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Assessing the toxicity of a reconstituted water simulating streams influenced by mountaintop mining in central Appalachia

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ASSESSING THE TOXICITY OF A RECONSTITUTED WATER SIMULATING STREAMS INFLUENCED BY MOUNTAINTOP MINING IN CENTRAL APPALACHIA

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Master of Science
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by
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Approved by
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APPROVAL OF THESIS

We, the faculty supervising the work of Benjamin David Browning, affirm that the thesis, *Assessing the Toxicity of a Reconstituted Water Simulating Streams Influenced by Mountaintop Mining in Central Appalachia*, meets the high academic standards for original scholarship and creative work established by the Master of Science in Environmental Science and the College of Engineering and Computer Sciences. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

3/30/21

Dr. Scott Simonton,
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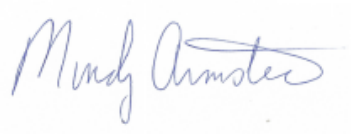

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Freshwater ecosystems in Central Appalachia experience increased concentrations of manganese (Mn) and total dissolved solids from the runoff of surface mines and valley fills. Biological communities have been impacted by these surface mining operations and it has been suggested that the increase in total dissolved solids may contribute to these negative effects, but standard laboratory toxicity tests have not found increased concentrations of total dissolved solids to have such negative effects as seen in the field. The elevated total dissolved solids in mining influenced streams may only be toxic in conjunction with another toxicant that is presence in these systems such as manganese. This study's primary goal was to determine the toxic effects of a simulated mine water representative of elevated ionic conditions in mining influenced streams of Central Appalachia on the fathead minnow (*Pimephales promelas*) and examine potential cumulative effects with manganese. Breeding colonies were exposed to different concentrations of the simulated mine water then toxicity tests were performed with manganese on the embryos and larvae. The adverse effects of the combination of toxicants were determined using traditional and non-traditional toxicity testing endpoints. This study found that fathead minnow larval growth was decreased in a concentration of 10 mg Mn/L in conjunction with a 50% dilution of the simulated mine water, but no effect was observed at higher simulated mine water concentrations most likely because of the increased water hardness that is known to reduce both sulfate and manganese toxicity. The concentration of sulfate in the simulated mine water was more toxic when combined with manganese in comparison to historic data for sulfate toxicity. These findings could be applied to mitigation and restoration efforts for streams affected by mountaintop mining operations in Central Appalachia.

CHAPTER 1

INTRODUCTION

Mountaintop Mining Affecting Streams in Central Appalachia

In Central Appalachia, surface mines in the form of mountaintop mining is the dominant driver in land cover change (USGS, 2016). Active and reclaimed mines compose 5.43% of the region's land cover and 5.8% of watersheds in the region have catchments in active or reclaimed mining areas (Townsend et al., 2009). Mountain top mining exposes coal seams using explosives and heavy machinery to remove up to 300 vertical meters of earth laying on top of these shallow coal deposits. The resulting waste rock, called overburden, is pushed downhill into adjacent valleys to create fills, resulting in headwater streams being buried (Lindberg et al., 2011). Over 11,000 km^2 of forest have been converted to surface mines and more than 2,000 km of streams have been buried with the overburden of these operations in the Appalachian region (Bernhardt et al., 2011). These activities lead to major increases in salinity, metal concentration and fine substrate downstream which can persist long distances as valley fills and surface mines can affect entire watersheds (USEPA, 2011). Water draining from the toe of a valley fill may be the only source of flow for some first order streams, leading to minimal dilution when streams converge (Armstead et al., 2016).

Increased frequency of fine substrates (referred to as "fines" from here on) is prevalent in mining affected streams. Fines may come from the valley fills, from increased stream bank erosion caused by an elevated streamflow or from surface runoff from the mining activities. Valley fills can function as aquifers for headwater streams, providing slower more steady flow from infiltration into the fill and reduced seasonal temperature variation (Griffith et al., 2012). Additional geomorphic characteristics of headwater streams are affected by mountaintop mining

such as deeper maximum channel depth and increased bedrock exposure in streambeds (Jaeger, 2015).

The drainage from mountaintop mining is different from underground mines in the Appalachian region in that the discharge is alkaline instead of acidic in its untreated form due to the increased presence of carbonate minerals in the overburden placed in the fills. Many metals are relatively insoluble in alkaline water, meaning metals commonly associated with mine drainage such as iron are not commonly a problem. Selenium (Se) and manganese (Mn), however, are found in increased concentrations persistently in valley-fill influenced watersheds, along with a number of major ions (Griffith et al., 2012; USEPA, 2003).

Manganese in Appalachia

Manganese is abundant globally and is an essential element to plants and animals. Its toxicity to aquatic life was studied in the 80s and believed to be relatively benign at concentrations at or below 0.09 mg/L (IDEM, 1997). There are no current USEPA national water quality standards for aquatic life and only secondary drinking water standards for the metal. The secondary maximum contamination level (SMCL) for Mn is set at 0.05 mg/L which is meant to protect against black staining and metallic tasting water (USEPA, 2020). SMCLs are non-enforceable, which means they are only recommendations and do not have to be followed by drinking water providers. The only state in the Central Appalachian region with an additional water quality standard in place for Mn is West Virginia which places an MCL of 1.0 mg/L on Mn for five miles upstream of public and private drinking water supplies (USEPA, 2017). In the Appalachian region Mn in water primarily comes from the weathering of sedimentary rock which occurs in high amounts at surface mining sites (USEPA, 2003). Other sources of Mn in the region include smelting operations, steel production and other industrial processes (Lasier et

al., 2000). Manganese concentrations have been reported as high as 3.94 mg/L at mining discharges (USEPA, 2003).

Manganese is believed to be most toxic in aquo-ionic forms compared to its insoluble forms similar to other metals such as iron, however dissolved Mn will stay in suspension far longer than dissolved iron. Iron was found to precipitate out of natural waters samples within 48 hours while a small fraction of total dissolved Mn precipitated in a week (Handa, 1970). The toxicity of Mn decreases with the increase of water hardness and aquatic organisms show a wide range of sensitivity to the element (Stubblefield & Hockett, 2000).

Fathead minnows and other fish species are most sensitive to acute Mn toxicity in early life stages. The primary route of accumulation of Mn in fathead minnows is by direct exposure in solution, though exposure by ingestion also contributes to accumulation (Kwasnik, 1977). Past studies have found that Mn is acutely toxic to fathead minnow fry (<7 days old) at about 3.542 mg/L in soft water (26 mg $CaCO_3$/L), 9.346 mg/L in moderately hard water (100 mg $CaCO_3$/L), and 15.826 mg/L in very hard water (200 mg $CaCO_3$/L) (Stubblefield & Hockett, 2000). The EC 20 (the concentration in which 20% of the population is affected) for 7-day chronic tests on fathead minnow fry is 1.338 mg/L in soft water (26 mg $CaCO_3$/L), 5.120 mg/L in moderately hard water (100 mg $CaCO_3$/L), and 13.152 mg/L in very hard water (200 mg $CaCO_3$/L) (Stubblefield & Hockett, 2000). Studies have been conducted using complex effluents containing Mn where fathead minnow egg production was significantly diminished but no tests have been conducted using only Mn to determine its effect on egg production (Ouellet et al., 2013).

Toxicity of Total Dissolved Solids

Salinity is the concentration of all salts dissolved in a body of water and is usually measured as total dissolved solids (TDS) or as specific conductance (SC). Secondary salinization

of freshwater ecosystems has increased globally and may be due to a variety of anthropogenic activities including agriculture, irrigation, mining discharges, de-icing of roads, construction, saltwater intrusion and clearing of natural vegetation (Williams, 2001). Elevated ionic concentration caused by mining discharges has been correlated with significant impacts to macroinvertebrate community structure (Kennedy et al., 2005; van Dam et al., 2014). The Orders *Ephemeroptera* (mayflies), *Tricoptera* (caddisflies), and *Plecoptera* (stoneflies) are most sensitive to salinity and decreases are seen in species richness and relative abundance in streams when mining activities occur in the watershed (Black et al., 2004; Pond et al., 2008; Voss et al., 2017). Multiple studies have identified the major ions that increase TDS in mining influenced streams to be: calcium, potassium, magnesium, sodium, chloride, bicarbonate, and sulfate. Of these ions, sulfate, bicarbonate, magnesium and calcium ions are the dominate ions in alkaline mine discharges of the Appalachian region; potassium, sodium and chloride are in lower concentrations but still elevated in comparison to streams in unmined areas (Armstead et al., 2013; Griffith et al., 2012; USEPA, 2003). The relative toxicities of these major ions to one another when tested on *Ceriodaphnia dubia*, *Daphnia magna* and *Pimephales promelas*, the fathead minnow, has been found to be $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2-}$; while Na^{2+} and Ca^{2+} toxicity was primarily attributed to the corresponding anions. (Lasier et al., 2010; Mount et al., 1997; van Dam et al., 2010).

There are several challenges in determining the toxic effects of increased major ion concentrations. For one, charge balance demands that increased concentration of an ion be offset by ions of an equal and opposite charge, this makes it difficult to identify the toxic effects of an individual ion (Mount et al., 2016). Secondly, the specific major ion composition of water affects it's toxicity, so a measure of elevated TDS cannot be a predictor for the toxicity of a solution

(Kunz et al., 2013; Mount et al., 1997). Another issue is the ratio of major ions to one another in solution affects their toxicity. For example, increasing potassium concentration in water will reduce the toxicity of sulfate to fathead minnows (Wang et al., 2016). Increased water hardness (Ca and Mg ions) has been reported in numerous studies to have an ameliorative effect on the toxicity of many major ions and only in recent studies has it been observed that other major ions covary with hardness and may alter toxicity instead of hardness in some cases (Elphick et al., 2011; Lasier & Hardin, 2010; Mount et al., 2016; Soucek, 2007; Soucek et al., 2005; Soucek et al., 2011; Wang et al., 2016).

Coal wastes from surface mining in Central Appalachia contain pyrite (FeS_2) which will convert to sulfuric acid (H_2SO_4) when exposed to oxygen and water. Sulfuric acid dissociates into hydrogen and sulfate ions (SO_4^{2-}) in water, resulting in mining-influenced streams having high concentrations of sulfate ions and commonly the dominant ion in these alkaline mine effluents (Kennedy et al., 2005; Lindberg et al., 2011). The toxicity of sulfate is ameliorated by increased hardness and potassium concentration (Elphick et al., 2011; Lasier & Hardin, 2010; Soucek & Kennedy, 2005; Wang et al., 2016). Chloride concentrations have variable impacts on sulfate toxicity depending on the organism being studied (Soucek, 2007). The fathead minnow has been shown to be more chronically sensitive to sulfate toxicity than other test organisms, such as *Ceriodaphnia dubia* and *Lampsilis abrupta*, which demonstrate acute toxicity at lower ion levels. The most sensitive life stage of the fathead minnow to sulfate is during the transitional period when the fish hatches from its egg and enters the yolk sac stage (Wang et al., 2016).

Bicarbonate ions in alkaline mine waters originate from carbonate minerals in valley fills that made up non-coal rock formations which were removed to reach coal seams. Carbonate can also be added to acidic overburden or fills to neutralize the pH of the effluent (Banks et al., 1997;

USEPA, 2003). Concentration of bicarbonate ions is often measured as alkalinity and is a major factor in the buffering capacity of a solution to adjustments of pH (Armstead et al., 2016). The EPA has a recommended lower limit for an aquatic life water quality standard for alkalinity which is a continuous minimum concentration of 20 mg/L in freshwater systems. If the natural alkalinity of the water body is lower, then the alkalinity cannot be lower than 25% of the natural level (USEPA, 2019). Bicarbonate toxicity is unique from many other major ion toxicities in that it is not affected by increased water hardness (Lasier & Hardin, 2010). Just fertilized embryos of fathead minnows have been shown to be 1.5 times more sensitive to sodium bicarbonate than 4-day old larvae, supporting the idea that this life stage is most vulnerable to major ions (Skaar et al., 2006). Interestingly, a study observed fathead minnows in higher alkalinity water having a significantly higher egg production in comparison to those breeding in lower alkalinity water (Ouellet et al., 2013).

Calcium and magnesium ions are the two main components of a water's hardness (Soucek et al., 2011). Both major ions originate from carbonate minerals in valley fills which were overburden on top of coal seams and they both mitigate metal toxicity in organisms (Banks et al., 1997; Pagenkopf, 1983; USEPA, 2011). Research has shown that calcium ions are not major contributors to aquatic toxicity and their corresponding anions typically are the origin if high TDS toxicity occurs (Mount et al., 1997). Toxicity studies using varying Ca:Mg ratios have revealed that calcium may have a more significant effect on the toxicity of other major ions than overall water hardness (Mount et al., 2016). Magnesium ions are most toxic in soft waters with low TDS and experience significant reduction in toxicity when calcium concentration rises (Kennedy et al., 2005; van Dam et al., 2010).

Both potassium and magnesium are macro-minerals which directly support many organisms including the fathead minnow (USEPA, 2002b). Potassium enters surface waters from natural sources such as the weathering of granite and from several anthropogenic sources including wastewater treatment plants, agricultural runoff, glass and fertilizer production (Khatri et al., 2015; Skowron et al., 2018). Potassium toxicity has been shown to reduce significantly when sodium concentrations rise (Mount et al., 2016).

The EPA has a recommended water quality standard for chloride in freshwater of 860 mg/L and 230 mg/L for acute and chronic exposure respectively (USEPA, 2019). Osmoregulation for fathead minnows and many other freshwater organisms require chloride ions to function properly (Elphick et al., 2011). The concentration of chloride in Appalachian streams is typically low but can be increased by anthropogenic sources such as road salting, municipal wastewater discharge, industrial plant discharge, urban and agricultural runoff, and drilling oil and gas wells (USEPA, 1988, 2003). Chloride ion toxicity is reduced by increased hardness and sodium concentration, and sulfate concentration has a negligible effect even though chloride can affect its toxicity (Lasier & Hardin, 2010; Soucek et al., 2011).

Sodium concentration in the Appalachian region is typically very low. Increases of sodium concentration in mine effluents can be caused by treatment of the effluent with sodium hydroxide, which is used to increase pH and remove metals (USEPA, 2003). Sodium ions have a role in deficiency-related toxicity, but are not a major contributor to aquatic toxicity in high concentrations as the associated anion is usually takes the credit for toxic effects (Mount et al., 1997). In cases where high sodium concentrations have been the apparent cause of toxicity, raising calcium concentration would reduce its lethality (Mount et al., 2016).

WET Testing and Mixture Toxicity

With a number of chemical and geomorphic characteristics of headwater streams affected by mountaintop mining, it is difficult to explain exactly which alterations impact local fauna the most significantly (Griffith et al., 2012; Jaeger, 2015; Pond et al., 2008; USEPA, 2003). Whole effluent toxicity (WET) testing is conducted to assess complex effluents and their potential toxicity to aquatic life and is included in the National Pollutant Discharge Elimination System (NDPES) permitting process. WET testing procedures can also be used in toxicity identification evaluations (TIE) to identify the toxic components of an effluent (USEPA, 2002a, 2002b). Previous studies on mountaintop mining-influenced streams have shown the ionic components of these streams can cause toxic effects in aquatic organisms (Armstead et al., 2016; Kennedy et al., 2005; Kunz et al., 2013; van Dam et al., 2014).

The fathead minnow has become the most widely used small fish model for ecotoxicology research in North America since its initial use in studies in the 1950s (Ankley & Villeneuve, 2006). Its significant role in ecotoxicology research can be attributed to its ease of culturing in lab and the support for using fathead laboratory data to deduce effects in other fish species and extrapolate effects of substances *in situ* (Ankley & Villeneuve, 2006). The traditional endpoints in toxicity tests utilizing the fathead minnow are larval survival, larval growth, embryo survival and larval teratogenicity (USEPA, 2002a, 2002b). These traditional endpoints focus on the embryo-larval stages of the fathead minnow as this has been determined to be the organism's most sensitive life stage (Thorpe et al., 2007), but these endpoints exclude potential reproductive effects that could occur due to chronic exposure of the adult such as fecundity and fertilization rates. Further, the embryo/larval test is not routinely used in NPDES monitoring, in part because evaluation of deformity endpoints requires an advanced skillset. Using more advanced methods

to determine the reproductive capacity of fathead minnows previously researched, this study intends to implement non-traditional chronic endpoints to observe potential reproductive impairment in fathead minnows.

In addition to the antagonistic effects major ions have on the toxicity of one another as previously discussed, previous studies have shown examples of how components of complex mixtures can be additive, or cumulative and, in general, may be hard to predict from the effects of the individual components. For example, 17α-ethinylestradiol and ammonia at their respective no observable effect concentrations (NOAEC) were combined and caused significant mortality in fathead minnows (Armstrong et al., 2015). The effects of metals in coordination with major ions have shown variations in toxicity as well, often reducing toxicity with increased major ion concentrations but a few exceptions have been revealed. For example, an increase in potassium concentration increased the toxicity of copper to larval fathead minnows (Benoit et al., 1996).

Objectives

The primary goal of this study is to determine the toxic effects of a simulated mine water representative of elevated ionic conditions in mining influenced streams of Central Appalachia on *Pimephales Promelas* (fathead minnow) and examine potential cumulative effects with other mining related toxicants prevalent in such streams. This will be accomplished by exposing breeding colonies in different concentrations of the simulated mine water then performing toxicity tests with the secondary toxicant on the embryos and larvae from the breeding colonies reared in the concentrations of simulated mine water.

Manganese was the secondary toxicant chosen for this study as it commonly found in mining influenced streams as mentioned earlier. The potential for toxicity from the elevated

conductivity reconstituted water is to be evaluated using traditional and non-traditional toxicity testing endpoints. The specific objectives of this study are to:

1. Investigate the toxic effects of elevated ionic conditions representative of mining influenced streams in Central Appalachia on the fathead minnow using traditional and non-traditional toxicity test endpoints.
2. Determine whether the combined exposure to ionic stress and manganese creates synergistic, antagonistic or additive toxicity using traditional or non-traditional endpoints.
3. Determine if non-traditional toxicity endpoints can reveal toxic effects on the fathead minnow which are more sensitive than those revealed by traditional endpoints.

CHAPTER II

METHODS

Simulated Mine Water

The simulated mine water (MW) recipe was developed by Armstead et al. (2013) to represent the ratio of ionic constituents found in the aforementioned region. Reconstituted water was preferred over actual stream water for the testing since variability between tests and interference due to other potential stressors could be minimized. The percentage of ionic constituents is listed in Table 1 and the recipe used to achieve that is listed in Table 2.

Following the volumes in Table 2, the simulated mine water was made by mixing each individual salt in reverse osmosis (RO) water in separate beakers. All RO water in this study was obtained from a Barnstead E-PURE 4-Module System installed in the facilities in which the research took place. One container was filled with 30% of the target volume with RO water, then the calcium sulfate was added to this container and was aerated and stirred for at least 24 hours. A separate container was filled with 50% of the goal volume with RO water and the remaining salts were added to this container in the following order: magnesium sulfate, sodium chloride, potassium chloride, and sodium bicarbonate. This solution was aerated and stirred for at least 24 hours as well. After both containers had been aerated and stirred for 24 hours they were mixed together and were left to aerate and stir for another 24 hours. After the second 24-hour period the conductivity was measured, and RO water was added to dilute the solution to 2430 ± 100 µS/cm. Hardness, alkalinity, conductivity, and pH were measured and recorded for each replicate. The simulated mine water had a hardness of 1240 ± 280 mg $CaCO_3$/L, an alkalinity of 122 ± 44 mg $CaCO_3$/L, and a pH of 8.21 ± 0.72. The solution was covered and aerated when in storage and the conductivity was checked with each use to ensure evaporation did not concentrate the water.

Constituent	% of MW
Total Calcium	11.64
Potassium	0.61
Magnesium	7.98
Sodium	5.55
Chloride	1.26
Bicarbonate	13.51
Total Sulfate	59.45

Table 1. Total Percentage of Ionic Constituents in Simulated Mine Water (Armstead et al., 2013)

Total volume to be made (L)	Calcium Sulfate (g)	Magnesium Sulfate (g)	Sodium Bicarbonate (g)	Potassium Chloride (g)	Sodium Chloride (g)
1	0.86	0.68	0.32	0.02	0.02
10	8.6	6.8	3.2	0.2	0.2
20	17.2	13.6	6.4	0.4	0.4
100	86	68	32	2	2

Table 2. Recipe for Simulated Mine Water

Control Water

The water used in this study as a control was the USEPA's moderately hard synthetic water (EPA) which is detailed in section 7.2.3 of the USEPA's manual "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (2002).

Manganese Stock Solution

The Mn used in this study was purchased from Acros Organics in the form of manganese (II) chloride tetrahydrate. The stock solution of Mn was created by mixing 1g of Mn (II) chloride tetrahydrate in 100 mL of RO water in a volumetric flask. The stock solution was covered and stored in the same flask for use.

Breeding Colonies

Adult fathead minnows were purchased from Aquatic Biosystems Inc. and were about 6 months old when they arrived for testing. When the fish arrived, the conductivity of their shipping water was measured, and all the test tanks were made to be no less than half the shipping water conductivity so as not to immediately stress the fish. Tank water conductivity was slowly altered to acclimate the fish to test conditions over one week by changing over 6 liters of water per day in each tank until the appropriate test conditions were met. Once the appropriate levels were reached, conductivities were maintained by weekly 20% water changes, utilizing RO, EPA and MW waters as appropriate.

Fathead minnows were reared in four different solutions: 100% MW (Conductivity = 2400 µS/cm), 50% MW (Conductivity = 1200 µS/cm), 25% MW (Conductivity = 600 µS/cm), and EPA water. The EPA water breeding colonies served as the control. The tests were performed in two rounds because there was not sufficient equipment and researchers to perform all toxicity tests simultaneously. The first round of tests began in January of 2020 and consisted of the 100% MW and 50% MW breeding colonies. The second round of tests began in July of 2020 and consisted of the 25% MW and the EPA breeding colonies.

Each breeding colony concentration consisted of two 10-gallon tanks, each containing four female and one male adult fathead minnow. Two tanks were used for each concentration to increase daily egg production for toxicity tests and to ensure the study would not be interrupted by tanks that did not breed. Two spawning tiles, longitudinally halved sections of PVC, were also placed in each tank for fathead minnow breeding and egg collection. Each tank had a waterfall filter containing granulated active carbon and a small bubble stone as well. The active carbon was replaced monthly. Water changes conducted weekly or more as needed to maintain

proper water quality. Water temperature, conductivity, dissolved oxygen and pH were logged Monday through Friday and tank maintenance was recorded daily along with any clutches produced or fish mortality. Each side of the tanks was covered with black trash bags to minimize stress caused by researchers working in the laboratory and by my male fathead minnows in adjacent tanks. Each tank maintained a 16-hour light/ 8-hour dark cycle with fluorescent lights on a timer, the fluorescent lights were replaced with LED lights part way through the first round of tests to increase the reliability of the light source. An image of the breeding colonies is displayed in Figure 1.

The breeders were exposed to their respective concentration for 21 days before egg collection and toxicity testing with Mn began. Breeders remained at ambient laboratory temperature (20±2°C) until the 18th day in which heaters were used to raise water temperature to 25±2°C over the remaining three days. Individuals who died during the 21-day exposure were not replaced. Tank dissolved oxygen (DO) was maintained at ≥6.8 mg/L, pH ranged from 6.0 to 8.3 and conductivity was sustained within 200 µS/cm of the concentration's goal. Breeders were fed Finfish Starter #1 from Aquatic Biosystems Inc. twice daily between the hours of 8-11am and 3-6pm. Tanks were cleaned using turkey basters when detritus began to accumulate in the tanks.

Figure 1. Fathead Minnow Breeding Colonies. Image by Ben Browning 2021

Objective 1

To determine whether salinity concentration affected fecundity, fertilization or deformity rates, the number of eggs laid, fertilization rates of clutches, and deformity frequencies in larvae from different concentrations of simulated mine water were compared for the four salinity concentrations. Fertilization rate was also compared across groups of breeders exposed to the elevated conductivity to determine if time of year and/or replicates of fish affected fertilization rates.

Egg Collection and Maintenance

On the 20th day of MW exposure, a catchment system was added below each spawning tile to collect any eggs that did not adhere to the tile. The design was based on the system developed in Thorpe et al. (2007) and is displayed in Figure 2. Egg collection began after 21 days of MW exposure. Nests were checked for embryos at 11am during the egg collection period. Catchment systems were cleaned daily to remove debris and prevent fungal growth.

Nests with embryos were carefully removed along with their catchment systems and “Day-0” photos were taken of the clutch and the catchment with the date the clutch was laid. The eggs in both the clutch and catchment system were rinsed with EPA water to remove detritus then placed in a 2L beaker with 1500 mL of the appropriate conductivity water and one drop of methylene blue, labelled with the date, MW concentration, tank the clutch came from, and initials of the collector. The beaker was then set in a 20 ± 1 °C water bath and lightly bubbled, with the bubble stick placed behind the PVC tile. Bubble sticks were stored in a beaker containing water mixed with methylene blue when not in use to prevent fungal growth and to fix the bubbling rate to minimize egg disturbance.

A Day-3 photograph was taken for each clutch and for the catchment systems. Later on in the experiment, the catchment systems were removed after Day-0 photos because they were causing extensive fungal growth on the clutches and themselves. So the catchment systems only have Day-0 photos for a large portion of the dataset. Once Day-3 photos were taken, the clutches were placed in fresh water (without methylene blue) of the appropriate conductivity and placed back into the water bath and lightly bubbled once again.

Beakers were checked daily for fry. Fry from the same MW concentration and hatched on the same day were placed in a 2L beaker separate from the clutch and labelled with the day hatched, concentration of MW and clutch IDs. Clutches were removed from the water bath when there were no remaining eggs or only opaque, unfertilized eggs. After the daily egg/fry maintenance, egg/fry numbers were assessed to determine if enough were present to begin a toxicity test.

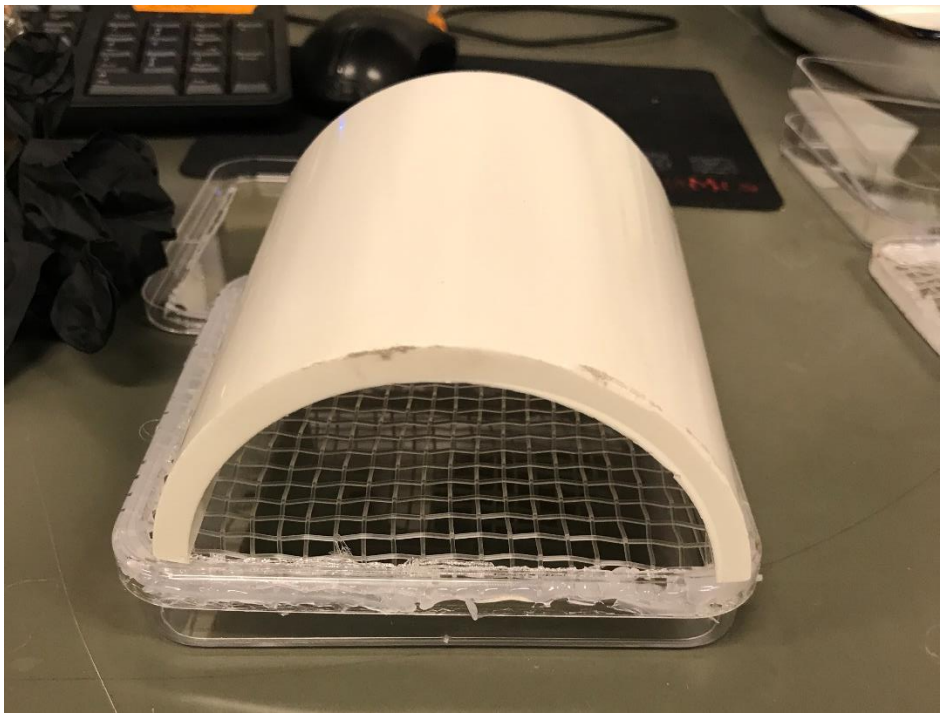

Figure 2. Nest Design. Image by Ben Browning 2021.

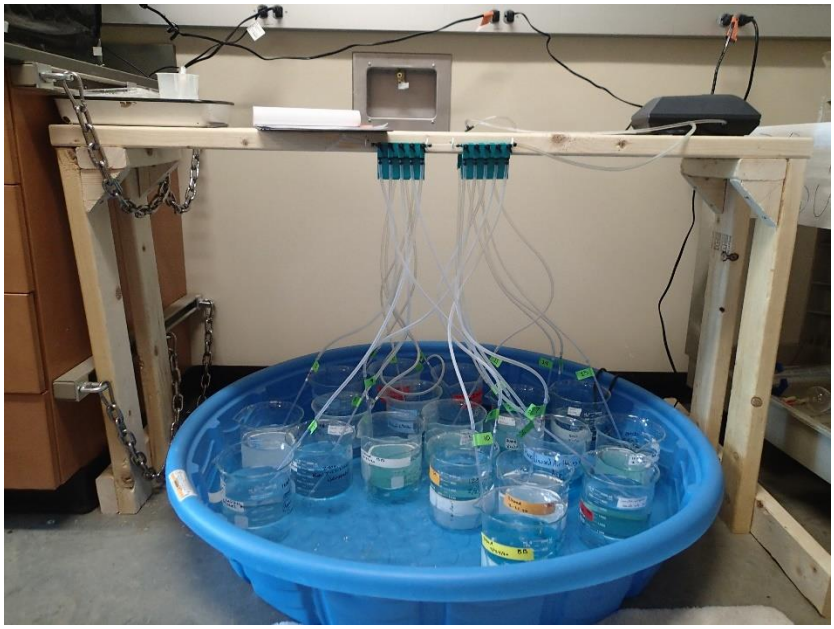

Figure 3. Water Bath for Clutch Incubation. Image by Ben Browning 2021.

Embryo-larval Tests

Embryo-larval tests followed the USEPA's *Method 1001.0: Fathead Minnow, Pimephales promelas, Larva; Survival and Teratogenicity Test; Chronic Toxicity* (USEPA, 2002b) unless otherwise stated. In order to adapt these methods to the experimental objectives, the following method deviations were utilized: due to the low number of spawns from one concentration at a time, the embryos from 1-2 spawns were collected together to provide a sufficient number for the test. Solution hardness and alkalinity were only measured on Day-0 and on the last solution renewal. Deformities were evaluated within four weeks of preservation. All embryo-larval tests were conducted using either the control water, 25% MW, 50% MW, or 100% MW as the test solution and Mn at 10 mg/L as the 100% concentration of the toxicant. A concentration of 10 mg Mn/L was chosen for the 100% concentration to prevent the chance of Mn to precipitate out of solution. This would not only decrease the concentration of Mn in a sample, it would also introduce a secondary mode of toxicity which could skew toxicity test results.

Deformity evaluation was conducted by an altered index presented in *A Teratogenic Deformity Index for Evaluating Impacts of Selenium on Fish Populations* (Lemly, 1997). The index was adjusted to have four different general categories of teratogenic deformities: spinal deformities, cranio-facial deformities, fin deformities, and yolk sac edema.

Figure 4. Examples of Deformed Larva. Images by Ben Browning 2021.
The left image is of a larva with yolk sac edema and the right image is of a larva with a spinal deformity.

Egg Counts

Eggs were counted using the Day-0 and Day-3 photographs taken of the clutches and catches. For Day-0 photos, the total number of eggs were counted. For Day-3 photos, the number of eggs which developed eyes were counted. Eggs that did not have eyes by Day-3 were deemed unfertilized for later statistics.

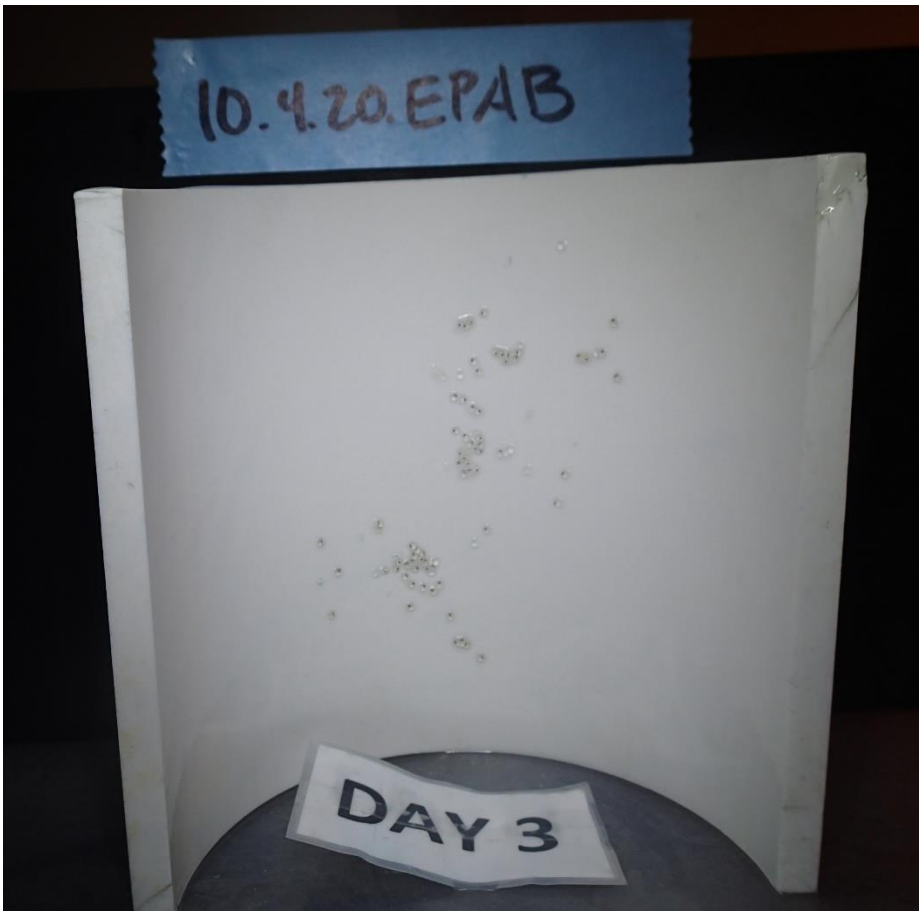

Figure 5. Day-3 Clutch Image Used for Egg Counts. Image by Ben Browning 2021.

Data Evaluation

The non-traditional toxicity endpoints used to examine the effect of the simulated mine water on the test organism was the fertilization rate of eggs in a clutch and the frequency of deformity types. The number of eyed eggs in a clutch in its Day-3 photo was divided by the total number of eggs in the same clutch in its Day-0 photo to yield a percentage of fertilized eggs. Clutches were then grouped by the concentration of the simulated mine water they originated from. Clutches that did not have Day-3 photos or could not be accurately counted due to fungal growths dissolving eggs were excluded from this analysis. Statistical analysis for the fertilization rate of clutches laid in different concentrations of the simulated mine water was conducted in the

program NCSS. Analysis of the fertilization rates was conducted by using the Kruskal-Wallis Test. For all statistical tests an alpha value of 0.05 was used.

The Kruskal-Wallis Test was also used to compare the fertilization rates of clutches coming from different replicates of breeding colonies to determine if time of year would affect fertilization rates. Four of these tests were run, one for each concentration of simulated mine water.

Frequency of deformity types was determined by grouping deformed larvae from the embryo-larval toxicity tests by the concentration of simulated mine water they were in, their test rep and the main type of deformity they had. The total for each type of deformity was then divided by the total number of deformed larvae from its rep to yield a proportion of the total deformities it represented. This proportion data was analyzed using the Friedman's Test to examine if the concentration of simulated mine water or the concentration of Mn had an effect on the frequencies of deformity types, and if the two toxicants had an interaction.

Objective 2

Standard acute, chronic and embryonic toxicity test endpoints with Mn or salinity as the primary toxicants were compared with dual contaminant exposures to evaluate whether higher concentrations of simulated mine water in conjunction with Mn will result in a synergistic, antagonistic or additive toxic effect.

Larval Acute Toxicity Tests

Larval acute tests followed the USEPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (USEPA, 2002a) unless otherwise stated. Each test was a 48-hour acute test. All larval acute tests were conducted using

either the control water, 25% MW, 50% MW, or 100% MW as the test solution and Mn at 10 mg/L as the 100% concentration of the toxicant.

Larval Chronic Toxicity Tests

Larval chronic toxicity tests followed the USEPA's *Short-term Methods for Estimating Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (USEPA, 2002b) unless otherwise stated. In order to adapt these methods to the experimental objectives, the following deviations were utilized: solution hardness and alkalinity were only measured on Day-0 and on the last solution renewal. All larval chronic tests were conducted using either the control water, 25% MW, 50% MW, or 100% MW as the test solution and Mn at 10 mg/L as the 100% concentration of the toxicant.

Data Evaluation

The standard toxicity test endpoints used to determine the combined effect of the simulated mine water and Mn were the no observable effect concentration (NOEC) and the lowest observable effects concentration (LOEC). Analysis of the standard toxicity tests were conducted using the program CETIS following USEPA methods (USEPA, 2002a, 2002b). The NOEC and LOEC were calculated by either the Steel Many-One Rank Sum Test or Dunnett Multiple Comparison Test depending on if the data met the test's assumptions.

Objective 3

The results obtained in objectives 1 and 2 were compared to historic data to evaluate if the non-standard endpoints tested in this study can reveal toxicity not observed in traditional endpoints.

Data Evaluation

The endpoints used in this study were compared to Mn and sulfate toxicity test results from past studies. The NOECs from past chronic toxicity tests and the LC50s from past acute toxicity tests on fathead minnows using Mn as the toxicant were collected to compare to the endpoints in this study to see if the mixture of the simulated mine water and Mn resulted in a synergistic, antagonistic, or additive effect compared to Mn alone. The results of the control water tests from this study were compared to historic data as well to evaluate the consistency in results. Since Mn and sulfate toxicity are hardness dependent, only Mn and sulfate tests with a reported hardness were used for comparison.

The NOECs and LOECs from past chronic toxicity tests on fathead minnows using sulfate as the toxicant were collected to compare to the endpoints in this study that used only the simulated mine water and to the endpoints generated using Mn in the simulated mine water. Toxicity data for sulfate was used to compare to the simulated mine water because the primary ion in the mixture is sulfate.

CHAPTER III

RESULTS

Objective 1

There was not a significant effect of the simulated mine water on the fertilization rate of clutches [Chi-squared = 0.55, p = 0.91]. Figure 6 displays the fertilization rates of clutches categorized by the concentration of simulated mine water.

There was no significant effect by the replicates of breeding colonies on the fertilization rates of clutches in the control [Chi-squared = 1.64, p = 0.65], 25% MW [Chi-squared = 7.66, p = 0.054], 50% MW [Chi-squared = 1.64, p = 0.20], or the 100% MW [Chi-squared = 1.43, p = 0.23] groups. Figure 7 displays the fertilization rates of clutches categorized by the replicates of breeding colonies from which they were collected.

There was a significant effect by the concentration of simulated mine water on the frequencies of spinal deformities [F-ratio = 12.66, p-value = <0.01] and fin deformities [F-ratio = 5.35, p-value = 0.02], but there was no significant effect by the simulated mine water on the frequency of cranio-facial deformities [F-ratio = 1.38, p-value = 0.25] or on the frequency of yolk sac deformities [F-ratio = 2.15, p-value = 0.10]. Figure 8 displays the frequency of deformity types based on the concentration of Mn and simulated mine water.

There was no significant effect by the concentration of Mn on the frequencies of spinal deformities [F-ratio = 1.03, p-value = 0.41], cranio-facial deformities [F-ratio = 2.23, p-value = 0.06], fin deformities [F-ratio = 0.84, p-value = 0.53], or yolk sac deformities [F-ratio = 0.85, p-value = 0.52].

There was no significant interaction between the concentration of simulated mine water and the concentration of Mn on the frequency of spinal deformities [F-ratio = 0.75, p-value =

0.73], cranio-facial deformities [F-ratio = 1.25, p-value = 0.26], fin deformities [F-ratio = 0.51, p-value = 0.93], or yolk sac deformities [F-ratio = 0.70, p-value = 0.77].

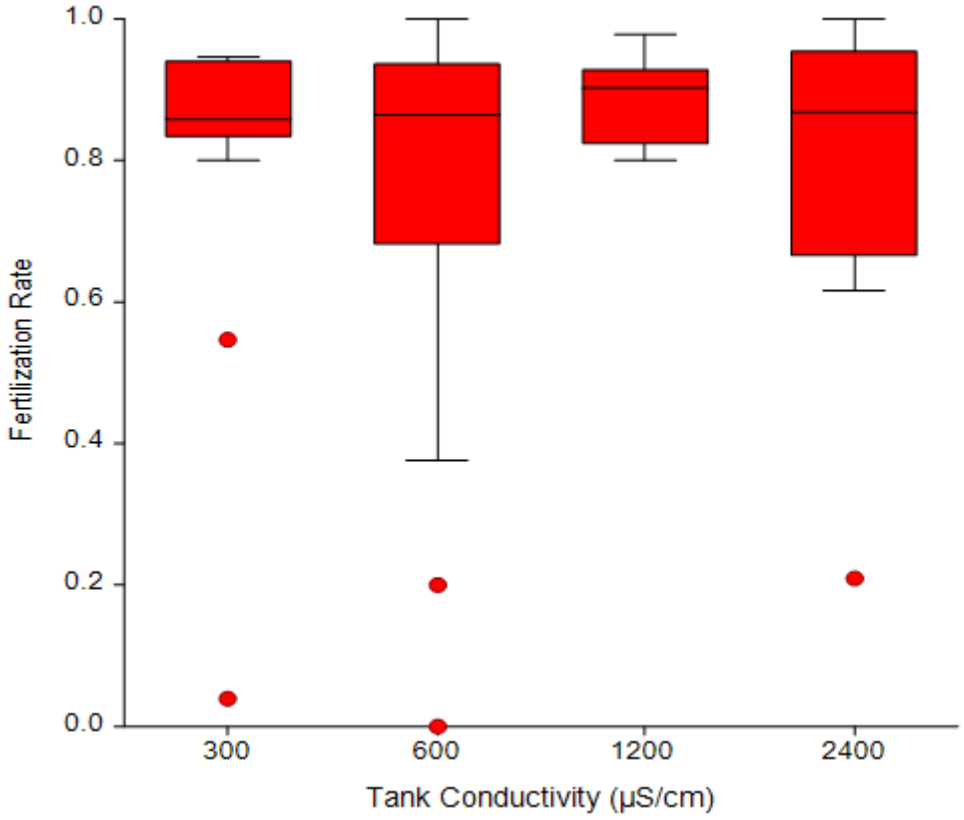

Figure 6. Box Plot of Clutch Fertilization Rates vs Tank Conductivity.
Figure 6 summarizes the fertilization rates of clutches from the three different concentrations of simulated mine water and the control (control conductivity = 300 µS/cm).

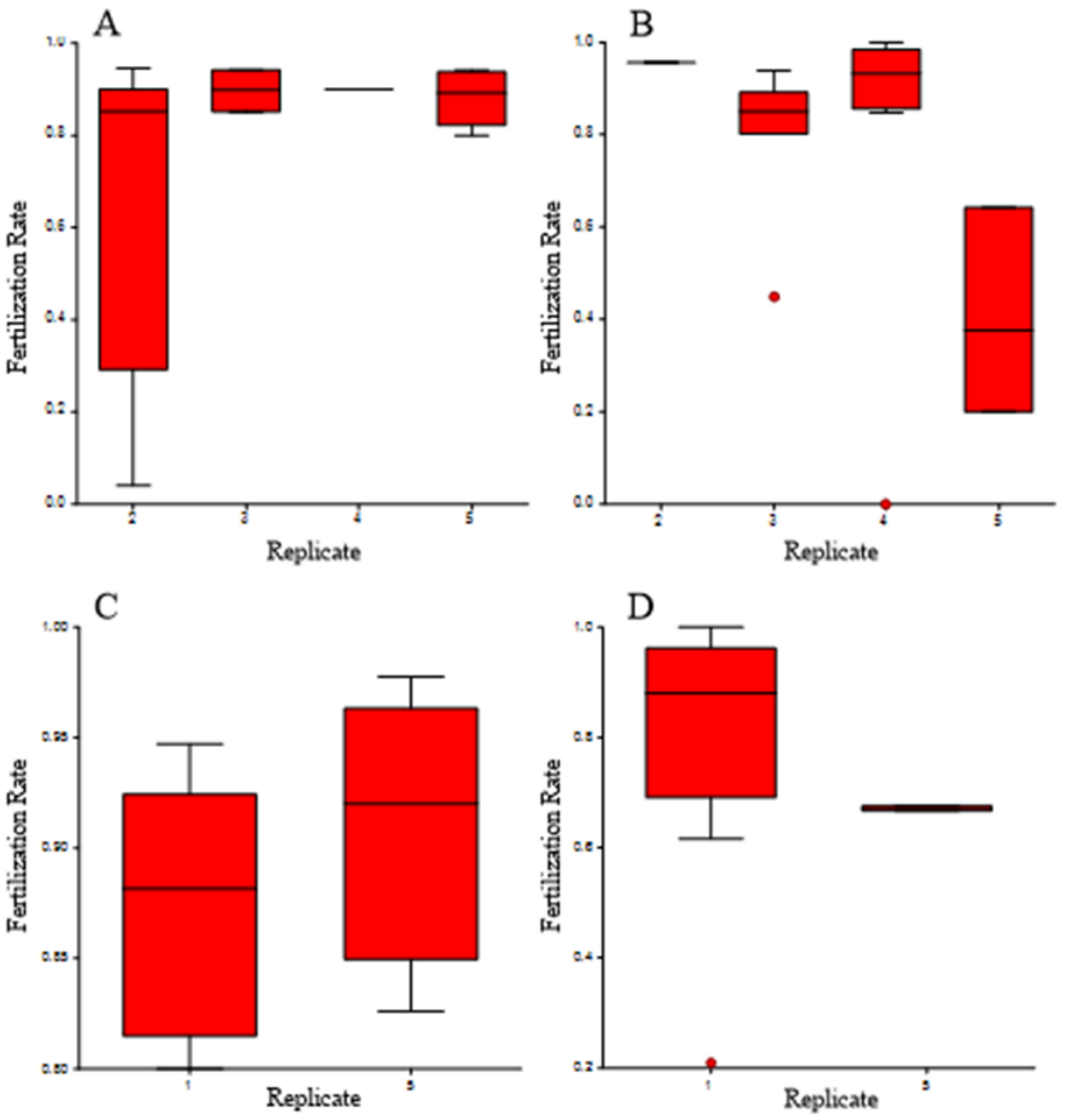

Figure 7. Box Plots of Fertilization Rates vs Replicate of Breeding Colonies.
Figure 7 illustrates [A] is all replicates of breeding colonies cultured with the control water. [B] is all replicates of breeding colonies cultured with the 25% MW. [C] is all replicates of breeding colonies cultured with the 50% MW. [D] is all replicates of breeding colonies cultured with the 100% MW.

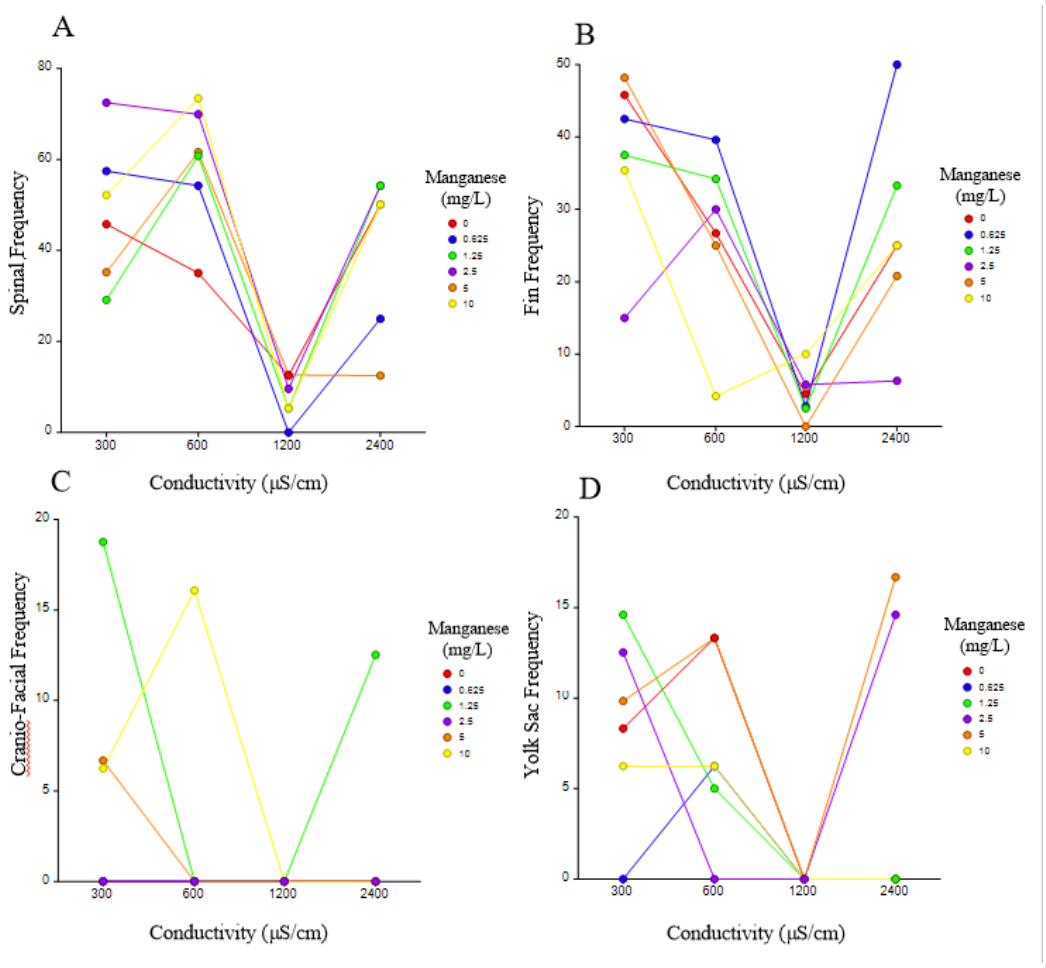

Figure 8. Frequency of Deformity Types.
Figure 8 summarizes the frequencies of each type of deformity as a fraction of the total deformed fish in a given test. [A] is the frequency of spinal deformities. [B] is the frequency of fin deformities. [C] is the frequency of cranio-facial deformities. [D] is the frequency of yolk sac deformities.

Objective 2

The 50% MW larval chronic toxicity test had a NOEC of 5 mg/L and a LOEC of 10 mg/L for the mean dry weight endpoint. All other traditional toxicity endpoints had NOECs of 10 mg/L and LOECs of >10 mg/L. Table 3 lists the results and tests used to derive the NOECs and LOECs.

Test Water	Toxicity Test	Endpoint	Statistical Test	NOEC (mg/L)	LOEC (mg/L)
EPA	Embryo	Survival	Steel Many-One	10	>10
EPA	Embryo	Proportion Normal	Dunnett	10	>10
EPA	Chronic	Survival	Steel Many-One	10	>10
EPA	Chronic	Mean Dry Weight	Dunnett	10	>10
EPA	Acute	Survival	Dunnett	10	>10
25% MW	Embryo	Survival	Steel Many-One	10	>10
25% MW	Embryo	Proportion Normal	Dunnett	10	>10
25% MW	Chronic *	Survival	x	x	x
25% MW	Chronic *	Mean Dry Weight	x	x	x
25% MW	Acute	Survival	Steel Many-One	10	>10
50% MW	Embryo	Survival	Dunnett	10	>10
50% MW	Embryo	Proportion Normal	Dunnett	10	>10
50% MW	Chronic	Survival	Steel Many-One	10	>10
50% MW	Chronic	Mean Dry Weight	Dunnett	5	10
50% MW	Acute**	Survival	Steel Many-One	10	>10
100% MW	Embryo	Survival	Dunnett	10	>10
100% MW	Embryo	Proportion Normal	Dunnett	10	>10
100% MW	Chronic	Survival	Steel Many-One	10	>10
100% MW	Chronic	Mean Dry Weight	Dunnett	10	>10
100% MW	Acute	Survival	Steel Many-One	10	>10

Table 3. Summary of the Standard Toxicity Tests.
This table presents the standard toxicity tests run and the statistical tests run to calculate the NOECs and LOECs for each endpoint. Tests marked with "*" were unable to be conducted due to lack of embryos from the appropriate breeding colonies. The test marked with "**" had two replicate tests ran at a later date to verify consistency in results and they yielded the exact same NOECs and LOECs.

Objective 3

The historic Mn data is displayed in Table 4. The historic sulfate data is displayed in Table 5. The historic sulfate data measured the amount of sulfate in a test by milligrams of sulfate per liter of solution. Table 6 displays the concentration of sulfate in the control water and the simulated mine water concentrations for comparison.

Species	LC50 (mg Mn/L)	NOEC (mg Mn/L)	Hardness ($CaCO_3^{2-}$/L)
Pimephales promelas	9.346	4.560	100
Pimephales promelas	15.826	7.860	200
Pimephales promelas	>45.000	-	396

Table 4. Fathead Minnows' Sensitivities to Manganese.
This table contains toxicity endpoints for Mn involving the fathead minnow. The hardness for each test is listed as well because Mn toxicity is hardness dependent (Stubblefield & Hockett, 2000). The LC50 endpoints are from acute toxicity test data. The NOEC endpoints are from chronic toxicity test data. This data was collected by a summary formed in Stubblefield & Hockett, 2000.

Species	Endpoint	NOEC (mg SO_4^{2-}/L)	LOEC (mg SO_4^{2-}/L)	Hardness ($CaCO_3^{2-}$/L)
Pimephales promelas	Survival	1300	2850	80
Pimephales promelas	Survival	2900	5250	320
Pimephales promelas	Mean dry weight	760	1300	80
Pimephales promelas	Mean dry weight	820	1400	320

Table 5. Fathead Minnows' Sensitivities to Sulfate.
This table contains toxicity endpoints for sulfate involving the fathead minnow, the hardness for each is listed as well because sulfate toxicity has been found to be hardness dependent (J. R. Elphick et al., 2011) This data was collected from Elphick et al., 2011.

Test Water	Sulfate Concentration (mg SO_4^{2-}/L)	Hardness (mg $CaCO_3^{2-}$/L)
EPA	120	100
25% MW	385	310
50% MW	770	620
100% MW	1540	1240

Table 6. Concentrations of Sulfate and Hardness in Each Test Solution.

CHAPTER IV

DISCUSSION

Objective 1

The different replicates of breeding colonies did not have statistically significant variation between their fertilization rates. Although the 25% MW tanks in replicate 5 of breeding colonies were close to being significantly different than the other replicates [Chi-squared = 7.66, p = 0.054]. The different breeding colony replicates had large variations in the number of clutches they produced, the 25% MW tanks in replicate 2 and the control tanks in replicate 4 only had one clutch recorded while the 100% MW tanks in replicate 1 had 17 clutches. This variation in numbers of clutches per replicate could have affected the range of fertilization rates and with larger sample sizes the tanks may have been even more similar in fertilization rates between replicates. The findings of this analysis mean fertilization rates did not change between breeding colony replicates due to variation in season or the biological variability of individual fish. If it was found to be the contrary, analyzing the fertilization rates of clutches from different breeding colony replicates would not be an effective analysis because additional variability from individual fish or seasonal variation could mask the potential effects of the simulated mine water. Knowing that the breeding colony replicates did not affect fertilization rates, the fertilization rates of the different concentrations of simulated mine water tanks were compared to each other and the control. It was determined that no concentrations of simulated mine water had a significant difference in fertilization rates of clutches between each concentration or the control.

The frequency of deformity types was compared against the concentration of simulated mine water and by the concentration of Mn to determine if either of these toxicants affected the types of deformities seen in fish. Such information could aid in understanding how these

toxicants are affecting aquatic organisms. It was found that the frequency of fin [F-ratio = 5.35, p-value = 0.02] and spinal [F-ratio = 12.66, p-value = <0.01] deformities was affected by the concentration of the simulated mine water. In Figure 8 plot A and B, the frequency of both deformities is observed as much lower in the 50% MW compared to the other concentrations and the control. The frequency of cranio-facial deformities was close to a significant effect by Mn [F-ratio = 2.23, p-value = 0.06] but trends were not as easily observable as in the plot of values as with the two previously mentioned ones. No cranio-facial deformities were observed in the 50% MW test in any concentrations of Mn which could have affected the analysis of this variable, giving it a low p-value. Additionally, there were no interactions between the concentrations of the simulated mine water and Mn that resulted in a significant effect to the fertilization rates of clutches. These findings on frequency of deformity types are difficult to interpret since there was no significant increase in the number of deformed fish by either Mn or the simulated mine water in any of the embryo-larval tests. Further investigation is necessary to explore how the 50% MW is reducing the frequency of spinal and fin deformities or if the concentration of simulated mine water is correlated with an unmeasured factor in this study.

Objective 2

The only traditional toxicity test that showed any result other than a NOEC of 10 mg Mn/L and a LOEC of >10 mg Mn/L was the chronic toxicity test using the 50% MW in conjunction with Mn. It found that the mean dry weight of the larvae had a NOEC of 5 mg Mn/L and a LOEC of 10 mg Mn/L. These results mean that the 10 mg Mn/L in conjunction with the 50% MW caused a significant decrease in the growth of the fish larvae. This reduction in growth is evidence of a potential synergistic or additive effect between the two toxicants. The lack of similar growth reduction in the 100% MW tests could be due to the increased hardness of the test

solution which will decrease the toxicity of sulfate and Mn. (Elphick et al., 2011; Stubblefield & Hockett, 2000) This in conjunction with the only significant findings from Objective 1 being with the 50% MW leads to interest in further study into this specific concentration of simulated mine water and why significant effects are caused by it but not higher conductivities.

Objective 3

Both Mn and sulfate toxicity are inversely proportional to hardness according to Stubblefield & Hockett, 2000 and Elphick et al., 2011. Due to the high hardness of the simulated mine water, which is listed in Figure 6, historic data is not readily available for direct comparison. Available data with the highest hardness that could be found was used for the best comparison possible.

Stubblefield & Hockett, 2000 had a NOEC and LC50 for Mn toxicity lower than the one determined in this study at a hardness of 100 mg $CaCO_3^{2-}$/L, their data is listed in Figure 4. Stubblefield's toxicity test at a hardness of 200 mg $CaCO_3^{2-}$/L had a lower NOEC than the one determined in the control tests of this study. These differences in NOECs and LC50s between the historic data and the findings of this study's control tests could be a result of different ages of the test organisms or different compositions of test solution. Stubblefield's other toxicity results listed in Figure 4 coincide with the findings of this study and except for the 50% MW chronic endpoint of mean dry weight which had a NOEC of 5 mg Mn/L and a LOEC of 10 mg Mn/L, hypotheses of why this was found is discussed after the comparisons to historic sulfate data.

These comparisons of historic data to findings of this study lead to the conclusion that the simulated mine water could have an antagonistic effect on Mn, decreasing its toxicity. To support this idea further testing would be required at higher concentrations of Mn to yield LOECs and LC50s for further comparison to historic data. Toxicity tests at higher concentrations

of Mn would not be as environmentally relevant though because Mn will begin to precipitate out of solution, causing other forms of toxicity non-specific to Mn.

Results of sulfate toxicity studies from Elphick et al., 2011 are listed in Figure 5. Elphick conducted chronic toxicity tests at similar hardnesses to both the control and 25% MW tests in this study and they yielded NOECs and LOECs similar to those found in this study. Results from Elphick's tests diverge from those in this study when comparing the mean dry weight endpoint's NOECs and LOECs. Elphick found the LOEC for mean dry weight was 1300 mg SO_4^{2-}/L at a hardness similar to the 25% MW test while this study found a LOEC of 770 mg SO_4^{2-}/L when this was combined with 10 mg Mn/L at a hardness double of the Elphick test in the 50% MW test. This could mean Mn has an additive or synergistic effect in coordination with the simulated mine water that inhibits fathead minnow growth. What is interesting is this additive or synergistic effect does not appear in the 100% MW which had sulfate concentration and hardness double of that in the 50% MW. This could be due to the higher hardness in the 100% MW test.

These results show that the combination of Mn and the simulated mine water may have an antagonistic effect to Mn toxicity but an additive or synergistic effect to the simulated mine water toxicity depending on the water hardness. To further investigate this potential additive or synergistic effect on sulfate toxicity in conjunction with Mn, chronic toxicity tests would need to be conducted at higher concentrations of sulfate while maintaining a consistent hardness.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

March 23, 2021

Ben Browning
613 14th Street
Huntington, WV 25701

Dear Mr. Browning:

This letter is in response to the submitted thesis abstract entitled *"Toxicity Evaluation of Simulated Mining Discharge to Early Life Stage of Fathead Minnows."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #708. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director