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Laboratory Investigation on the Effects of Conductivity on the Sensitive Early Life Stages of Fishes from the Appalachian Region

Logan Ryan Beach

LABORATORY INVESTIGATION ON THE EFFECTS OF CONDUCTIVITY ON THE SENSITIVE EARLY LIFE STAGES OF FISHES FROM THE APPALACHIAN REGION

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Master of Science
In
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by
Logan Ryan Beach
Approved by
Dr. Scott Simonton, Committee Chairperson
Dr. Mindy Armstead
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Marshall University
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APPROVAL OF THESIS

We, the faculty supervising the work of [Logan Ryan Beach], affirm that the [thesis/dissertation], [*Laboratory Investigation On The Effects Of Conductivity On The Sensitive Early Life Stages Of Fishes From The Appalachian Region*], meets the high academic standards for original scholarship and creative work established by the Environmental Science and the College of Information, Technology and Engineering. 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.

Dr. Scott Simonton,
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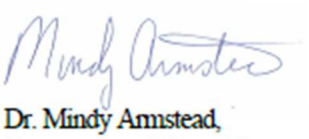
Committee Chairperson

6/5/20
Date



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ABSTRACT

While it is known there is a link between land disturbance and elevations in ionic constituents in streams, the relationship between elevated conductivity and aquatic taxa impairment is harder to define. Multiple field studies demonstrating correlations between conductivity and fish or benthic macroinvertebrate communities have not described the mechanisms of impairment and impairment has not been demonstrated with traditional toxicity testing. In an effort to explore more sensitive sub-lethal endpoints for evaluation of instream effects of mining effluent, chronic toxicity testing was conducted on eggs and early life stages of trout species and the fathead minnow, utilizing a simulated mining discharge with elevated conductivity. Chronic toxicity testing conducted with the native taxa and sub-lethal endpoints were utilized to evaluate the relationship between conductivity and organism fitness without the variability associated with field studies. Embryo-larval and standard chronic larval toxicity testing was conducted on sensitive life stages of brook trout (Salvelinus fontinalis) and rainbow trout (Oncorhynchus mykiss) using a high sulfate synthetic mine effluent. Testing was also conducted using the standard test organism, fathead minnows (Pimephales promelas). Comparison of the response between taxa and between life stages of individual taxa were made. Conductivities ranged from 100-2400 µS/cm in the exposures with mortality and teratogenesis being the endpoints in embryo-larval testing. Embryo exposures were initiated at fertilization in the fathead minnow tests and at 3 days old for rainbow trout, with both having EC50s greater than 2400 µS/cm. Generally, there was little sensitivity in the embryo or larval exposures with endpoints consistently >2400 µS/cm. Estimated effect concentrations (IC20s) were variable between the species and the life stages indicating that not only are the tolerance levels of each species different, but the tolerance of the life stages of each species is also variable.

CHAPTER 1

INTRODUCTION TO SALINITY AND ITS IMPACTS ON APPALACHIAN FISHES

Salinity Introduced

Salinity is the measured amount of salts that occur in a water sample. Salinity is naturally occurring and variable on earth within many different types of environments. Normal background salinity levels are, however not of huge concern. The main concern with salinity is the rising salinity in freshwater ecosystems by anthropogenic activities. These anthropogenic activates then cause salinization, which is the process of dissolved salts increasing in a given area (Kefford et al. 2016). The salinity levels of an ecosystem can rise from human disturbances such as mining discharge, farming, removal of vegetation, industrial waste and the application of salt to roadways (Canedo-Arguelles et al. 2013). These disturbances introduce dissolved solids into the waterways through overland flow and/or groundwater discharging.

When the salts become dissolved into the water, they can cause major disruptions within various aquatic assemblages. The disruptions can come with various consequences depending on the organism in question. Each organism has its own individual range of salinity tolerance in which it can function properly (CWT, 2004). Certain salinity levels are needed in freshwater ecosystems to aid in the fertilization of eggs, the inflation of the swim bladder and larval growth (Boeuf and Payan, 2001). For these reasons it is imperative that levels of salinity are monitored in the freshwater systems.

Some of the specific salts that are common in the Appalachian region are calcium, magnesium, potassium, sulfate, potassium and bicarbonate (Armstead et al. 2016). Once the salts arrive in the waterways their amounts can be measured by looking at the conductivity of a given sample. The measuring of conductivity is done in μS/cm. When it comes to measuring electrical

conductivity and total dissolved solids, they are positively related to each other in terms of indicating the salinity or mineralization of a sample. Charged ions of dissolved solids contribute to the conductivity in this aqueous relationship. Therefore, conductivity is generally a common variable to easily measure for the estimation of the dissolved solids (Gustafson and Behrman, 1939).

Basis for Study

One of the areas of main interest regarding increased salinity in aquatic systems in the Appalachian region is the coal mining areas the region is host to. It has been known for a while the effects of coal mining are generally not appealing to the human eye or health, but within the last 10-15 years some connections started to be made to the overall health of the waterways that these mining wastes find their way into (Johnson and Hallberg, 2005). Recently connections have started to come together in terms of how the salts that are entering the water may affect the health of all living organisms in each area.

Laboratory testing of surrogate organisms has not been demonstrating impairment in the range of conductivity generally found in coal mining regions (Kennedy et al. 2005). For this reason, a study was undertaken to evaluate the potential for impairment which standard toxicity testing failed to demonstrate. Due to the complex nature of performing field studies, the relationship was examined within a laboratory setting with controlled variability. Two lines of investigation included looking at more sensitive endpoints in surrogate test organisms, such as the egg and larval stages of life and evaluating the sensitivity of native taxa with respect to elevated salts. With the history of mining in the region, there is a critical need to better understand the effect of elevated conductivity on native stream organisms.

Objective of Study

With the previous studies being cited giving a glimpse as to what has been investigated already, it was felt that the subject could be expanded in several ways. The study was set up to investigate and evaluate the effects of elevated conductivity levels associated with the heavy mining in the Appalachian Region and a variety of stream organisms that reside in this area, within a laboratory setting. Several direct objectives had been set in place to investigate the effects.

1. To successfully rear salmonid eggs in a laboratory setting for toxicity use.
2. To successfully induce the reproductive cycle of the fathead minnow and gain egg clutches for test use while in high sulfate water.
3. Examine the relative effects of mining- related conductivity on surrogate test organisms and native stream taxa.
4. Compare the response of sensitive life-stages of surrogate and native fish to mining-related conductivity, particularly egg and larval stages.

CHAPTER 2

RESEARCH MATERIALS & METHODS

Introduction to Methods

Specifically, the sensitive life stages of the rainbow trout, brook trout and fathead minnows were exposed to a high sulfate simulated mine effluent with the elevated ionic concentrations representative of ratios and concentrations found in mining uninfluenced streams. Sensitive egg stages of fish were evaluated by having the eggs laid directly into the elevated conductivities to evaluate the potential effects on the most sensitive stages of the reproductive cycle: fertilization, hardening and development. The endpoints looked at included: percentage of eggs hatched, teratogenesis of larval stage and growth in the larval stages and hatch time of the egg stage.

Reconstituted stream water (Armstead et al. 2013) indicative of streams in the surface coal mining region receiving alkaline discharges was utilized in the toxicity testing. For every toxicity test being conducted four concentrations of the reconstituted water were used, along with USEPA's moderately hard water and diluted USEPA moderately hard water.

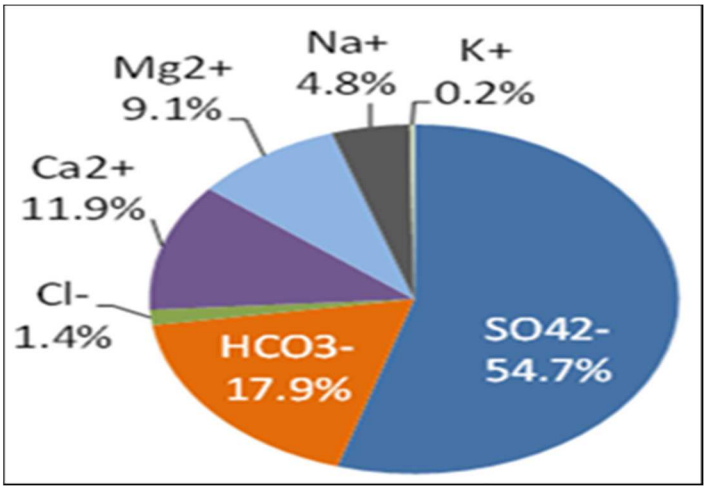

Figure 1 Salt Ratios in Simulated Mine Effluent (Armstead et al. 2013)

The fish used within the test were acquired from a few different sources. The rainbow trout used in the test were acquired from Cold Springs Trout Farm and the brook trout were acquired from Paint Bank Hatchery in Virginia. All life stages of the fathead minnows were acquired from Aquatic Biosystems. Before any of the fish were introduced to any of their test systems all parts of the test system were cleaned.

Rainbow & Brook Trout Egg Test

During initiation of the test a cool bath system was designed to help regulate proper water temperatures for the test. The bath was kept at a constant 55 degrees Fahrenheit +/- 5. The cool bath was then separated into 6 different chambers through the use of Tupperware containers to create the six sections for the concentrations. Each Tupperware container was then given four mason jars (replicates) and pump which provided the fresh water moving over the eggs and aeration throughout the test.

After setup was complete the jars were each given 15 pre-fertilized eggs at random. 20% water changes of the individual tubs were then administered on a weekly basis along with daily water chemistry being conducted out of each concentration. If a certain concentration fell out of its given range an alteration was made and noted to reset the concentration to its proper range of conductivity.

At 11:00 A.M. all jars were investigated for signs of hatching or death. If any larval trout had fully exited the egg at that time, the fish would be removed from the jars and placed in the freezer until they reached a state of torpor. The larval trout would then be transferred to pre-marked vials and introduced to 70% ethanol to preserve them for deformity evaluation. After all preserving was done for the day the jars would be cleaned of any egg debris as an attempt to

prevent any growth of fungus. Tests were run until all eggs had hatched or become solid white showing signs of death within the embryo.

Upon test completion the larval fish were examined for several different types of deformities. The larval fish were examined for the following deformities: craniofacial, spinal, yolk sac edema and anything else that would have prevented the fish from sustaining normal living functions. Evaluations were done by one member of the team and then QA/QC was done by another. Recordings were also kept to show how many larval trout were hatched out of each concentration for later comparison.

Figure 2 Trout Egg Test System. Image by L. Beach, 2020

Rainbow Trout Larval & Alevin Test

The larval test was conducted with 24-48-hour old rainbow trout. To initiate the test 2-liter beakers were filled with the six different concentrations of simulated mine effluent and four replicates were made of each. The 2-liter beakers were placed within a cool bath that maintained a constant temperature of 55-degree Fahrenheit +/- 5 degrees. The beakers were stored under 250

+/- 10 lux lighting with a 16h hour light and 8-hour night photoperiod. Daily 20% water changes were conducted, and daily water quality variables were recorded throughout the test. Upon completion of the 7-day test survival was evaluated using USEPA's WET Analysis Spreadsheet v1.6.1. When final tallies were taken for the survival the fish were then dried in the oven for the purpose of inspecting the growth of the trout. Methods for the test were taken in combination from the EPA's short-term methods and Rainbow Trout and Brook Trout 7-Day Survival and Growth Test Method (Lazorchak and Smith, 2007). The previously mentioned test set up was again used on 10-14-day old alevin stage fish and were evaluated the same way.

Figure 3 Larval & Alevin Trout Bath. Image by L. Beach, 2020

Fathead Reproductive/Embryo Toxicity Test

To initiate the test fathead minnows were separated into 6 ten-gallon tanks, with randomly selecting 1 male and 4 females to represent each tank. The sides of the tanks were

covered in black plastic to not stress the fish. The tanks were then gradually brought up to their given conductivity gradually as not to shock the fish over a three-day period. Additionally, the tank heat and photoperiod were manipulated at the initiation of the conductivity being raised to mimic that of spawning season, allowing the water to hold a constant 77 degrees Fahrenheit +/-5 and the photoperiod consisting of 16 hours light and 8 hours dark. Five-inch pvc pipe was cut in 4-inch sections, then cut in half to make nests. Four nests were kept in each tank throughout the running of the test and swapped out when nests were found. Fish were fed ½ tsp of feed twice daily and twenty percent water changes were conducted on a weekly basis and water chemistry (list chemistries) were conducted daily. Filters were cleaned, and charcoal was replaced on a monthly basis.

Nests were checked daily for eggs at 11:00 A.M. If eggs were found, that nest was replaced with an empty nest. Due to the large amount of eggs in each clutch, only a small subset (15 embryos) were kept on the tile nest. The eggs were stored in the corresponding effluent concentration in a 2-liter glass beaker. Eggs were slowly bubbled, and water changes were daily conducted on the eggs. Water quality was also recorded daily for the nests. Tanks were kept breeding until 4 reps of each concentration were collected.

Once the eggs hatched, the larval fish were collected and placed in the freezer until they reached a state of torpor. Larvae were then preserved in 10% formalin solution for 24 hours. After the 24 hours in the formalin solution the fatheads were then rinsed and stored in 70% ethanol. The preserved larval fish were examined for several different types of deformities. The larval fish were looked at for any of the following deformities: craniofacial, spinal, yolk sac edema and anything else that would have prevented the fish from sustaining normal living functions. Evaluations were done by one member of the team and then QA/QC by another.

Written records of the date the test was started were recorded and the date in which the egg clutches were laid was also recorded. These dates were then analyzed to see if any differences in reproductivity were found throughout the different concentrations. The date was also recorded for when the nest was laid and when all the viable larval fish were hatched from the nest. Length of hatch was then analyzed throughout the 6 different concentrations.

Figure 4 Fathead Minnow Reproductive System Layout. Image by L. Beach, 2020

Figure 5 Fathead Minnow Nest Layout. Image by L. Beach, 2020

Larval Fathead Minnow Test

The larval fathead minnow was initiated in two 6-well plates, as shown in Fig. 6, containing chambers for each replicate. A separate well plate was introduced for each concentration. The less than 24-hour old larvae were placed into their respective well plates at random and ran for a period of 7 days. The well plates were placed under 250 +/- 10 lux florescent lighting with a 16-hour day and 8-hour night photoperiod throughout the test. The larval fish were fed Artemia on a daily basis and water was changed daily with water quality also being done and recorded. Survival was evaluated using USEPA's WET Analysis Spreadsheet v1.6.1.

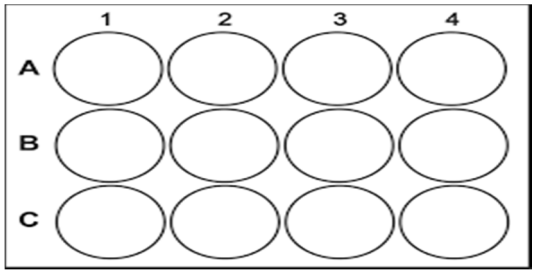

Figure 6 Well Plates

CHAPTER 3

RESULTS & DISCUSSION

Rainbow & Brook Trout Egg Test Results

During the brook trout portion of the test an unsuccessful fertilization of the eggs took place. Due to this reason no results are present for the brook trout. The graph for Fig. 7, shows the rainbow trout egg hatch was significantly reduced at all concentrations when compared to the EPA's reconstituted moderately hard water.

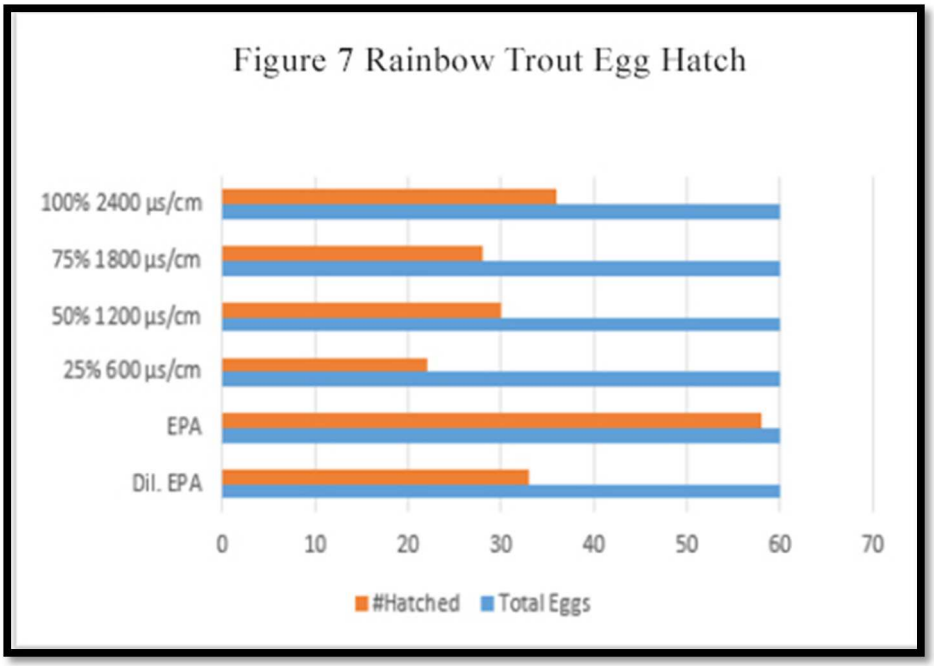

Figure 7 Rainbow Trout Egg Hatch

The figure also shows there is a statistical significance when comparing the control (300 µs/cm) and each of the higher concentrations (600 µs/cm – 2400 µs/cm) and the diluted EPA water at (100 µs/cm). Also, in the data collected in posttest examination, Fig. 8, shows us deformities within the larval trout were found to be much more present at higher concentrations than the control. A slight increase in deformities was also found in the diluted EPA water.

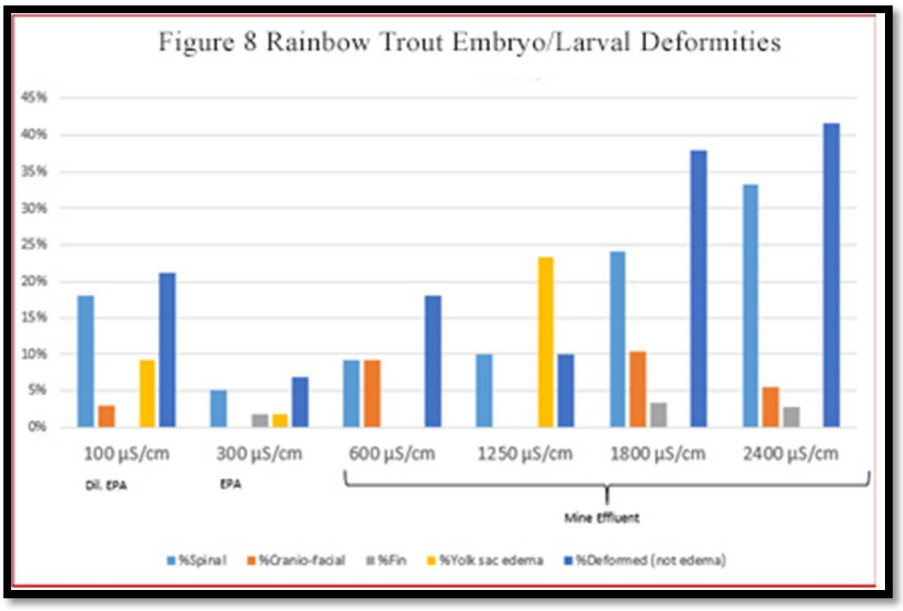

Figure 8 Rainbow Trout Embryo/Larval Deformities

Rainbow Trout Larval & Alevin 7-Day Chronic Results

The graph for Fig. 9, shows during the <24-hour old larval rainbow trout egg test the first sign of a statistical difference was at the 1800 µs/cm concentration. The chart shows there was a bounce back at the 2400 µs/cm concentration but not enough to rise above the trigger point. A possible reason for the slight increase in survival could possibly be linked to the overall health and fitness of the fish placed into those replicates. In Fig. 10 we see the larval rainbow trout showed absolutely no significant signs of statistical difference within the growth of the larvae during the 7-day chronic test.

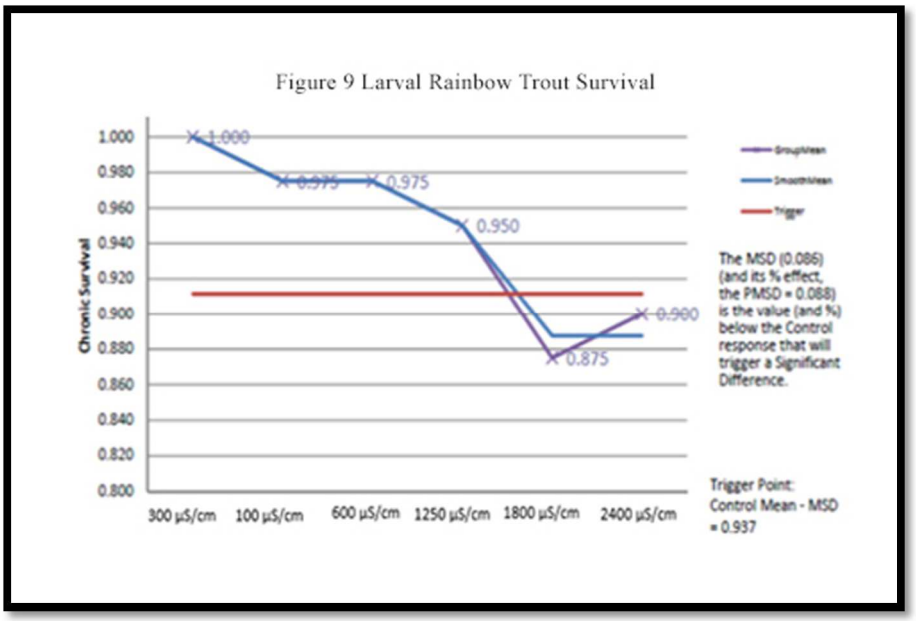

Figure 9 Larval Rainbow Trout Survival

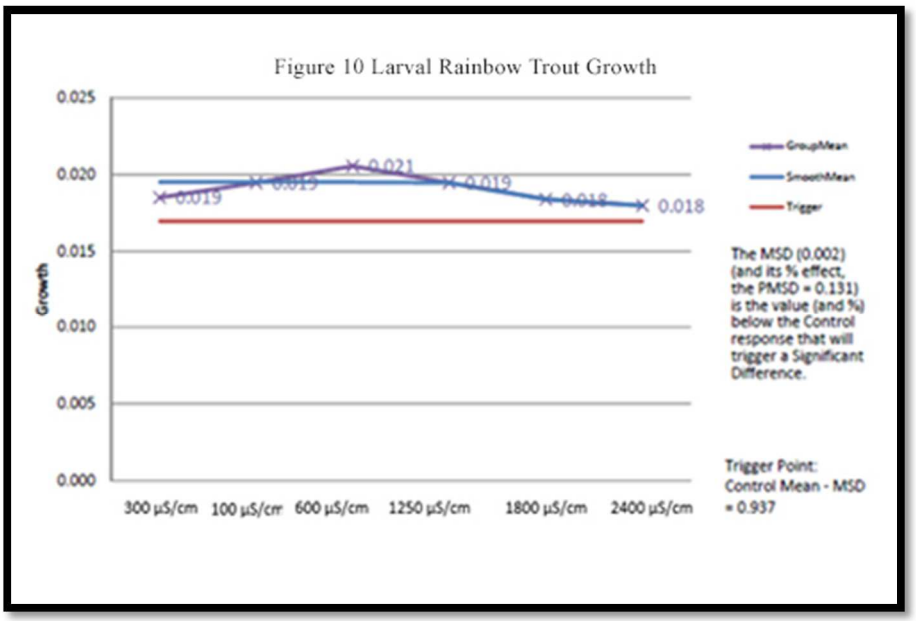

Figure 10 Larval Rainbow Trout Growth

When looking into the survival of the 10-14-day old rainbow trout, Fig. 11, shows us that the fish tended to be more sensitive in this specific test as the effects of the higher conductivity was greater. For statistical reference we see the alevin trout had an LOEC of 1250 µs/cm during the 7-day chronic test.

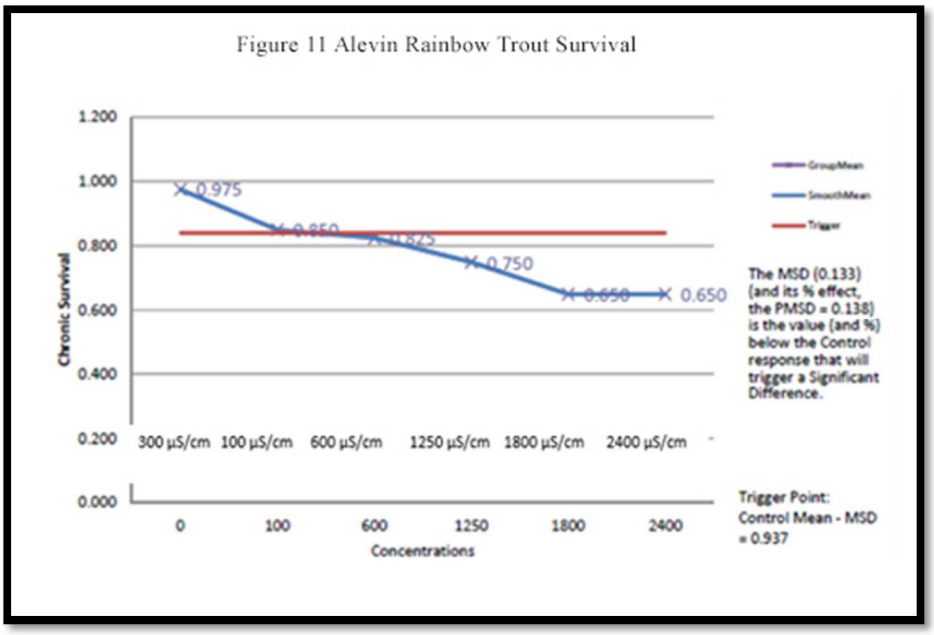

Figure 11 Alevin Rainbow Trout Survival

Again, like with the larval trout a growth observation was made as shown in Fig. 12. The graph shows us the alevin stage also was not affected statistically in terms of growth by the higher conductivity concentrations.

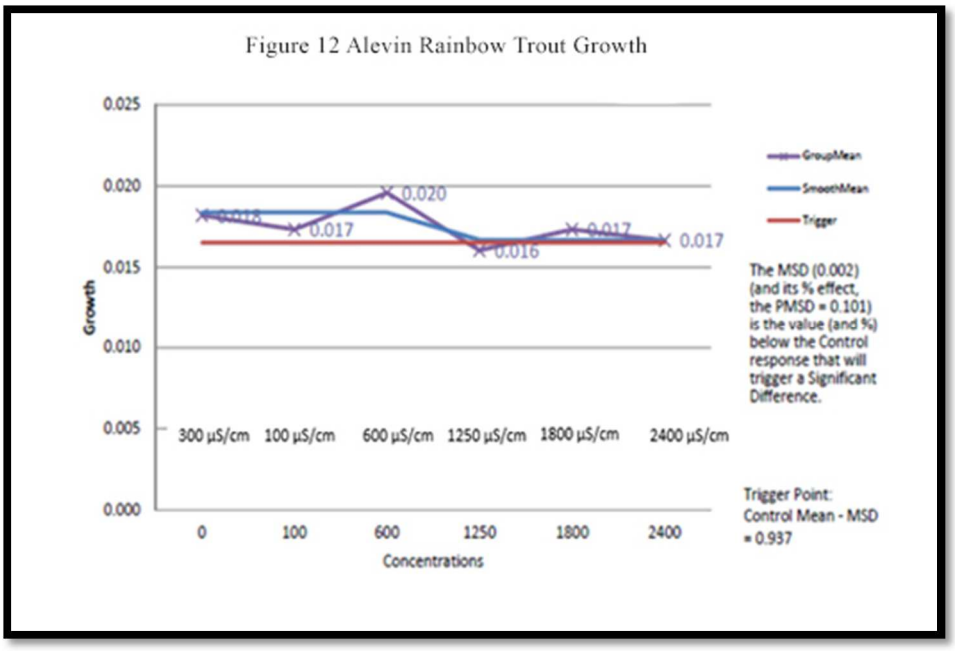

Figure 12 Alevin Rainbow Trout Growth

Fathead Reproductive/Embryo Toxicity Test Results

It was found during the reproductive test involving the fathead minnow that lower concentrations were more successful in producing egg clutches throughout the test. Though the test did yield a proper number of replicates throughout all concentrations the lower concentrations and the EPA water control produced more throughout the testing time. Also noticed in the test was a slight upward trend in the amount of time taken to completely hatch all eggs in the higher concentrations, as shown in Fig. 13.

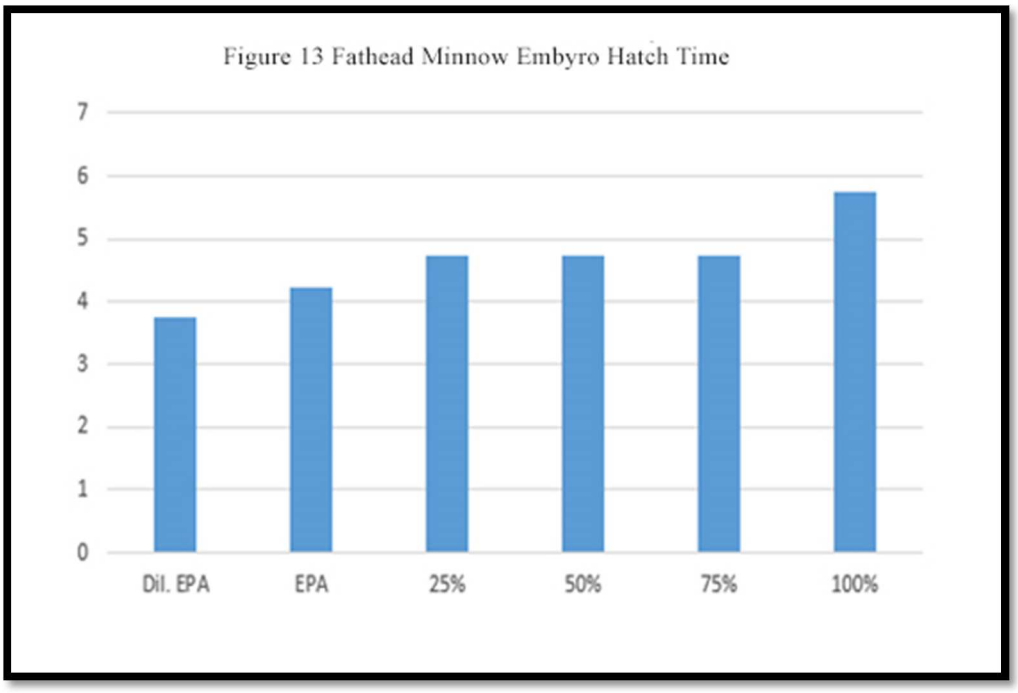

Figure 13 Fathead Minnow Embryo Hatch Time

Some effects of the higher concentrations, as seen in Fig. 14, were also seen in terms of how successful each nest was with hatching. The lower concentrations successfully reared around 83% of all their eggs. The higher concentrations however remained around 58% successful in terms of hatching.

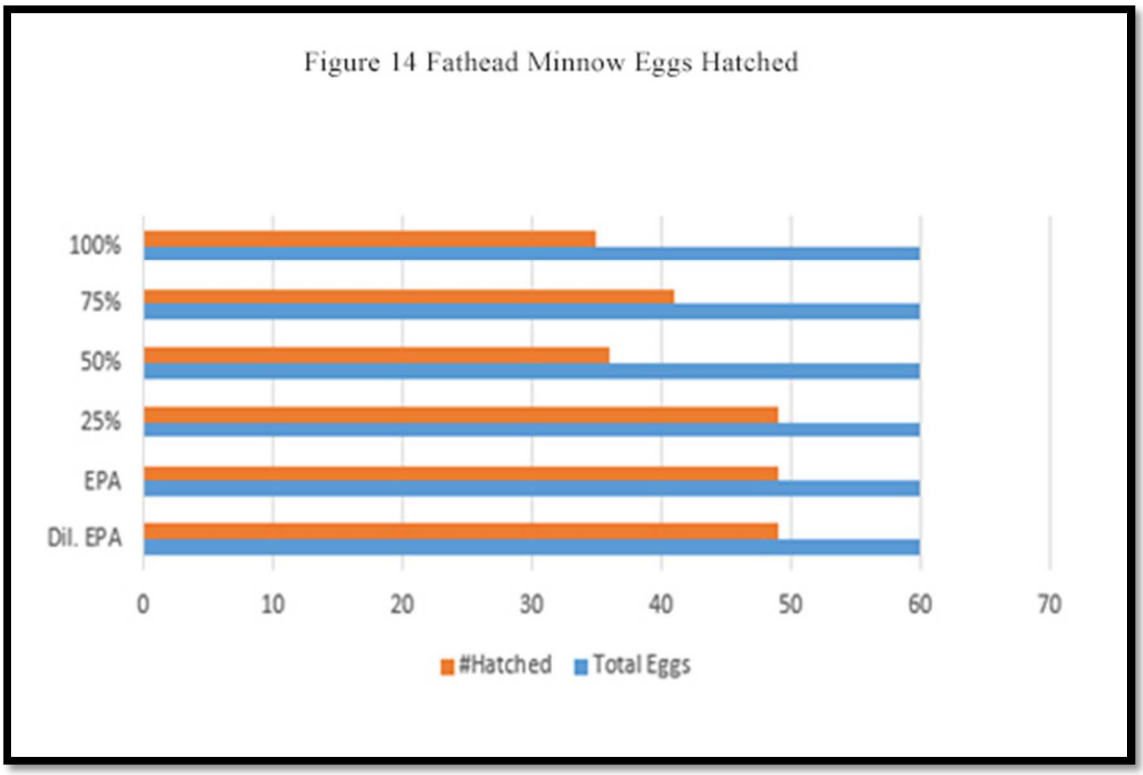

Figure 14 Fathead Minnow Eggs Hatched

Fathead Minnow Larval 7-Day Chronic Results

The larval fathead minnows fared the best out of any of the tests completed. As shown in figure 15, there was no statistical difference within the survival of the fathead minnows throughout the test. There also was no statistical difference within the growth of the fathead minnows, which was determined by weight. The differences in growth of the fathead minnow are shown in Fig. 16. Overall, through both endpoints there was no sign impairment for the larval fathead minnows.

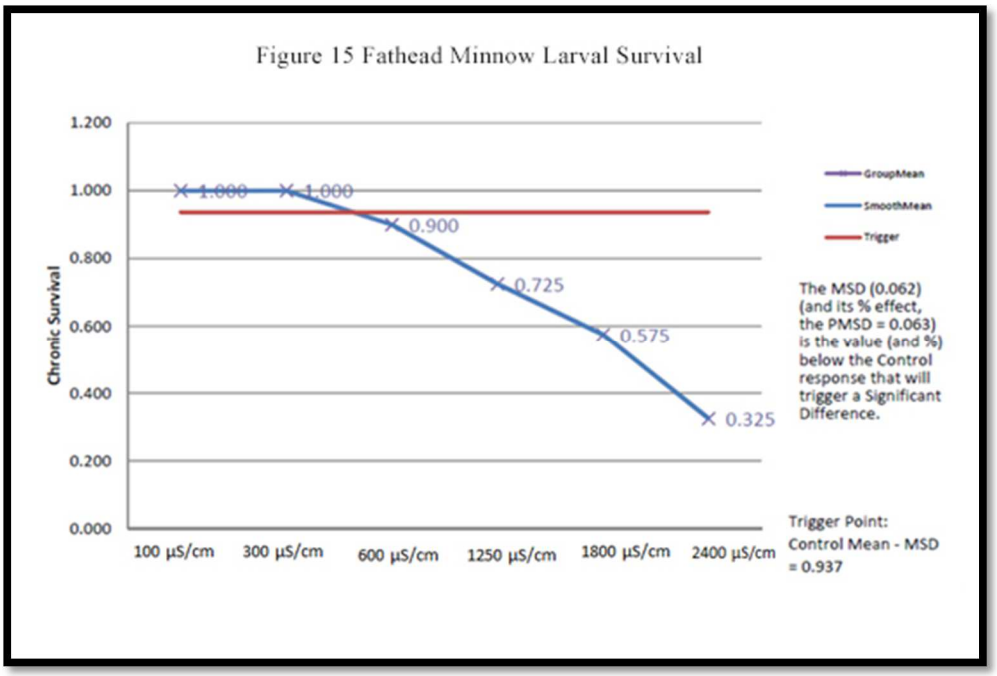

Figure 15 Fathead Minnow Larval Survival

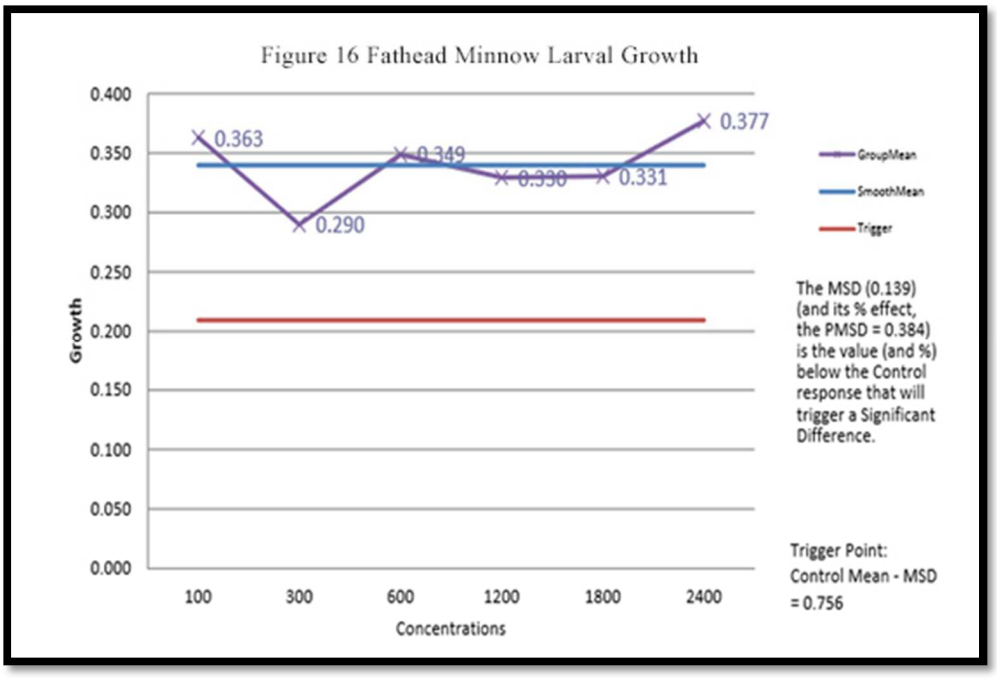

Figure 16 Fathead Minnow Laval Growth

Discussion

The fathead minnows overall outperformed the rainbow trout in every test while being introduced to the high conductivity effluent. Though we cannot compare reproductivity between the two species, we can assume that exposing breeding adults to the mine effluent more realistically represents stream exposures than traditional embryo larval testing. With this exposure scenario, effects of elevated conductivity are demonstrated at concentrations generally believed acceptable for sulfate exposures. The older rainbow trout experienced more mortality than the younger, which could have been caused by handling stress or lack of food during the time since hatching. When the eggs are exposed to the elevated conductivity, survival becomes the most sensitive endpoint as compared with growth which may be specific to the osmoregulatory stressor acting on processes such as hardening or may result from the toxicity of the individual salts. Additional work is planned to determine the mechanism for this impairment. Teratogenic deformities in the control water were less than 10%, by convention the threshold for acceptable rates. However, all other test concentrations, including the diluted water control, demonstrated deformity rates greater than the threshold indicating a narrow range of conditions favorable to development. These evaluations can provide information needed for understanding when the impacts occur during organism development. Further analysis will be conducted to evaluate teratogenic deformities with fathead minnows as well as conducting comparable analyses with brook trout larvae to further understand the influence of high sulfate effluents to sensitive life stages to populations of freshwater aquatic organisms in the Appalachian region.

CHAPTER 4

CONCLUSIONS

Summary

To summarize the research, a final look at the research will be taken in terms of the objectives. The ability to rear salmonid eggs for use in the tests proved to be successful and got better with time as new requirements for the eggs were learned throughout testing. The same situation took place with the fathead minnows that were setup in tanks and used for reproduction to collect eggs from. Beginning stages of reproduction were slow, but learning how the fathead minnows reacted to the high conductivity solution helped the tests and results improved.

In terms of the effects on the species used in testing, the fathead minnow tended to be a little less sensitive that the rainbow trout in all aspects of the test. It must also be noted that when looking at the results of the test, the rainbow trout eggs were not introduced to toxicity before being laid; whereas, the fathead minnow eggs came from subjects that had already been placed into the high conductivity mixture.

I feel that further research should be conducted on the basic principles of this test as salinization of our freshwater systems is a growing issue. I have a few main points I feel should be looked at into the future. The first main point is I would like to see what would take place with the rainbow trout eggs if the surrogates were to spawn in the simulated mine effluent like the fathead minnows did. A second area of interest would be to rerun all tests with the knowledge learned from all the tests as some things have not really been tested before in a way like we had. It also would be beneficial to run the reproductivity with both species and to see how much of the simulated mine effluent the surrogates absorb and possibly how much is in the eggs if possible.

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APPENDIX A

IRB LETTER

MARSHALL UNIVERSITY.
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Office of Research Integrity

December 13, 2018

Logan Beach
124 Beechwood Estates
Scott Depot, WV 25560

Dear Mr. Beach:

This letter is in response to the submitted thesis abstract entitled *"Laboratory Investigation on the Effects of Conductivity on the Sensitive Early Life Stages of Fishes from the Appalachian Region."* 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 #661. 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