below LOD	1.17	1.36
2.5	0.63	0.84	1.87	1.66
5	0.89	1.70	4.11	3.30
7.5	3.30	5.05	4.20	2.45
10	5.53	7.89	4.47	2.11
12.5	9.04	8.18	3.46	4.32
15	2.46	1.36	12.54	13.64
17.5	5.54	11.28	11.96	6.22

As previously mentioned, the control used when analyzing cells in a monolayer with the graphite furnace were wells in a cell culture plate that had no cells (no monolayer) with the

addition of copper. It was then determined that this control would not be adequate as the surface of the cell culture plate is a much different surface than the cell culture plate when a cell monolayer is present. Thus, the control could have bound copper much more or much less, and in different ways than when cells were present. Ultimately, it was determined that this control was not an adequate control as a cell culture plate alone, with no cells, has a much different surface that is not exposed during titration when cells are present. Due to this, data modelling could be performed using the data from Figures 22 and 23 above. For the RTgill-W1 cell line, model fits were performed using both pavement cells (PVCs) and mitochondrial rich cells (MRCs) from primary rainbow trout gill cells. This was used as the model for this study as it had not yet been performed for cells from a continuous cell line, calculated using parameters from Smith *et al*. (2017). The MRC and PVC bound calculations were done assuming the same number of cells as the RTgill-W1 tests. The LSgill-e analysis was completed at a lower cell density due to the size and growth rate of the cell line. It can be seen below in Figure 24 that neither the RTgill-W1 or LSgill-e cell line performed like either of the predicted models. This was strongly indicative that the continuous cell lines do not have the same binding capacity and much weaker, than that of primary rainbow trout gill cells. When looking at primary rainbow trout gill cell cultures, they are composed mainly of PVC (~85%) and MRCs compose only about 15% of gill cells (Bury et al. 2014). Both MRCs and PVCs are said to be involved in ion regulation, however, MRCs play a more important role in these processes (Evans et al. 2005; Lai et al. 2015). Due to the role of MRCs in ionoregulation it can be hypothesized that they would play an important role in the binding of copper in comparison to PVCs (Smith et al. 2017). As seen in Figure 24 below, the MRC model (solid black line) for primary rainbow trout gill cells, has much more bound copper than then PVC model (solid red line) which was expected as

previously discussed. When looking at the monolayer data for both the RTgill-W1 and LSgill-e cell lines, the majority of the samples (with the exception of three that had equivalent binding to primary PVC rainbow trout gill cells), showed much less copper binding than both PVCs and MRCs. All of the continuous cell line culture samples had an order of magnitude less binding than the MRCs. This was significant as it is MRCs that are said to be important for copper binding, an indication that both the RTgill-W1 and LSgill-e continuous cell lines would not be a good alternative to primary gill cell testing. The majority of cell line samples were also below the PVC cell culture model indicating very, very weak copper binding. When looking at both the RTgill-W1 and LSgill-e cell lines, it can be said that the RTgill-W1 cell line (blue squares) showed slightly more binding capability than the LSgill-e cell line (orange circles) as the majority of the samples examined were closer to the PVC model than the LSgill-e cells.

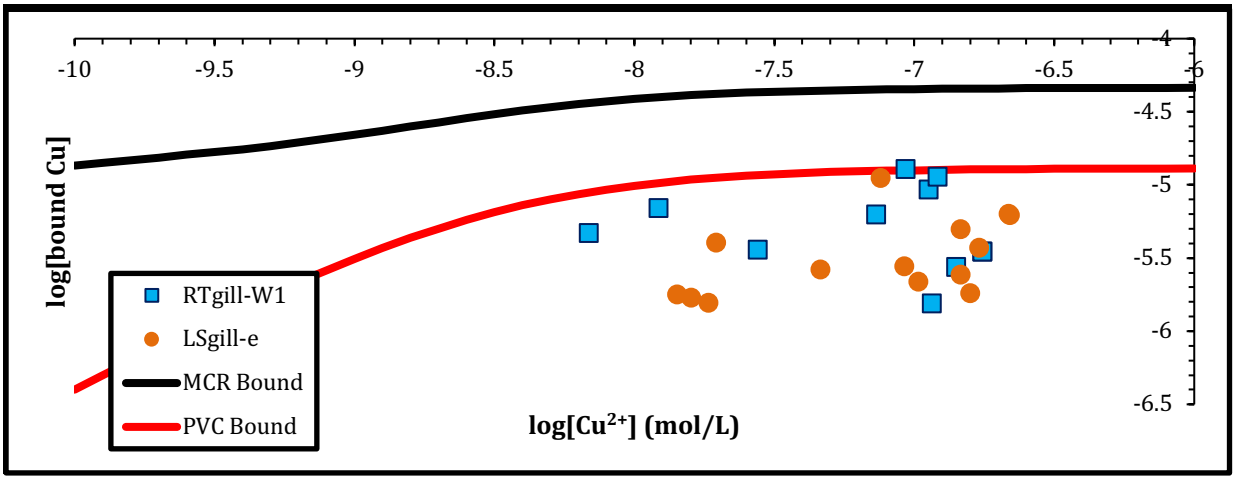


Figure 24. Copper titration curve as measured free copper versus calculated bound concentration of copper. The blue squares represent RTgill-W1(plating density = 493,000) titrations and the orange circles represent LSgill-e titrations (plating density = 317,000). The solid black line represents the mitochondrial rich cell (MRC) model from primary rainbow trout gill cells and the solid red line indicates the pavement cells (PVC) model from primary rainbow trout gill cells calculated using parameters from Smith *et. al*. (2017).

3.3.2 Graphite Furnace Analysis for Cells in Suspension

Next, copper binding was examined for both RTgill-W1 and LSgill-e cells in suspension. The calibration curve used for analyzing all the samples for cells in suspension experiments can be seen below in Figure 25. Working standards with copper concentrations of

0.5 µM, 1 µM, 2 µM, 5 µM, 10 µM, and 15 µM were analyzed and from here the calibration curve was produced. The calibration curve has an R^2 value of 0.9832 and a line of best fit equation of y=0.0852x+0.0797 was used to determine the concentrations of free copper found in each sample and from here, the concentrations of bound copper and bound copper/cell was able to be determined.

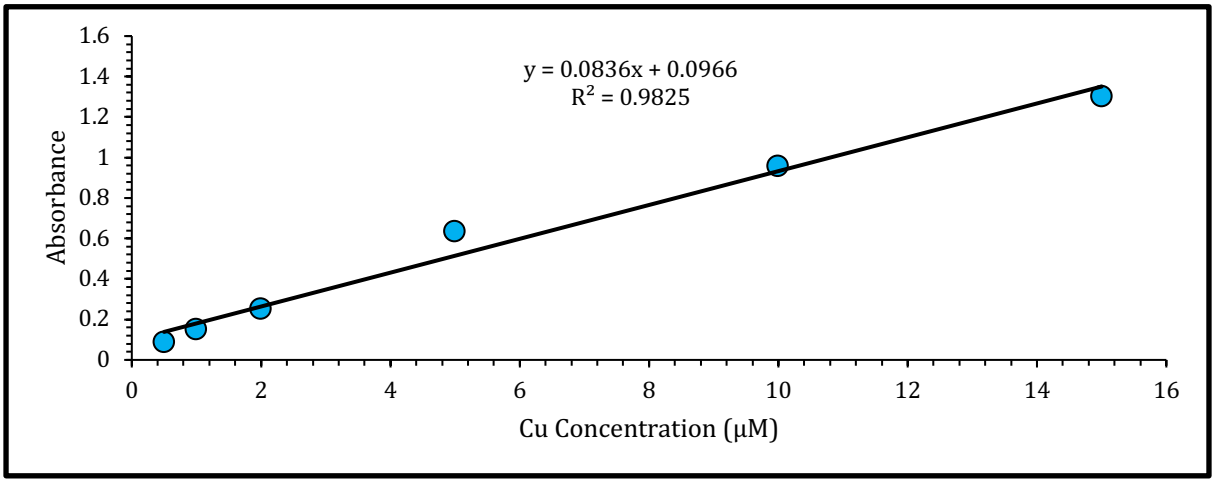


Figure 25. Calibration curve for determining free copper concentrations in RTgill-W1 and LSgill-e suspension samples. All standards were acidified using HNO_3 to 0.2% in titration media prior to analysis. The calibration curve had an R^2 value of 0.9832 and a line of best fit equation y=0.0852x+0.0797.

As can be seen below in Figure 26, the black line indicates the 1:1 line for the total amount of copper added to the concentration of free copper in the sample, indicating that all RTgill-W1 samples analyzed in this experiment bound copper due to being below this 1:1 line. The exact concentrations of measured free copper, bound copper and the concentration of copper/cell can be seen in Table 16. As can be seen in column 3, as the concentrations of total copper increased, the concentration of bound copper also increased. However, also performed in this experiment was the addition of copper to samples containing no RTgill-W1 cells, only titration media, as a control. These samples can be seen below in Figure 26 (red squares). When looking at this data, it was found that there was a significant amount of copper binding to the actual vessel and that the binding observed in the sample with RTgill-W1 cells, it cannot be said

whether this copper was actually bound to the cells or to the vessel, or both. For example, when 2.5 µM of total copper was added to the sample, 1.5 µM was analyzed to be bound to the cell line. However, when the experiment was performed with media alone and no cells in the sample, the concentration of bound copper was determined to be 3.24 µM. This is not possible as there cannot be more copper bound to the vessel than what was added to the sample. In order to accurately determine how much copper was bound to the RTgill-W1 cells, a vessel needs to be used that does not bind copper, such as Teflon which is discussed in further detail in section 4 below. The concentration of copper/cell can also be seen below in Table 16; however, this may not be entirely accurate as once again, it cannot be certain whether or not this copper was bound to the vessel or to the RTgill-W1 cell line. It was also found that after the incubation period, the live/dead cell counted dropped drastically to 47% live and 53% dead in the sample with 15 µM copper. This loss of cell viability could also be why cell binding was much lower than expected.

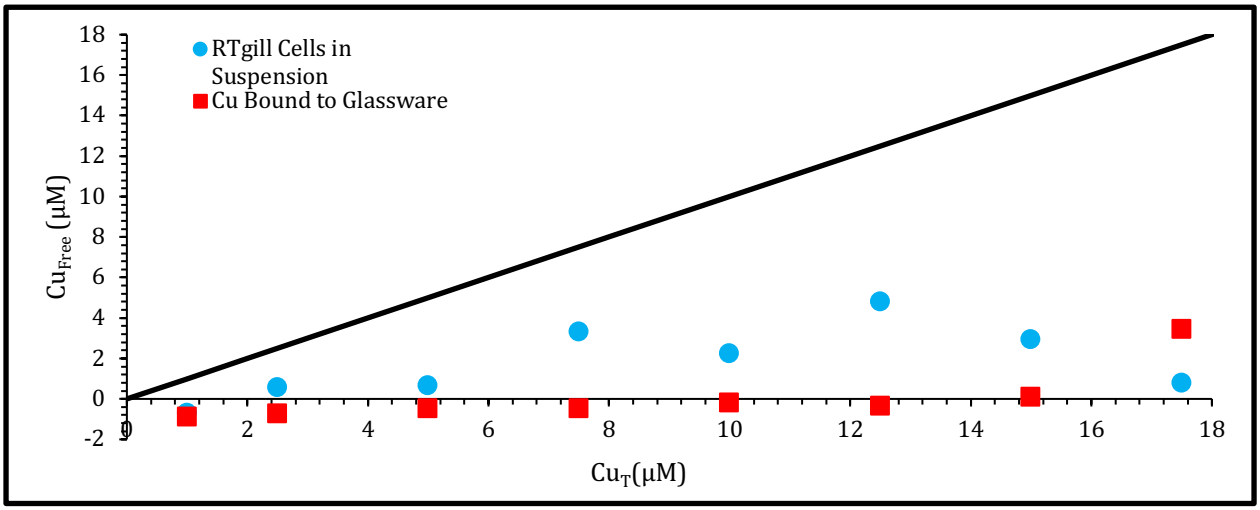


Figure 26. Graphite furnace analysis for copper binding to RTgill-W1 cells in suspension. Increasing concentrations of $CuSO_4$ were added to the RTgill-W1 (~ 500,000 cells/mL) cells in suspension and left to incubate for three hours prior to analysis. The black line indicates the 1:1 line of free Cu to total Cu, free [Cu] (blue circles) and bound [Cu] to microcentrifuge tube with no cells present (red circles).

Table 16. GFAAS results for copper binding to RTgill-W1 cells in suspension.

Total [Cu] Added (µM)	Measured Free [Cu] (µM)	Bound [Cu] (µM)	Bound [Cu] (Vessel) (µM)	[Cu]/Cell (µM)
1	Below LOD	1.69	1.89	3.78×10^{-6}
2.5	Below LOD	1.50	3.24	5.0×10^{-6}
5	0.550	4.33	5.48	10.0×10^{-6}
7.5	0.668	4.18	7.99	15.0×10^{-6}
10	3.320	7.77	10.21	20.0×10^{-6}
12.5	2.235	7.71	12.85	25.0×10^{-6}
15	2.921	12.08	14.93	3.0×10^{-5}
17.5	0.768	16.73	14.08	3.5×10^{-5}

As can be seen below (Figure 27), the blue circles represent the concentration of free copper in the samples with the LSgill-e cells while the red squares represent the concentration of free copper in the samples with no LSgill-e cells, only copper added to titration media. Similar to the RTgill-W1 cell line, the exact concentrations of free copper, bound copper and concentration of copper/cell can be found in Table 17 below.

When looking at the concentrations of free copper in the LSgill-e samples, there is none. This is an indication of strong binding capabilities of cells from the LSgill-e cell line. This can also be verified when looking at the concentrations of bound copper to the cells. It can be seen that in Table 17, that in a lot of the samples (samples with 1µM, 2.5 µM, 5 µM, 7.5 µM, 10 µM, and 15 µM of copper added) the amount of bound copper is greater than the total concentration of copper that was added to the sample, which is impossible. This could be due to human error when adding the concentrations of total copper to the samples, or due to incorrect analysis of free copper in the samples. Also similar to the RTgill-W1 cell line, a large amount of copper binding to the vessel was observed which will be discussed in further detail in section 4.

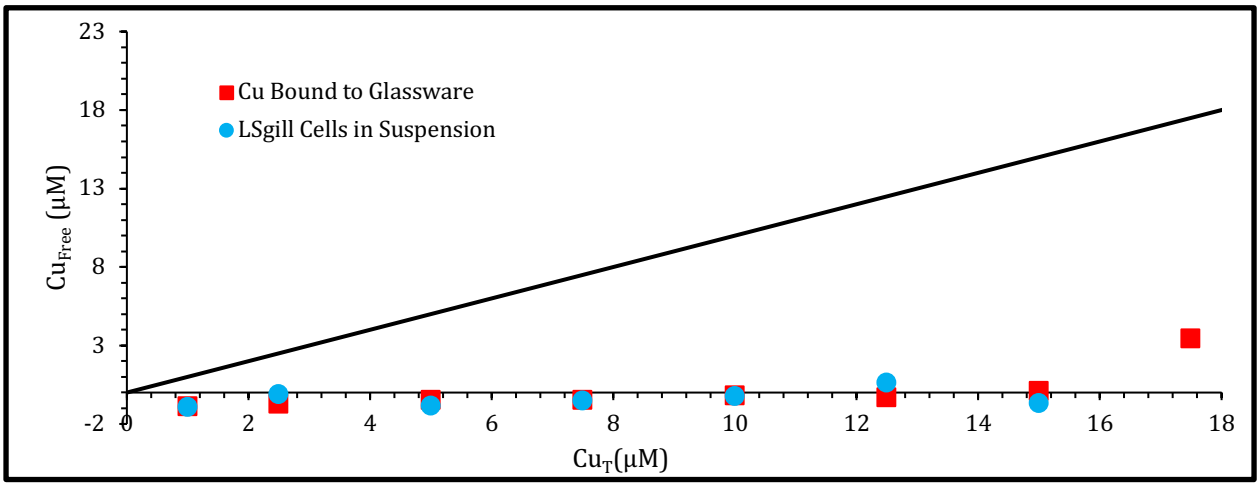


Figure 27. Graphite furnace analysis for copper binding to LSgill-e cells in suspension. Increasing concentrations of $CuSO_4$ were added to the LSgill-e cells in suspension (~ 400,000 cells/mL) and left to incubate for three hours prior to analysis. The black line indicates the 1:1 line of free Cu to total Cu, free [Cu] (blue circles) and bound [Cu] to glassware with no cells present (red circles).

Table 17. GFAAS results for copper binding to LSgill-e cells in suspension.

Total [Cu] Added (µM)	Measured Free [Cu] (µM)	Bound [Cu] (µM)	Bound [Cu] (Vessel) (µM)	[Cu]/Cell (µM)
1	Below LOD	1	1.89	2.5×10^{-6}
2.5	Below LOD	2.61	3.24	6.25×10^{-6}
5	Below LOD	5.85	5.48	12.5×10^{-6}
7.5	Below LOD	8.04	7.99	18.75×10^{-6}
10	Below LOD	10.26	10.21	25×10^{-6}
12.5	0.20	11.88	12.85	31.25×10^{-6}
15	Below LOD	15.70	14.93	3.75×10^{-5}

When comparing the concentrations of free and bound copper between the RTgill-W1 and LSgill-e cell lines, and not taking into account the binding to the vessel, it can be said that the LSgill-e cell line has a stronger affinity for copper than the RTgill-W1 cell line. This was observed when looking at the free copper, as the RTgill-W1 cell line samples had a larger concentration of free copper in each sample compared to the LSgill-e cell line and the calculated concentrations of bound copper per sample was much higher in the LSgill-e cell line compared to that in the RTgill-W1 cell line. This also stands true when looking at the concentration of copper/cell which was much greater for the LSgill-e cell line than the RTgill-W1 cell line.

However, as mentioned previously, this needs to be further investigated with a vessel that has no binding affinity for copper to truly get sufficient results.

3.3.3 Graphite Furnace Analysis for Cells in a Cell Culture Insert

After analyzing both the RTgill-W1 and LSgill-e samples in a monolayer and in suspension using the graphite furnace, both cell lines were examined in a cell culture insert, as cultured epithelia. As mentioned in section 1.3, cells in a cell culture insert should more closely resemble that of a living fish in comparison to cells in a monolayer or suspension. For these experiments, the cells were plated in a cell culture insert where both the apical and basolateral side of the cells had access to media. The cells were grown in the inserts for 21 days where the TEER (transepithelial electrical resistance) values were monitored consistently throughout the experiment. Measuring TEER values is a widely used technique to measure the integrity of tight junctions in cell culture models (Srinivasan et al. 2015).

For these insert experiments, the inserts used were 75mm Transwell with 0.4 μm pore polycarbonate membrane insert, TC-treated from Sigma-Aldrich Canada Co. These inserts were quite large with a surface area of 44 cm^2. Typically, when the RTgill-W1 cell line is grown in cell culture insert, they have a stable TEER value of ~200 $\Omega \cdot cm^2$ in a culture area of 0.47cm^2 (Schnell et al. 2016). As can be seen in Figure 28 below, for the RTgill-W1 cell line, stable, reproducible TEER values were observed after 21 days, staying very consistent with the TEER value at 14 days of ~1700 $\Omega \cdot cm^2$. Due to the consistent TEER values for the RTgill-W1 cell line after 21 days, the experiment began after 21 days of RTgill-W1 cells in culture. The LSgill-e cells were also grown in the same cell culture inserts with a surface area of 44 cm^2. The LSgill-e cell line has never previously been grown in cell culture inserts so there is no indication of a typical TEER value for this specific cell line. The LSgill-e cells were grown in the cell culture

insert for 21 days, the same duration as with the RTgill-W1 cell line which can be seen in Figure 29 below. It was observed that the TEER values for the LSgill-e cell line were not as high or stable as the RTgill-W1 cell line however after 21 days, TEER values were consistent around ~1600 $\Omega \cdot cm^2$. This could be an indication of a stronger monolayer for the RTgill-W1 cell line than LSgill-e cell line, with more tight junctions *ie*. a less leaky monolayer. This will be further examined below as copper (II) sulfate was added to both cell lines in these cell culture inserts.

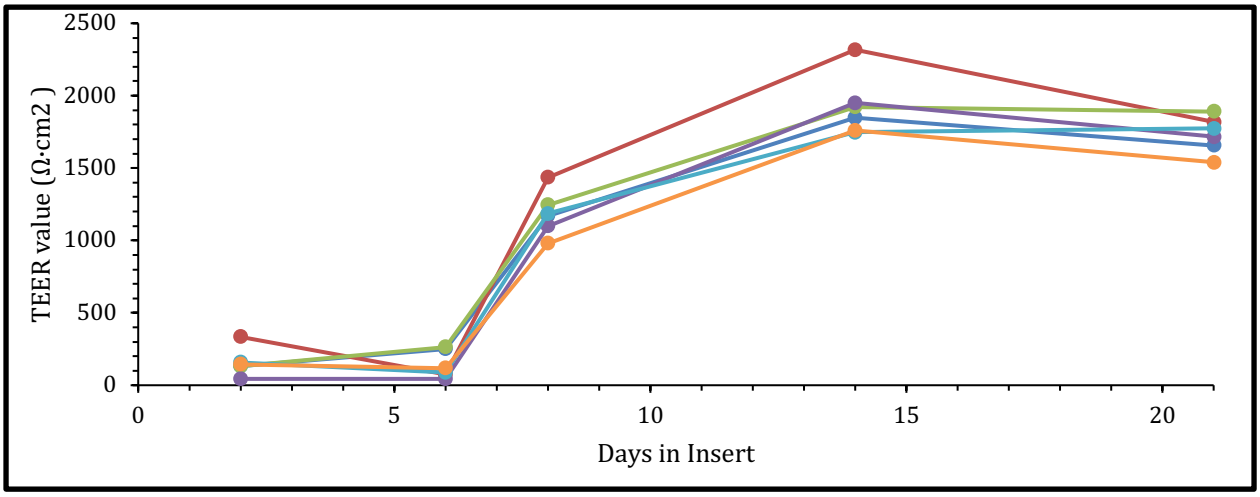


Figure 28. TEER values of RTgill-W1 cells in a 44 cm^2 cell culture insert. The graph displays the TEER values for six replicates of RTgill-W1 cells in cell culture inserts taken at day 2, 6, 8, 14, and 21 after plating the cells in the inserts at day 0 at a cell density of 3.77×10^6 cells/mL. Both the apical and basolateral portions of the insert were L-15 media supplemented with 10% FBS and 1% penstrep until the time of the experiment where the apical surface media was changed to titration media.

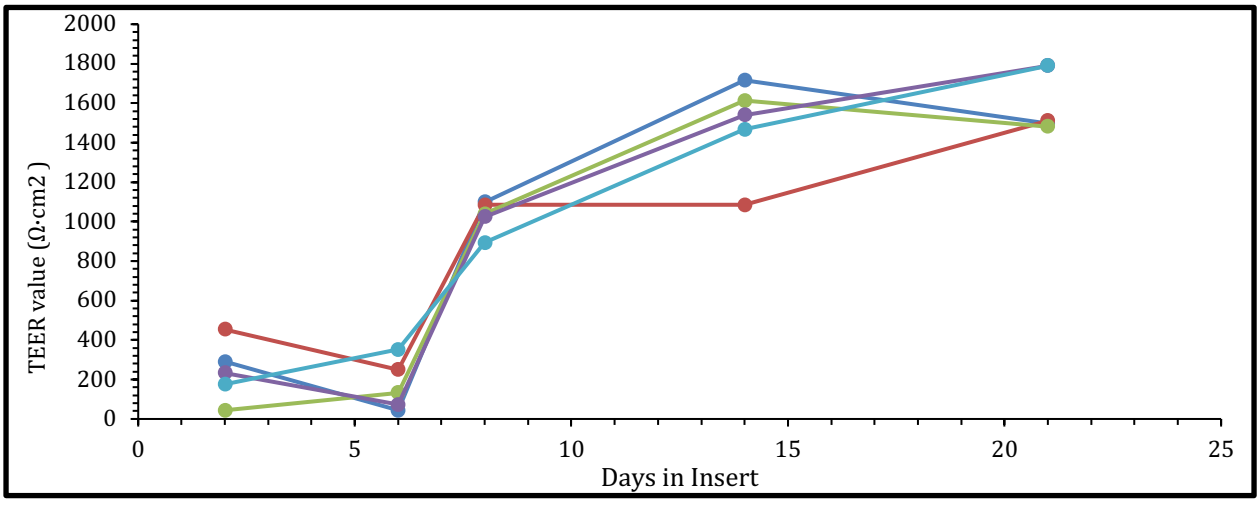


Figure 29. TEER values of LSgill-e cells in a 44 cm2 cell culture insert. The graph displays the TEER values for five replicates of LSgill-e cells in cell culture inserts taken at day 2, 6, 8, 14, and 21 after plating the cells in the inserts at day 0 at a cell density of 1.47×10^6 cells/mL. Both the apical and basolateral portions of the insert were L-15 media supplemented with 10% FBS and 1% penstrep until the time of the experiment where the apical surface media was changed to titration media.

Prior to analyzing the samples from the cell culture inserts in the graphite furnace, standard samples were analyzed in order to produce a calibration curve to determine concentrations of free and bound copper in the samples. This calibration curve can be seen below in Figure 30. Working standards with copper concentrations of 2 µM, 10 µM, 25 µM, 50 µM, 100 µM, 150 µM, and 225 µM were analyzed and from here the calibration curve was produced. However, it was determined that these copper concentrations were much too high as absorbance values were much greater than 1, proving the samples were too concentrated. Due to this, some samples had to be diluted and a new calibration curve was produced with new standards which can be seen below in Figure 31. This calibration curve had an R^2 value of 0.9856 and a line of best fit of y=0.054x-0.0272 used to calculate the free concentrations of copper in the samples which will be discussed in further detail below.

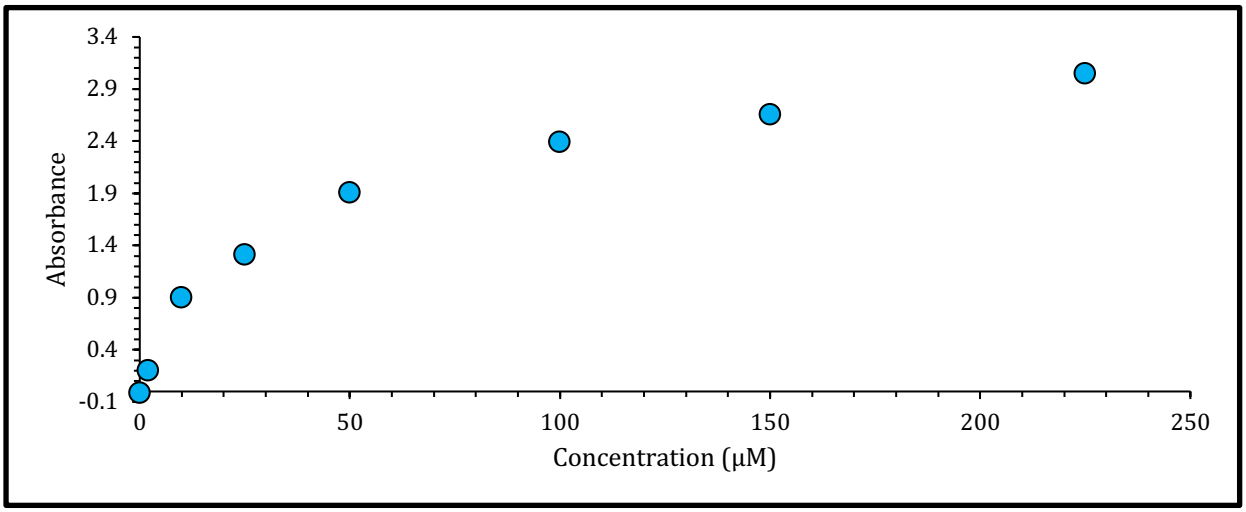


Figure 30. Calibration curve for both RTgill-W1 and LSgill-e cells used to determine the amount of free copper in cell culture inserts. All standards were acidified using HNO_3 to 0.2% in titration media.

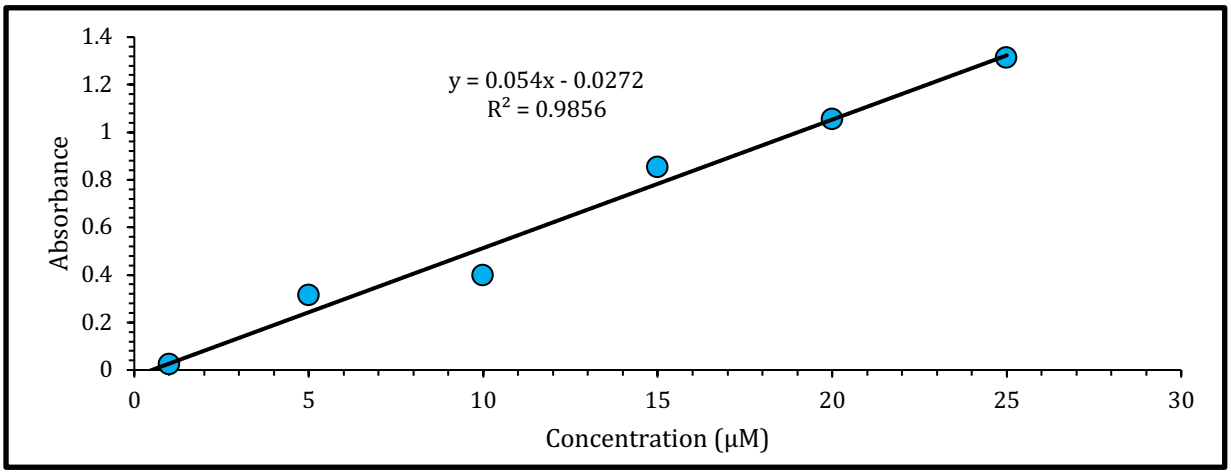


Figure 31. Calibration curve for both RTgill-W1 and LSgill-e cells used to determine the amount of free copper in cell culture inserts. All standards were acidified using HNO_3 to 0.2% in titration media.

At the start of the experiment, both the RTgill-W1 and LSgill-e cells were detached from the cell culture flasks, and a live/dead cell count was performed. The RTgill-W1 cells were 85% live and 15% dead and the LSgill-e cells were 89% live and 11% dead when seeded onto the cell culture insert in L-15 media, this was considered day 1. On day 1, as the cells were freshly plated, new L-15 media was added to the basolateral side to a final volume of 13 mL and new L-15 media was added to the cells on the apical side to a final volume of 9 mL. On day 2, the TEER values were measured and media on the apical side was replaced to remove any dead, floating cells. On day 6, TEER values were measured again and following this measurement, both the apical and basolateral L-15 media was replaced with fresh media. On day 8, TEER values were measured again. On day 14, TEER values were measured and both the apical and basolateral L-15 media was replaced with fresh media. Finally, on day 21, TEER values were measured, the basolateral L-15 media was replaced with new L-15 media, while the apical side with the cells was washed with titration media and then titration media with increasing concentrations of copper (II) sulfate was added. Due to the limited number of inserts available for this preliminary experiment, the control was a blank insert with no cells with 125 µM copper

(II) sulfate added to determine whether the insert with no cells present still bound copper. The cells were exposed to concentrations of copper ranging from 2 μM – 200 μM for three hours prior to analysis. For analysis in the graphite furnace, samples from both the basolateral and apical side of the insert were examined.

The amount of copper remaining in the media on the apical side was analyzed and can be seen below in Figure 32 for the RTgill-W1 cell line. This is the amount of free copper that was not bound to the RTgill-W1 cells or that had not passed through to the basolateral side of the insert. The media on the basolateral side of the insert was also analyzed to fully examine if copper had passed through from the apical side and to examine what copper has bound to the cells, if any, on the apical side of the insert. However, this particular insert experiment was only performed once, and the results were not as expected. Below in Table 18 the amount of free copper that was analyzed in the basolateral media from the graphite furnace can be seen. No copper (II) sulfate was added directly to the basolateral media so it can be assumed that all copper (II) sulfate accounted for on the basolateral side had passed through from the apical side. On the apical side of the insert for RTgill-W1 cells, it was observed that for all of the concentrations of copper added, binding was observed as evidenced by the data below the 1:1 line. However, high concentrations of free copper were analyzed indicating less bound copper to the cells in the insert.

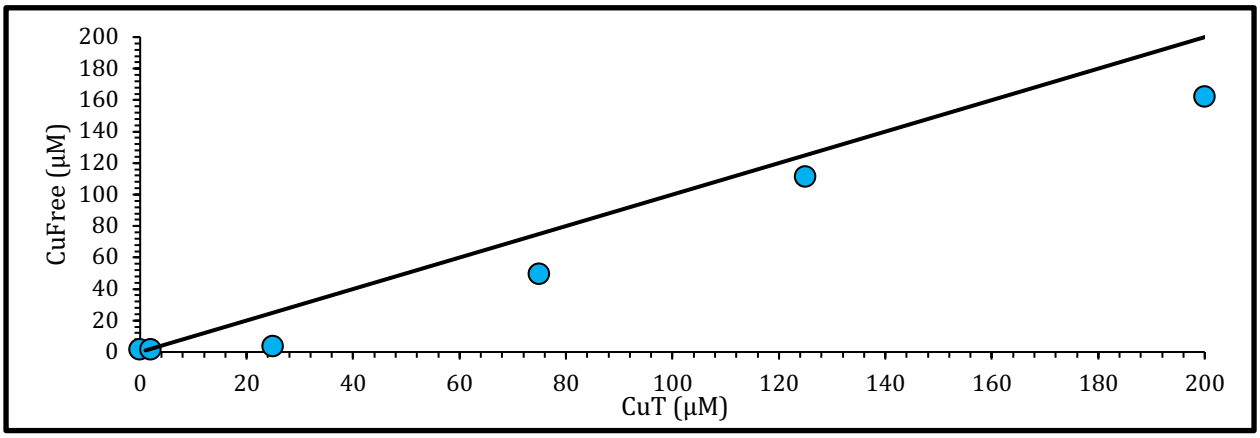


Figure 32. Sorption data for copper binding to RTgill-W1 cells grown in a cell culture for 21 days. Copper (II) sulfate was added to the top of the insert in concentrations of 2, 25, 75, 125, and 200 µM and the free copper concentration was analyzed in the graphite furnace after a three-hour incubation period. The black line indicates the 1:1 line of free Cu to total Cu.

In Table 18 below, it can be seen that a lot of free copper was accounted for in the basolateral side of the insert. This can be due to a number of reasons, which would need to be further examined in future experiments. The high concentrations of free copper on the bottom could be due to human error. The insert used had three separate large spaces between the side of the insert and the bottom chamber which made it easy for measuring TEER values. However, when moving the insert to the incubator for the three-hour incubation period, media from the top could have passed through to the bottom, causing an increased copper concentration in the basolateral chamber. Another reason for this could have been that the cells created a tight monolayer that was able to pass the copper through from the apical side to the basolateral side, in a way in which would be observed in the whole organism from the gill to the blood. The basolateral side had L-15 media which could have been too complex for the graphite furnace to analyze which could produce a false concentration of copper present. Another possibility could be that the monolayer tight junctions were not as tight as expected and copper was able to leak through without being transported inside the cells. Further experiments would need to be

performed to determine how these high concentrations of copper were observed in the basolateral chamber.

Table 18. Concentration of copper (II) sulfate measured on the basolateral side of the cell culture insert with RTgill-W1 cells. Total Cu concentrations of 2, 25, 75, 125, and 200 μM were added to the apical side of the insert in titration media. After three hours, both the apical and basolateral free [Cu] were analyzed using the graphite furnace. The amount of free [Cu] measured on the basolateral side of the insert can be seen below. No Cu was directly added to the basolateral side of the insert.

Total [Cu] Added to Apical Side (μM)	**Free [Cu] Measured on Basolateral Side (μM)**
2	1.34
25	2.52
75	36.69
125	84.61
200	123.06

After analyzing the samples from the RTgill-W1 cell line, the same was completed for the LSgill-e cells in the cell culture inserts. This can be seen in Figure 33 below. As can be seen in the apical chamber, with the addition of 2 μM copper, the concentration of free copper was 1.45 μM, when 50 μM total copper was added, the free copper concentration was 39.38 μM, when 125 μM total copper was added, the free copper was 141.70 μM and when 200 μM total copper was added the free copper was 158 μM. It was observed that with all four of these samples, the concentrations of free copper were quite high indicating very little binding to the cells which would also indicate very little copper being passed through the monolayer by the cells from the apical chamber to the basolateral chamber. It was noticed that one sample had a free copper concentration that was higher than the concentration of total copper added to the sample, which is theoretically impossible. The high concentrations of copper that were analyzed in the basolateral chamber can be seen below in Table 19. The possible reasons for these high concentrations observed in the basolateral side of the inserts was discussed previously and needs to be examined further in future research.

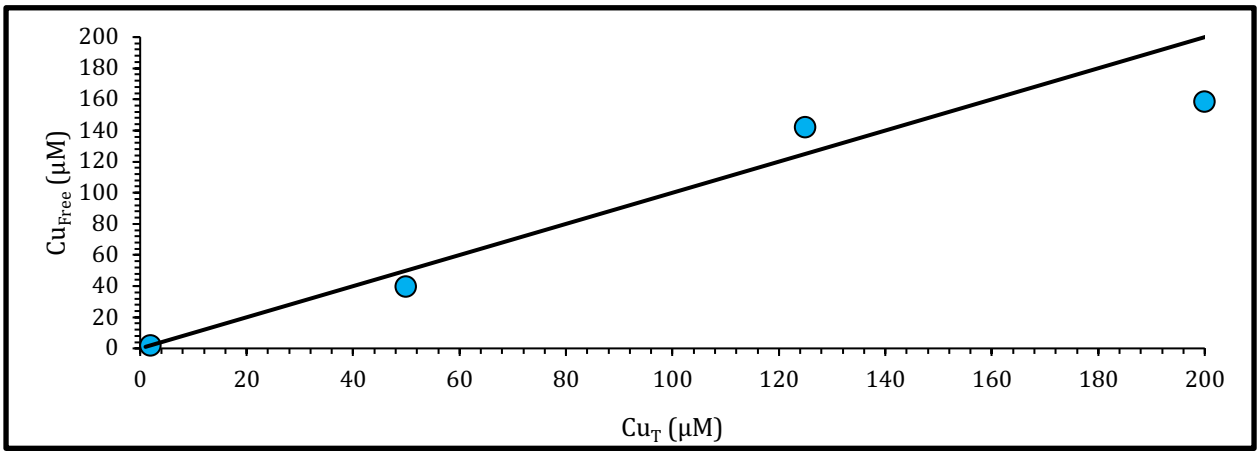


Figure 33. Sorption data for copper binding to LSgill-e cells grown in a cell culture for 21 days. Copper (II) sulfate was added to the apical side of the insert in concentrations of 2, 50, 125, and 200 µM and the free copper concentration was analyzed in the graphite furnace after a three-hour incubation period. The black line indicates the 1:1 line of free Cu to total Cu.

Table 19. Concentration of copper (II) sulfate measured on the basolateral side of the cell culture insert with LSgill-e cells. Total Cu concentrations of 2, 50, 125, and 200 µM were added to the apical side of the insert in titration media. After three hours, both the apical and basolateral free [Cu] were analyzed using the graphite furnace. The amount of free [Cu] measured on the basolateral side of the insert can be seen below. No Cu was directly added to the basolateral side of the insert.

Total [Cu] Added to Apical Side	Free [Cu] Measured on Basolateral Side
2	1.34
50	31.80
125	70.31
200	116.69

Due to the potential error that could have occurred with the measured copper on the basolateral side, for the purpose of this discussion, it will be ignored. The comparison between the measured free copper in the apical side of the inserts for both the RTgill-W1 and LSgill-e cell lines can be seen below in Table 20. For the lowest concentration of total copper added, more free copper was found in the LSgill-e cell line indicating less binding however, the values were very close for both cell lines. When higher concentrations of copper were added, 125 µM, more free copper was found in the LSgill-e cell line however when 200 µM of copper was added, more free copper was found in the RTgill-W1 cell line. Thus, from this insert experiment it can

be said that no strong conclusion can be drawn to the binding capabilities of either cell line.

Further insert experiments for both cell lines would need to be performed.

Table 20. Comparing the concentration of free copper in the apical side of cell culture inserts for the RTgill-W1 and LSgill-e cell lines. The cells were incubated with various concentrations of copper (II) sulfate for three hours prior to analysis using the graphite furnace. The greyed-out squares indicate concentrations of copper that were not examined for that particular cell line.

Total [Cu] Added to Apical Side (µM)	Measured Free [Cu] on Apical Side (µM)	
	RTgill-W1	LSgill-e
2	1.42	1.45
25	3.44	
50		39.39
75	49.56	
125	111.30	141.70
200	161.94	158.22

4. Conclusions and Future Work

4.1 Conclusions – Cell Viability Toxicity Assays

Cell viability and toxicity was examined throughout six different experiments using two different cell lines: RTgill-W1 and LSgill-e. To begin, the titratable range for copper (II) sulfate that was non-toxic for both the RTgill-W1 and LSgill-e cell line was established to ensure greater than 80% cell viability. In L-15 media, the RTgill-W1 cell line remained 80% viable when exposed to copper (II) sulfate concentrations from 0 μM - 500 μM for both metabolic activity and cell membrane integrity. When exposed to a copper concentration of 975 μM in L-15 media, the RTgill-W1 cells are 100% dead. For the LSgill-e cell line in L-15 media, viability remained greater than 80% for both metabolic activity and cell membrane integrity with copper concentrations from 0 μM – 500 μM. Thus, it was established that for both cell lines, in L-15 media, it was appropriate to use copper (II) sulfate concentrations between 0 μM – 300 μM with cells remaining at or greater than 80% viable.

The effect of cell density when exposed to copper (II) sulfate in L-15 media was examined for the RTgill-W1 cell line. It was determined that with cell densities of 25,000 cells /well, 20,000 cells/well, 15,000 cells/well, and 10,000 cells/well exposed to concentrations from 1 – 200 μM, no effect was observed indicating that cell density had no effect on cell toxicity for the RTgill-W1 cell line in L-15 media with copper concentrations from 1 μM – 200 μM.

The media composition was switched to titration media, a simpler media consisting of only sodium chloride and calcium chloride to more closely mimic freshwater. Cell toxicity for the RTgill-W1 cell line was examined by exposing the RTgill-W1 cells in a monolayer to titration media for up to 24 hours. It was found that cell viability did not drop below 80% and so further experiments in titration media could be performed.

Examining cell viability and toxicity for both the RTgill-W1 and LSgill-e cell lines in titration media when exposed to copper (II) sulfate. For the RTgill-W1 cell line, at a copper concentration of 50 μM 80% viability was observed and at 200 μM, only 5% viability was observed. Much lower viability was observed for the RTgill-W1 cell line in titration media compared to L-15 media, due to the absence of copper binding ligands protecting the cells. However, this was not true for the LSgill-e cell line as even when exposed to copper concentrations up to 500 μM, cells still remained greater than 80% viable. It is possible that due to the mucus secretion of the LSgill-e cell line, they are further protected from cell toxicity in comparison to the RTgill-W1 cell line, a non-mucus secreting cell line.

4.2 Conclusions – Ion Selective Electrode

Following the establishment of a titration range, ISE titrations with copper were performed in order to aid in the determination of biotic ligand model parameters. ISE titrations were completed for RTgill-W1 and LSgill-e cells that were in a monolayer and in suspension. It was determined that for both cell lines in both monolayer and in suspension, the electrode consistently failed to stabilize.

4.3 Conclusions – Graphite Furnace

After trying to determine biotic ligand model parameters using an ISE with unsuccessful results, a graphite furnace was then used to aid in the determination of these parameters.

First, the binding of copper to both RTgill-W1 and LSgill-e cells in a monolayer was analyzed using a graphite furnace. Binding was observed for both cell lines, with the LSgill-e cell line having higher concentrations of bound copper for some total copper concentrations, and RTgill-W1 having higher bound copper concentrations than others. However, the extent of this

binding is uncertain due to a significant amount of copper binding to the vessel. Due to this, BLM parameters could not be determined, and further research needs to be performed.

Next, the binding to both the RTgill-W1 and LSgill-e cells in suspension was analyzed using a graphite furnace. Copper binding was observed to both the RTgill-W1 and LSgill-e cells in suspension. More free copper was present in the RTgill-W1 samples than in the LSgill-e samples indicating more bound copper/cell for the LSgill-e cell line. However, similar to the cells in the monolayer, large amounts of copper bound to the vessel so it cannot be said whether the copper was actually bound to the cells or to the vessel.

Lastly, the binding to both the RTgill-W1 and LSgill-e cells to copper was analyzed for both the apical and basolateral chambers of a cell culture insert. It was found that with lower copper concentrations, more free copper was observed in the LSgill-e cell line indicating higher binding capacity observed in the RTgill-W1 cell line. However, with higher additions of total copper, at 125 μM, the LSgill-e cell line had more free copper (less bound) than the RTgill-W1 cell line but with 200 μM of total copper added, the RTgill-W1 cell line had more free copper (less bound) than the LSgill-e cell line. From this data, it cannot be concluded whether or not the RTgill-W1 or LSgill-e cells have higher binding capacities.

4.4 Future Work

Due to unforeseen circumstances including a global pandemic, there were some experiments that were unable to be completed due to lab time being cut short. Some of these experiments can be found listed below and if completed in the future, would serve as great complementary work to this thesis.

4.4.1 Cell Viability Assays

1. RTgill-W1 cell viability was examined using two fluorescent indicator dyes: alamar blue CFDA-AM to see the effect, if any, that cell density has on cell viability when exposed to copper (II) sulfate. In the future, examining the effects of cell seeding density on the LSgill-e cell line when exposed to Cu would be beneficial to see if it acts the same as the RTgill-W1 cell line or if the results differ.
2. Cell viability was examined for the RTgill-W1 cell line exposed to titration media for different time points to ensure viability at or greater than 80%. In the future, this should also be done for the LSgill-e cell line to ensure that this is also the case for this cell line. This would ensure that toxicity observed for the LSgill-e cell line was from the copper (II) sulfate alone and not from the titration media.
3. Cell viability was only analyzed for RTgill-W1 and LSgill-e cells in a monolayer. In the future, this should be done for cells in suspension and in cell culture inserts as well.

4.4.2 Ion Selective Electrode

As was discussed earlier in section 3.2, there was very little success when performing ISE titrations for both RTgill-W1 and LSgill-e cell lines when in a monolayer and when in suspension. Future experiments that could potentially increase the success of these ISE experiments can be found listed below and if completed, would be great complimentary work to this thesis.

1. Increase the total concentration of copper (II) sulfate added to the samples. Ion selective electrodes depend upon the activities of the free ions in solution resulting in a potential that is measured. This measured potential is directly proportional to the concentration of copper in the sample. It is possible that the concentrations of copper added to the samples

in this research where not high enough for the electrode to stabilize. With more copper added to the sample, more free ions would be present, producing higher potentials and hopefully, a stable ISE. However, further investigation would need to be performed as increased copper concentrations could also kill the cells.

2. Increase the cell density. This would be done by drastically increasing the number of cells in all of the samples, both monolayer and suspension experiments. By increasing the cell density in the samples, the binding capacity would in theory, be increased and with more binding observed, a stable electrode.
3. Increase both the total concentration of copper added to the sample and increase the cell density. This would most likely be the best option. With higher concentrations of copper and a higher binding capacity due to more cells in the sample, it is likely that the electrode would stabilize allowing for the determination of biotic ligand model parameters in future research.

4.4.3 Graphite Furnace

The overall goal of this research was to determine biotic ligand model parameters for both the RTgill-W1 and LSgill-e cell lines and compare them to that of previous research to determine whether or now continuous cell lines are a good alternative to primary gill cells and whole organism toxicity testing. However, due to complications in the experiments as discussed in section 3.3 above, biotic ligand model parameters were not determined. Further experiments need to be performed to fully determine the validity of continuous cell lines for toxicity testing. The experiments listed below would be great complementary work to this thesis and would help in the determination of the biotic ligand model parameters for both cell lines.

1. First, for all experiments it is essential to perform all experiments in triplicate. Due to time constraints the experiments performed in this research were only performed once so the data's reproducibility is uncertain.
2. Proper controls would also need to be used to fully examine the data from the graphite furnace for the cell culture insert experiments. A control of copper binding to an insert with no cells present was used to determine whether or not binding occurred to the vessel. This is not ideal as when cells are present, the surface is completely different for copper binding than when cells are not. It is possible that the cell culture has a higher affinity for the copper than when the cells are present so a better control would need to be examined.
3. For the graphite furnace data, the concentration of bound copper was determined by subtracting the free copper concentration from the total copper concentration added to the sample. This is not ideal due to binding that could be seen to the vessel, or copper that could have been lost due to human error or other circumstances. In future work, bound copper could be directly measured by digesting the cells and then analyzing the exact amount of copper bound to the cells.
4. For all monolayer, suspension, and cell culture insert experiments performed and analyzed using the graphite furnace, copper binding to the vessel was consistently observed. This severely effects the data as it is unknown how much copper is actually bound to the cells or bound to the vessel. To correct for this, Teflon containers can be used to ensure no copper is binding to the vessel and all binding accounted for is to the gill cells.
5. For the cell culture insert experiments completed in this research, the integrity of the monolayer was analyzed and assessed by measuring TEER values. Another method that

could be used is by using lucifer yellow to quantitatively assess the permeability of the cell monolayer (Alkie et al. 2019).

6. As a positive control for this study, primary rainbow trout gill cells should be run in cell culture inserts analyzed using the graphite furnace.
7. For the cell culture insert experiments, L-15 media was used on the basolateral side and a sample was run through the graphite furnace to determine the amount of copper that had transported through. This media could have been too complex resulting in false concentrations of copper presented. This could be examined in future work by standard additions.

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Appendices

Appendix A

L-15/ex media (Schirmer et al. 1997) is a media modified from the original Leibovitz 15 culture media (L-15) that was originally developed for the RTgill-W1 cell line for experiments with short exposure times (Yue et al. 2015).

The standard operating procedure for making the L-15/ex media was generously provided by Dr. Vivian Dayeh from the University of Waterloo which can be found below (Leibovitz 1963; Schirmer et al. 1997).

SOP for L-15/ex Media

For preparation of 500mL of L-15/ex media, the following ingredients were combined:

- 440 mL tissue culture grade water
- 30 mL solution A
- 5 mL solution B
- 15 mL solution C
- 5 mL galactose
- 5 mL pyruvate

For preparation of solution A:

- 80 g NaCl, 4.0 g KCl, 2.0 g $MgSO_4$-$7H_2O$, 2.0 g $MgCl_2$-$6H_2O$
- Final volume to 600 mL in tissue culture grade water

For preparation of solution B

- 1.4 g $CaCl_2$
- Final volume to 100 mL in tissue culture grade water

For preparation of solution C

- 1.9 g Na_2PO_4, 0.6 g KH_2PO_4
- Final volume to 300 mL in tissue culture grade water

For preparation of galactose

- 90 mg/mL
- Dissolve 9.0 g in 100 mL tissue culture grade water
- Filter sterilize using 0.22-micron filter

For preparation of pyruvate

- 55 mg/mL
- Dissolve 5.5 g in 100 mL of tissue culture grade water
- Filter sterilize using 0.22-micron filter

For this research, cell membrane integrity and metabolic activity were examined using two fluorescent indicator dyes, CFDA-AM and alamar blue to examine cell viability in L-15/ex media. L-15/ex media, due to its composition was expected to have very few copper binding ligands and thus, offering very little to no protection to the RTgill-W1 cell when exposed to concentrations of copper (II) sulfate.

RTgill-W1 cells were grown in cell culture flasks until confluent and then detached using Gibco TrypLE Express trypsinizing solution. Once detached, the cells were centrifuged for five minutes to pellet, counted using the Countess II FL Automated Cell Counter and then resuspended in L-15 media. Cells were then plated in a 96-well plate at a density of 20,000 cells/well in L-15 media with a final volume of 100 μL/well. Once plated, the cells were left to attach overnight at 20°C. The following day, the cells were rinsed with L-15/ex media and copper (II) sulfate was added ranging in concentrations from 10 μM – 450 μM in L-15/ex media with a no copper control. The cells were left to incubate for 24 hours at room temperature. After

the incubation with copper, the copper and media were removed, the cells were rinsed with PBS and cell viability was then determined using alamar blue and CFDA-AM. After an hour-long incubation with the fluorescent indicator dyes, the plates were read on a fluorometric multiwell plate reader and cell viability was analyzed as a percentage of the control.

As can be seen below in Figure 34, this curve is representative that the L-15/ex media has very few copper binding ligands and cell toxicity is observed with the RTgill-W1 cell line. With copper (II) sulfate concentrations up to 50 µM, cell viability remains greater than 80% indicating very few cytotoxic effects. However, when Cu concentrations reach 200 µM or greater, cell viability begins to drop to less than 70% and then at 500 µM, cell viability drops to 0%, almost 100% cell death. L-15/ex media was examined only for one experiment due to the composition of the media. It was determined that titration media more closely resembles "real" freshwater than the L-15/ex media so further investigation using L-15/ex media did not occur for the LSgill-e cell line or for duplicate or triplicate trials with the RTgill-W1 cell line.

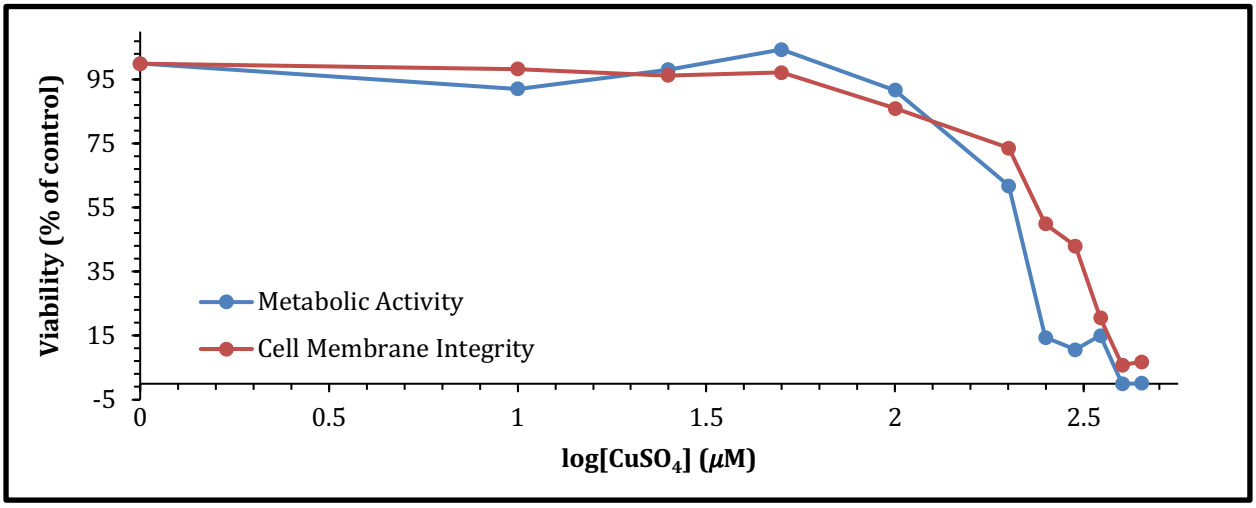

Figure 34. Viability of the RTgill-W1 cell line in a monolayer in 96-well plates after 24h incubation with copper (II) sulfate in L-15/ex media. Cell viability was assessed using two fluorescent indicator dyes, alamar blue (metabolic activity) and CFDA-AM (cell membrane integrity). Each concentration was performed only once with a cell density of 20,000 cells/well.

In Table 21 below, the concentration of copper/cell can be seen along with the percent viability for alamar blue and CFDA-AM when exposed to multiple different copper concentrations in L-15/ex media. As expected, it can be observed that when more copper is added to the sample, the concentration of copper per cell increases which causes a much lower cell viability. This decrease in cell viability is caused as the concentration of copper per cell is higher, having a much more detrimental impact on cell viability, as toxic concentrations of copper are reached, killing the cells.

Table 21. Alamar blue and CFDA-AM results for cell viability for RTgill-W1 cells in a monolayer in L-15/ex media.

Total [Cu] (µM)	**Viability (% of control)**		**[Cu]/Cell (µM)**
	Metabolic Activity	**Cell Membrane Integrity**	
10	92.08	98.35	5.0×10^{-4}
25	98.07	96.30	12.5×10^{-4}
50	104.42	97.20	25×10^{-4}
100	91.68	86.00	50×10^{-4}
200	61.79	73.61	10×10^{-3}
250	14.47	49.98	12.5×10^{-3}
300	10.64	43.07	15×10^{-3}
350	15.05	20.55	17.5×10^{-3}
400	-0.026	5.84	20×10^{-3}
450	0.144	6.88	22.5×10^{-3}

Appendix B

In spring 2019, an immunology conference, North American Comparative Immunology Workshop (NACIW) was in Waterloo and I was given the opportunity to present a poster at this conference.

As mentioned in section 1.1 Copper in Aquatic Environments, fish are likely to be exposed to copper in freshwater environments. Also, in freshwater environments fish, more specifically rainbow trout, are likely exposed to copper during viral infections. Thus, it is important to understand what role copper plays in the antiviral process in these animals. For this study, in Figure 35 below, copper (II) sulfate was used to examine whether or not exposure to copper makes the RTgill-W1 cells more susceptible to virus infection infected with viral hemorrhagic septicaemia virus (VHSV)-IVa.

Viral susceptibility was measured by first treating the cells with a non-toxic concentration of copper (II) sulfate, 300 µM, for 7 hours, prior to infection. After which, cells were infected with (VHSV)-IVa titers until toxicity was observed and viability was measured using alamar blue and CFDA-AM. The dose of copper used, 300 µM, was found to be non-toxic which can be seen in section 3.1. The RTgill-W1 cells were plated at a density of 20,000 cells/well. The CFDA-AM and alamar blue assay was performed after cytopathic effect was visually observed under a microscope. This can be seen below in Figure 35. Cytopathic effect was observed 6.5 days post-infection. It can be seen that with no virus and copper present, the RTgill-W1 cells are epithelial like, with no rounding and all cells can be seen attached to the culture flask. However, on the right, after 6-days of being infected with the VHSV-IVa virus, the cells can be seen very round, not epithelial like and detached from the flask. This can be a strong indicator that the cells are dead most likely caused by the virus.

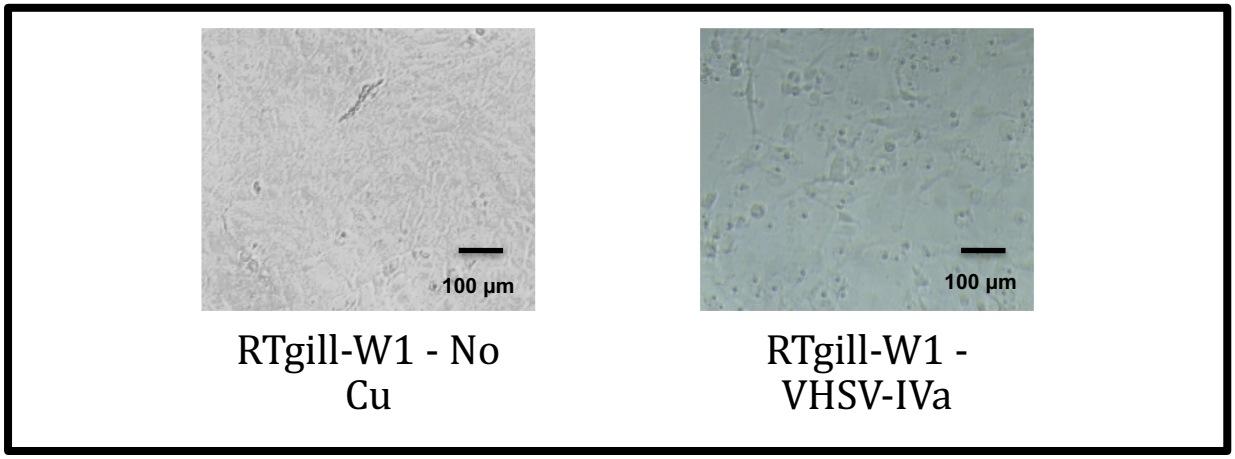

Figure 35. RTgill-W1 cells under a microscope at 100X magnification before and after VHSV-IVa infection. Cells were grown in L-15 media (right) and when confluent exposed to 300 µM copper (II) sulfate for 7 hours prior to infection. After the incubation with copper, cells were infected with VHSV-IVa and alamar blue and CFDA-AM assays was performed 6.5 days post-infection.

Below in Figure 36, viability curves examining the effect of exposing RTgill-W1 cells to copper (II) sulfate prior to infection with VHSV-IVa. When looking at metabolic activity, as indicated by alamar blue, it can be seen that cell viability is higher when cells have no copper exposure prior to infection (black line) that when the cells are exposed to copper prior to infection (green line). This could indicate that for metabolic activity, copper interferes with the immune response of the RTgill-W1 cells making them more susceptible to infection. When looking at cellular membrane integrity, as indicated by CFDA-AM, it can be seen that viability remained consistent when exposed to copper prior to infection (blue line) and when no copper exposure occurred (red line). It is possible that when exposed to copper prior to virus infection, the cells are unable to proliferate and therefore become more suspectable to virus infection. However, this experiment was only completed once so more experiments would need to be performed in order to fully examine whether or not copper exposure makes RTgill-W1 cells more susceptible to virus infection.

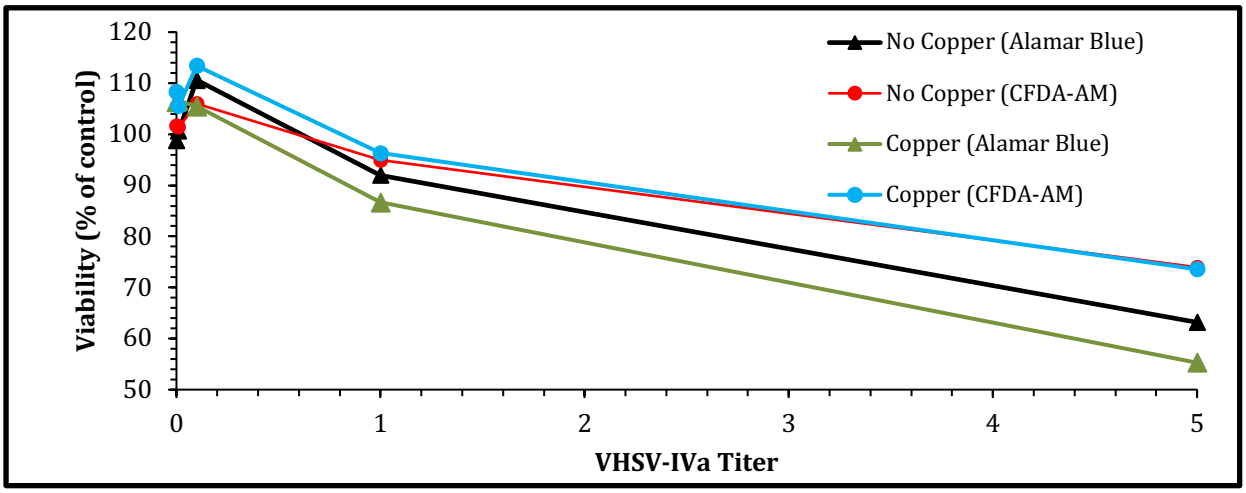

Figure 36. Viability curve examining the effect of 300 µM copper (II) sulfate on RTgill-W1 cells exposed to increasing titers of VHSV-IVa. Cells were treated with copper for 7h in L-15 media prior to infection. Cells were incubated with cell viability dyes 6.5 days post-infection.

Appendix C

Prior to completing ISE titrations with the RTgill-W1 and LSgill-e cell lines, practice titrations were performed by titrating in increasing concentrations of copper (II) sulfate to salicylic acid. These practice titrations were performed in order to gain experience working with the ISE and to ensure the ISE was working.

Seen below in Figure 37, was the first practice titration completed using the ISE. Copper (II) sulfate was added to the salicylic acid sample in concentrations of 20 µM, 50 µM, 70 µM, and 100 µM. After each addition of titrant, the electrode was allowed to stabilize until the measured potential was within 0.1mV of each value for five consecutive minutes. After the first addition of titration, the electrode stabilized after approximately 48 minutes. The total copper concentration was then increase to 50 µM and the electrode stabilized after approximately 28 minutes after the addition of copper. The total copper concentration was then increased to 70 µM and after stabilizing, the final addition of copper was added to a total concentration of 100 µM. This entire titration took approximately 144 minutes. This titration proved to work with the electrode stabilizing after each addition of titrant and from here, another practice titration was performed.

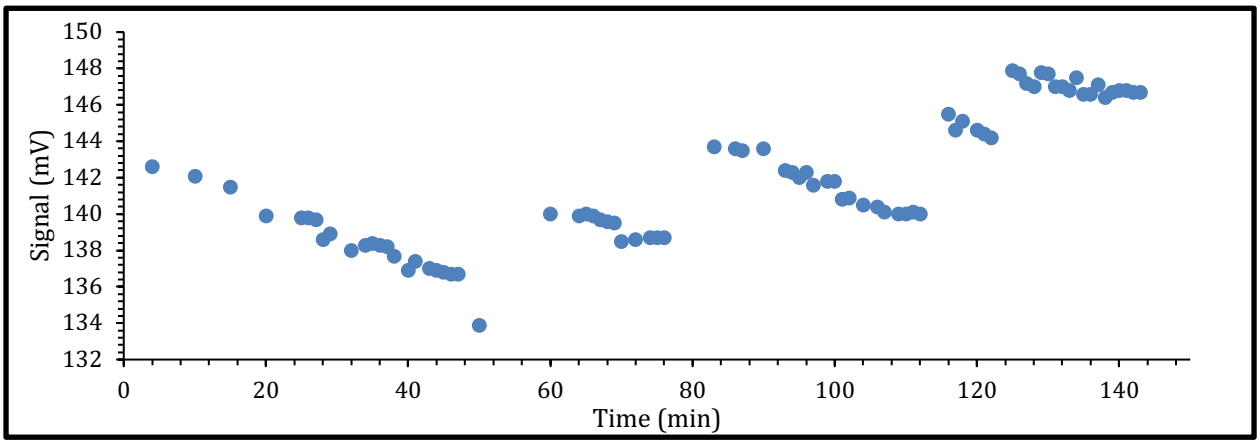

Figure 37. Salicylic acid practice titration measured as the signal (mV) from the free ion, Cu^{2+}, versus time it took for the signal to stabilize. Copper (II) sulfate was added from 20 µM – 100 µM and the pH was maintained at 7 ± 0.1.

Found below in Figure 38, is the next ISE titration that was performed. Copper was added to the salicylic acid solution with a concentration range from 20 µM – 130 µM. Once again, after each addition of titrant, the electrode was allowed to stabilize until the measured potential was within 0.1mV of the previous value for five consecutive minutes. After stabilization following the final addition of titrant, the sample was acidified to a pH of ~3.4 to allow for the calculation of free and bound copper in the sample. This can be seen below in Figure 39.

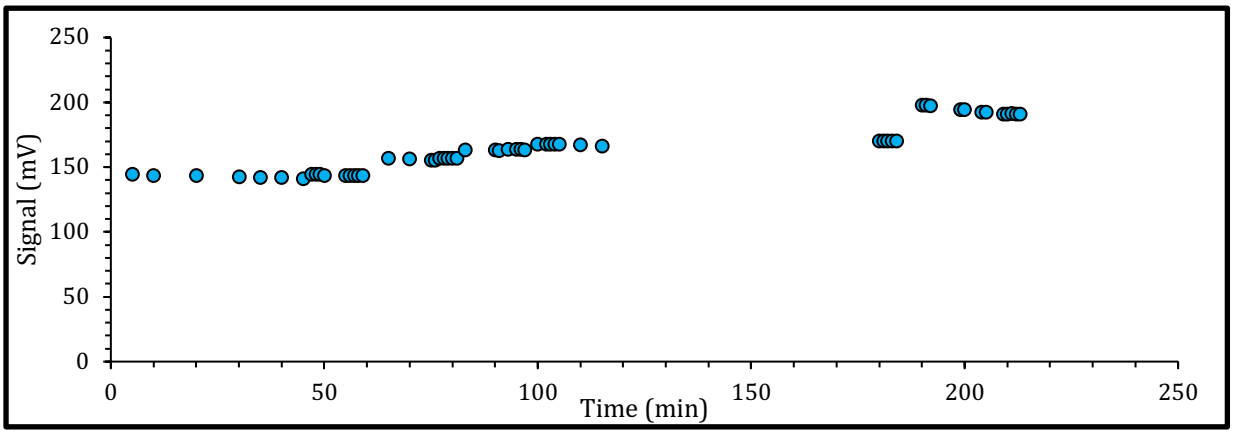

Figure 38. Salicylic acid practice titration measured as the signal (mV) from the free ion, Cu^{2+}, versus time it took for the signal to stabilize. Copper (II) sulfate was added from 20µM – 130µM and the pH was maintained at 7 ± 0.1 with acidification at the end maintaining a pH of ~ 3.4.

For this salicylic acid titration, total copper concentrations added were 20 µM, 50 µM, 100 µM, and 130 µM. Following the first addition of titrant, the concentration of free copper present in the sample was 3.37E-6 µM with 2.00E+1 bound to salicylic acid. Next, when 50 µM of total copper was added, there was 9.28E-6 µM of free copper and 5.00E+1 bound. When 100 µM of copper was added, the concentration of free copper in the sample was 2.12E-5 and the concentration of bound copper to salicylic acid was 1.00E+2. Lastly, when 130 µM of total

copper was added to the sample, 2.59E-5 µM of the copper was free and 1.30E+2 µM was bound. This is indicative of the strong binding capacity observed by salicylic acid and due to the success observed with this titration, ISE titrations were then performed using RTgill-W1 and LSgill-e cells in the samples. This can be found in section 3.2.

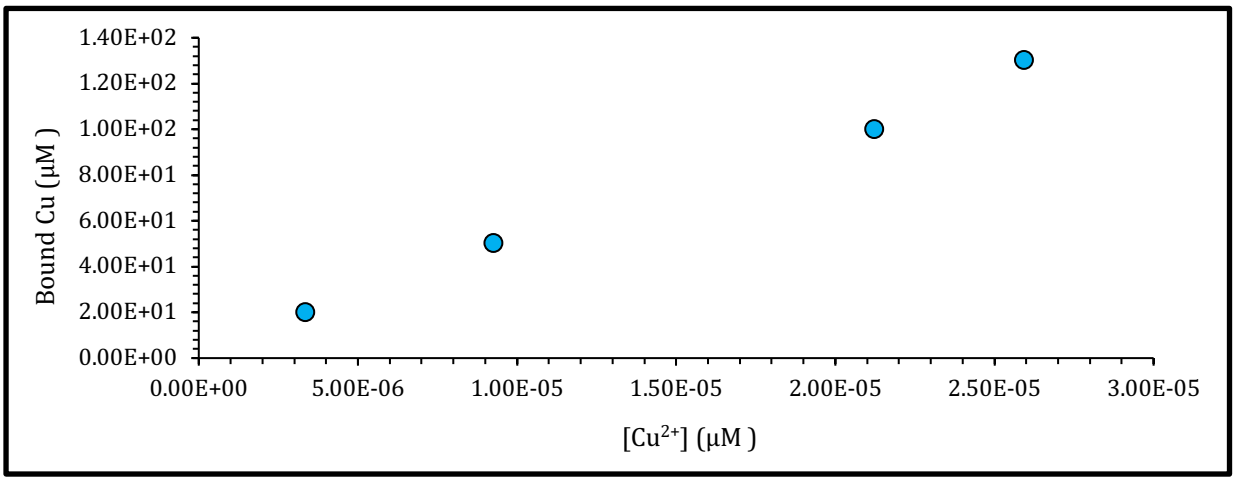

Figure 39. Salicylic acid practice titration data measured as the free ion, Cu2+, plotted against the calculated bound metal. The blue circles represent titrations of salicylic acid with copper (II) sulfate. Copper was titrated in at 20µM, 50µM, 100µM, and 130µM.