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# Ephemeroptera Culturing Methods: An Analysis on Rearing and Toxcity Testing on Sensitive Early Life Stages of Native Mayfly Taxa

Geneve Rainette Edwards
edwardsgeneve@gmail.com

# EPHEMEROPTERA CULTURING METHODS: AN ANALYSIS ON REARING AND TOXCITY TESTING ON SENSITIVE EARLY LIFE STAGES OF NATIVE MAYFLY TAXA

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Geneve Rainette Edwards
Approved by
Dr. Scott Simonton, Committee Chairperson
Dr. Mindy Yeager-Armstead
Mandee Wilson M.S.

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**APPROVAL OF THESIS**

We, the faculty supervising the work of Geneve Rainette Edwards, affirm that the thesis, *Ephemeroptera Culturing Methods: An Analysis on Rearing and Toxicity Testing on Sensitive Early Life Stages of Native Mayfly Taxa*, 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.

D. Scott Simonton

Dr. Scott Simonton,
College of Information, Technology and Engineering
Committee Chairperson Date 3/31/2022

Dr. Mindy Yeager-Armstead,
Department of Natural Resources and the Environment
Committee Member Date 3/31/2022

Amanda L. Wilson

Mandee Wilson M.S.
Department of Natural Resources and the Environment
Committee Member Date 3/31/2022

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## ABSTRACT

Standard toxicity testing organisms are utilized for regulatory purposes and often ecological risk assessments. Ephemeroptera taxa have been reported to be more sensitive to aquatic contaminants than the standard toxicity testing organisms currently used in determining effects on aquatic ecosystems. Establishing methods for culturing native Ephemeroptera taxa will provide a more sensitive test organism to determine the toxicity of contaminants and will be more representative of the responses of native taxa. Additionally, it will provide a test organism at the most sensitive life stages. The objective of this research is to develop methods for culturing and testing of native Ephemeroptera in the laboratory. Two different experiments were conducted to try to establish culturing and testing methods: temperature fluctuation effects on egg development and preliminary high sulfate toxicity tests on eggs and nymphs. Eggs were collected from native Ephemeroptera via oviposition or dissection and exposed to various temperature and high sulfate toxicity treatments during incubation. During the temperature test the eggs would either be put in a constant temperature or moved from a low (10°C) to a high temperature (20°C or 24°C). Incubation duration, hatch rate and hatch length were evaluated to see if incubation temperature manipulation can be utilized to provide consistently available juvenile Ephemeroptera for toxicity testing. The results showed no significant difference between the temperature treatments and egg development, which shows the low storage method can be utilized and will allow for less frequent field collections and more testing year-round due to increased holding time for eggs. In the preliminary high sulfate toxicity test, both eggs and nymphs were exposed to a simulated mine effluent representing exposure to elevated conductivity in mining influenced Appalachian streams. Endpoints evaluated were hatch rate, hatch duration, incubation period, survival and growth of juveniles exposed to elevated

conductivity post-hatch. Multiple taxa were evaluated including Ephemeridae (*Hexagenia* sp.), Heptageniidae (*Epeorus* sp.) and Baetidae (*Acentrella* sp. and *Baetis* sp.). The significant difference between the natural water control, the reconstituted water control and all treatment groups was hypothesized to originate from the natural conditions being optimal for genus-specific survival. These mechanisms of greater development and survival in the natural water have not been confirmed but are under further investigation.

# CHAPTER 1

# INTRODUCTION INTO EPHEMEROPTERA TESTING

## Ephemeroptera Life History

Ephemeroptera is an order of over 2,100 aquatic insect species commonly known as mayflies. The name Ephemeroptera is derived from the Greek "ephemera" meaning short-lived, and "ptera" meaning wings (Meyer, 2020). Mayflies are known for mass emergence and short adult lifespans. They are mostly found in unpolluted habitats with fresh, flowing water. About 700 of the species can be found in North America (Ecospark, 2022). Mayflies are considered a keystone species and can indicate the conditions of the aquatic system they inhabit.

Mayflies go through three life stages: egg, nymph, and adult (Figure 1). Their life cycles involve both aquatic and terrestrial phases (Brittain, 1990; Wilbur, 1980). During mating, males form a swarm and the females fly up to meet them to facilitate fertilization. Once copulation has occurred, the female will fly down to the water and oviposit eggs. Eggs can take a few days to months to hatch depending on environmental conditions and species of mayfly. Once hatched the nymphs will begin grazing on diatoms and algae going through multiple instars as they grow bigger. The nymph will then swim to the surface of the water where it will emerge from the water as a sexually immature subimago. This instar only occurs in mayflies and can be identified by the opaque wing coloring. Within the next few hours, the subimago will shed its exuvia one last time to reach sexual maturity as an imago which can be identified by clear wings (Figure 2). This final instar is only for reproduction and egg dispersal.

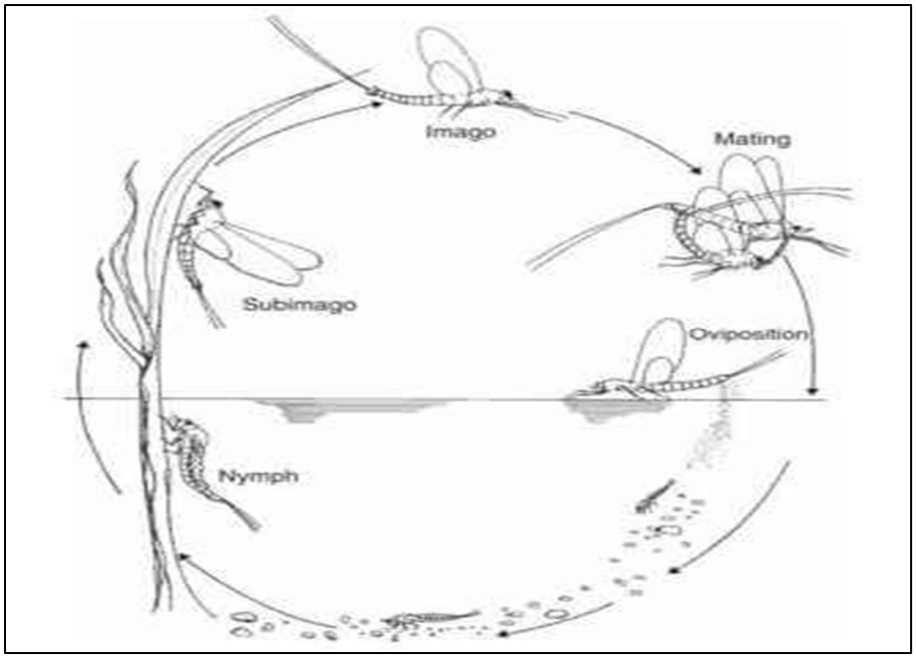


**Figure 1 (Ephemeroptera (Mayflies) (Insects), 2007)**

**Figure 2 Male & Female Baetidae Imago. Image by Geneve Edwards**

Not all female mayflies need to mate to produce offspring. Some genera of mayflies are parthenogenetic which is a form of asexual reproduction in which females can develop unfertilized eggs into embryos that will grow into genetic clones of the mother.

**Temperature Effects on Egg Development**

There are many environmental factors that contribute to the life history of mayflies, such as temperature. Water temperature is a major influence on egg development. It has been shown with mayflies that the relationship between water temperature and length of egg development is codependent, therefore they are more temperature dependent than other macroinvertebrates (Brittain, 1990). It has also been shown that the effects of temperature on egg development (incubation days, hatch rate, hatch success & hatch time) was direct and strongly dependent on temperature (Brittain, 1990; Parnrong & Campbell, 2003; Jackson & Sweeney, 1995).

In several studies conducted on temperature effects on egg development, the eggs were incubated in the laboratory with temperatures ranging from 3℃-22℃ (Parnrong & Campbell, 2003; Humpesch, 1980; Jackson & Sweeney, 1995). At 22℃, the eggs required about 10 days incubation before hatching, but at 9℃ an incubation period of about 2 months was required (Parnrong & Campbell, 2003). Hatching time and hatching success were also temperature dependent, with a large proportion of the eggs hatching at 19℃ and 22℃, with the proportion decreasing as the incubation temperature was reduced (Parnrong & Campbell, 2003). Hatching occurred at 9℃, 14℃, 19℃ and 22℃, but not at 4℃, even after the eggs were incubated for four months; however, some egg development occurred at 4℃ when the embryos formed visible ocelli after three weeks of incubation (Parnrong & Campbell, 2003). Hatching time decreased with increasing temperature and the relationship between the two variables within the temperature range 3.5℃-22℃ was well described by the power law (Humpesch, 1980; Jackson & Sweeney, 1996; Parnrong & Campbell, 2003). The number of degree-days (i.e., incubation temperature multiplied by incubation time) for hatching was linearly related to water temperature (Humpesch, 1980; Jackson & Sweeney, 1995; Parnrong & Campbell, 2003). Approximately 440

degree-days were required for eggs to hatch at 10℃, whereas only 260 degree-days were required at 20℃ (Parnrong & Campbell, 2003).

Time from egg development to hatch can range from a few days to over a month (Jackson & Sweeney, 1995). It has been shown in the previously stated studies that hatch can occur at a range from 4℃ to over 22℃ (Brittain, 2000; Humpesch, 1984). Most studies on the effect of temperature on egg development have been carried out in the laboratory at constant temperatures, while the effect of fluctuating temperatures on development is uncertain and may differ from species to species (Brittain, 2000; Sweeney, 1978; Humpesch, 1982). One study found that eggs can be stored at 4℃, transferred into a 10℃ incubator for 3 days, and then moved into a 25℃ incubator until hatching, if freshly collected eggs are not available (Weaver et al., 2015). In another study on toxicity testing on mayflies, prior to the start of the test, a vial containing eggs from 3 females was moved from 10℃ environmental chambers to a 25℃ chamber to encourage hatching of eggs (Soucek & Dickinson, 2015). With this being stated, the research I conducted is to determine if temperature fluctuations can be used to manipulate hatch to provide for a more consistently available source of test organisms.

**Salinity Effects on Macroinvertebrates in Appalachia**

Salinity is the measure of the concentration of dissolved salts in water. The salinity of water is expressed most as specific conductivity. Specific conductivity is the ability of a material to conduct an electric current measured in microSiemens per centimeter (µS/cm) standardized to 25°C (USEPA, 2011). Salinity is increasing in freshwater due to anthropogenic effects and is becoming a major environmental concern, while its effects on benthic macroinvertebrate communities is still under investigation.

In studies done on the patterns of Ephemeroptera taxa loss on Appalachian streams results have shown that mayfly communities are disappearing from streams where mining disturbance has occurred (Fritz et al., 2010; Pond, 2010; Merrick et al., 2006; Chambers & Messinger, 2001). Mayfly richness and relative abundance were significantly higher at reference sites compared to mining sites (Pond, 2010; Chambers & Messinger, 2001). The average proportion of mayfly richness to total benthic taxa richness in the sample was 20% at reference sites, 17% at residential sites, 14% at mined/residential sites, and 6% at mined sites (Pond, 2010). Specific conductance and sulfate concentration of stream water were most strongly correlated with effects on invertebrate communities (Kefford et al., 2012; Hassell et al., 2006; Chambers & Messinger, 2001). Although basin size and physiography were important in structuring communities, coal mining was the greatest anthropogenic influence in basins of less than 128 $mi^2$ (Chambers & Messinger, 2001).

Appalachian field studies have shown that mayflies have been reduced from some streams with the loss attributed to elevated dissolved solids, mostly derived from mining (Fritz et al., 2010; Pond, 2010; Merricks et al., 2006). Establishing methods to culture mayflies in lab would provide sensitive test organisms to evaluate high conductivity effects on native mayflies.

**Toxicity Testing**

The assessment of ecological risk assessment provides information for environmental policies based upon the most comprehensive scientific information available (Bauernfeind & Moog, 2000). There are standard toxicity testing organisms utilized for regulatory purposes and ecological risk assessments. These test organisms, even though easily cultured, do not represent the most sensitive native taxa. Multiple studies have suggested that mayflies may be more sensitive to aquatic disturbances than the standard test organisms (Gerritsen et al., 2000;

Rosenberg & Resh, 1996). Mayflies are considered a sensitive aquatic bioindicator species which can indicate water quality (Bauernfeind & Moog, 2000). Mayflies can be used in toxicity testing to help establish a direct link between contaminants of concern and the effects they have on native taxa.

In *A Field-based Aquatic Life Benchmark for Conductivity in Central Appalachian streams* it states that waters in the Appalachian Region that are dominated by salts of $Ca^{2+}$, $Mg^{2+}$, $SO_4^{2-}$ and $HCO_3^-$ at a circum-neutral to alkaline pH (USEPA, 2011). It also suggests that a specific conductance benchmark of 300 µS/cm for the protection of aquatic life in streams and river in the region (USEPA, 2011). With the exposure of aquatic organisms to salinity being most often direct, aquatic insects, such as mayflies, have a lower salinity tolerance having been evolved in low-salt environments (USEPA, 2002). Whole Effluent Toxicity (or WET) testing can be used to measure contaminants of concerns' toxic effects on surrogate test organisms' ability to survive, grow, and reproduce (USEPA, 2002). This can show a dose-response relationship which assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response (USEPA, 2002). The results from WET testing can generate a dose-dependent response concentration (NOEC, LOEC) or point estimation (LC50, EC50, IC25). The application of sensitive mayfly species in laboratory research will help to advance the understanding between standard laboratory toxicity test results and field-based observations of community impairment (Soucek & Dickinson, 2015).

**Objectives**

The end goal of this research is to develop methods for culturing and testing of mayfly taxa in the laboratory. The specific objectives for the temperature study are to:

- To evaluate if temperature fluctuations effect incubation duration, hatch rate, and hatch length.

- Deduce if incubation temperature manipulation can be utilized to improve the availability of laboratory reared organisms for toxicity testing.

The specific objectives for the toxicity test are to:

- To establish toxicity testing methods for native Ephemeroptera taxa
- To evaluate high conductivity effects on sensitive life stages of Ephemeroptera

# CHAPTER II

# MATERIALS AND METHODS

## Mayfly Collection

Baetidae and Heptageniidae nymphs were collected from Mash Fork in Mercer County, West Virginia (Figure 3). The mayfly nymphs were collected by hand off of substrate located in riffles and runs of the stream. They were then placed in an aerated container with stream water, placed in a cooler, and returned to the laboratory for separation.


**Figure 3 Mash Fork Collection Site. Map by Mandee Wilson**

Adult Ephemeridae (*Hexagenia* sp.) mayflies were collected from the banks of the Kanawha River in Kanawha County, West Virginia. Adult mayflies were caught with nets during emergence and placed in holding chambers. Water was collected at both sites and filtered with a 54-micron sieve and continuously aerated to provided optimal dissolved oxygen.

Once back from field collection the mayfly nymphs were immediately counted, identified to the lowest practical taxon, and placed into the hexagon or stream culturing unit until emergence. The hexagon culturing unit is an aquatic plant culturing system with continuously flowing water simulating a stream (Figure 4). Filtered water from Mash Fork was added to the unit as needed. The unit contained a pump and chiller to keep the water constantly flowing, aerated, and at a regulated temperature. The culturing unit was fitted with lights on a 16hr/8hr light/dark cycle. A mosquito net was wrapped around the unit so upon emergence the mayflies could cling to it and be easily removed for egg collection.

**Figure 4 Hexagon Culturing Unit. Image by Geneve Edwards**

The stream culturing unit was constructed using natural rock substrate placed in a rectangular plastic tray propped at an approximately 15% grade (Figure 5). A water pump placed at the low end of the tray pumped water to the top to simulate flow. The culturing unit was fitted

with lights on a 16hr/8hr light/dark cycle. A mosquito net was wrapped around the unit so upon emergence the mayflies could cling to it and be easily removed.

**Figure 5 Stream Culturing Unit. Image by Geneve Edwards**

Upon emergence, adult Ephemeridae (*Hexagenia* sp.) were collected and placed in netted containers for holding until mating or ovipositing were facilitated (Figure 6).

**Figure 6 *Hexagenia* sp. in Holding Chamber. Image by Dr. Mindy Yeager-Armstead**

**Diatom Culturing**

In preparation for mayfly rearing and toxicity testing diatoms were cultured in lab. Substrate was added to the bottoms of the culturing units and *Navicula* sp. was added to provide a food source for the mayfly nymphs. Growing media containing 20 mL of Carolina Biological Supply Co. freshwater *Navicula* sp. starter culture Alga-Gro®, 13 mg sodium metasilicate, and 0.13 mL of both Proline® F/2 Algae Food Part A and Part B to 1 L of autoclaved EPA water was added to the units to facilitate growth.

**Mayfly Reproduction and Egg Collection**

Different methods were used to facilitate reproduction and collect eggs. If a male and female emerged simultaneously, they would be placed in a mating chamber, consisting of a 118 mL (4 oz) larval fish jar with Nitex© bridge and 5 mL of autoclaved natural water to encourage mating and oviposition. If a parthenogenic sub-imago female emerged it would be left in a vial with plastic on the top for air flow until final exuvia shed to imago. If a parthenogenic imago female emerged it would be placed in a mating chamber for a maximum of 3 hours to encourage natural oviposition. In all cases if natural oviposition did not occur then the female would be dissected to collect eggs. Ephemeridae adults were placed in breeding chambers overnight to facilitate fertilization. If oviposition did not occur, then the females were dissected.

**Incubators**

Three different incubators were set at 10℃, 20℃, and 24℃. Each incubator had a shaker set at ~55 RPM (Figure 7).


**Figure 7 Mayfly Eggs in Incubator. Image by Geneve Edwards**

**Egg Temperature Test**

During the temperature test, the egg would either be put in a constant temperature (controls) or moved from a low (10℃) to a high (20℃ or 24℃) temperature treatment (Figure 8). Once eggs were collected, a single clutch would be divided into 4 different watch glasses with 5 mL of natural water and given an identification number. Each clutch would be assigned a different temperature treatment. Treatment 1 was considered a control and the watch glass would remain into the incubator set at 20℃. In treatment 2, the watch glass would go into 10℃ and then be moved to 20℃. Treatment 3 is another control; the watch glass would remain in the incubator set at 24℃. In treatment 4, the watch glass would go into 10℃ and then be moved to 24℃. Treatments 2 and 4 were moved to respective temperatures after 2,4, or 6 weeks. The weeks were randomly chosen by a number generator to eliminate bias and repetition.

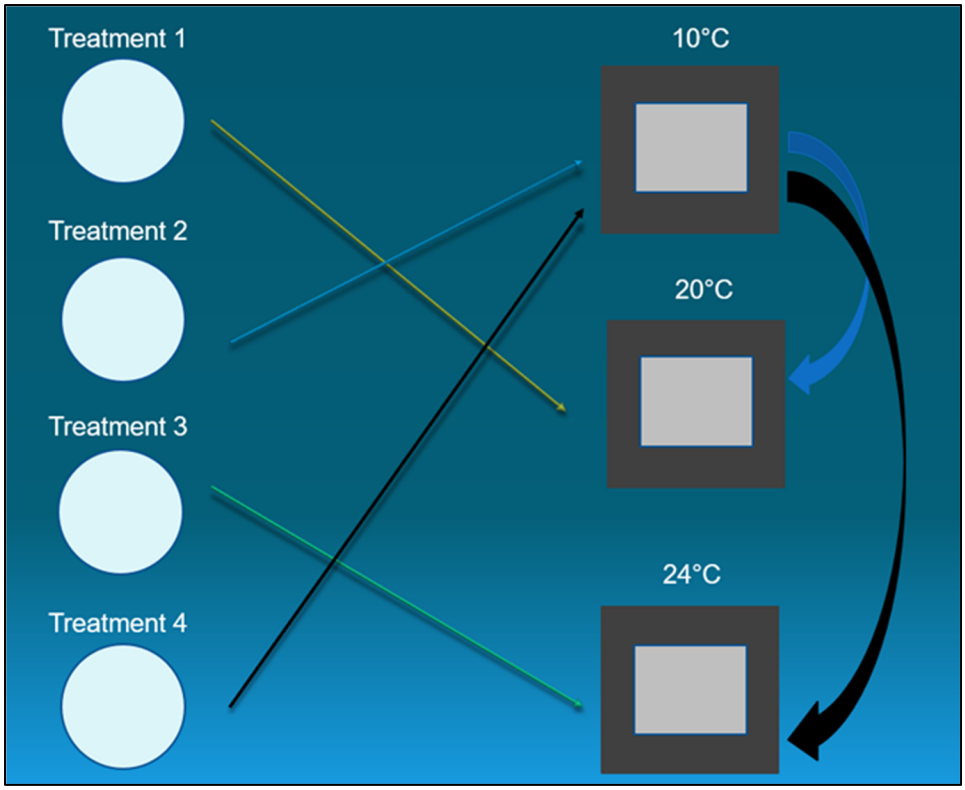


**Figure 8 Egg Temperature Treatments. Image by Geneve Edwards**

A 90% water change was conducted every day using autoclaved Mash Fork (~50 µS/cm) and Kanawha River Water (~180 µS/cm). The natural waters were constantly aerated in an incubator at 20℃.

**High Sulfate Simulated Mine Effluent Toxicity Testing**

The sulfate-dominated conductivity reconstituted effluent used in testing was developed to approximate ionic ratios found in the mining region of southern West Virginia (Armstead et al., 2013). The highest concentration of the simulated effluents had a specific conductivity of ~2,400 µS/cm. The high sulfate effluent contains:

- Calcium Sulfate (0.86 g/L)
- Magnesium Sulfate (0.68 g/L)
- Sodium Bicarbonate (0.32 g/L)
- Potassium Chloride (0.02 g/L)
- Sodium Chloride (0.02 g/L)

With the test concentrations being (Figure 9):

- 100% (~2,400 µS/cm)
- 75% (~1,800 µS/cm)
- 50% (~1,250 µS/cm)
- 25% (~600 µS/cm)
- EPA (~325 µS/cm)
- Diluted EPA (~100 µS/cm)
- Kanawha River (~180 µS/cm)
- Diluted Kanawha River (~100 µS/cm)
- Mash Fork (~50 µS/cm)

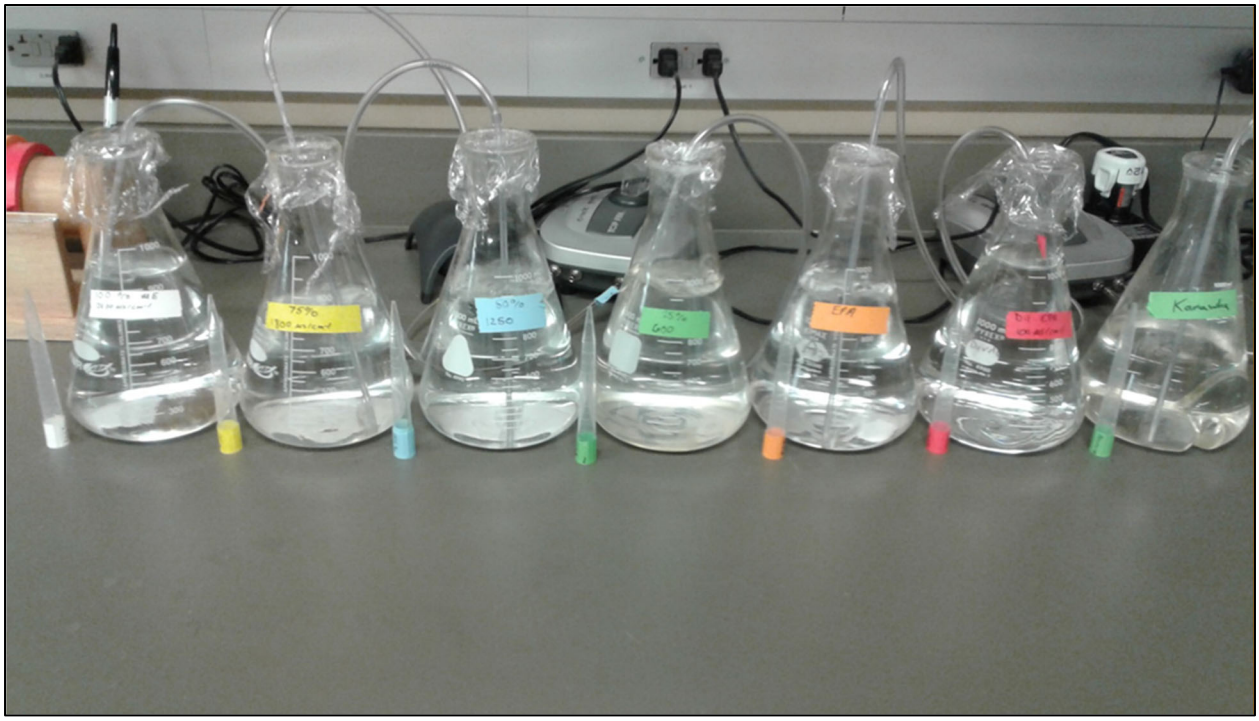


**Figure 9 High Sulfate Test Concentrations. Image by Geneve Edwards**

**Egg Toxicity Testing Maintenance**

Once eggs were collected via oviposition or dissection, each single egg clutch would be divided and placed into corresponding test concentrations and given an identification number (Figure 10). The test concentrations were placed into an incubation unit at 20℃ on a shaker set to ~55 RPM. A 90 % water change and egg count were conducted every other day to provide

fresh aerated effluent without handling the eggs too much. Egg clutches were terminated after 90 days if no hatch occurred within the past month.

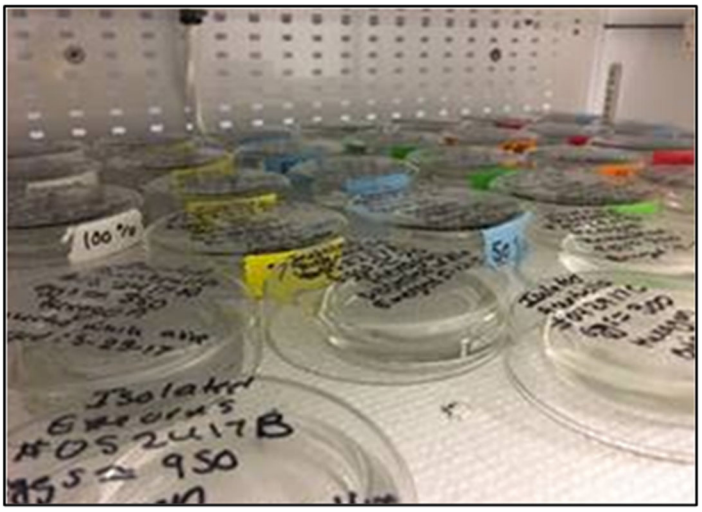

**Figure 10 Egg Toxicity Test. Image by Geneve Edwards**

**Nymph Toxicity Testing Maintenance**

Upon hatching, nymphs were placed in 6-well plates with 2-5 nymphs and 5 mL of test solution in each well. Each test concentration was assigned to a well plate. Growth was evaluated daily by the number of exuvia shed. Mortality was evaluated daily. A 90% water change was conducted every other day. Nymphs were fed a *Navicula* sp., *Selenastrum* sp., and YCT mixture every other day.

**Figure 11 Newly Hatched Baetidae Nymph. Image by Geneve Edwards**

# CHAPTER III

# RESULTS

## Temperature Test Results

| TWO-WAY ANOVA | | | |
|---|---|---|---|
| | df | F | Sig. |
| **Hatch %** | | | |
| Temperature | 1 | 1.23 | 0.293488 |
| Low_Temp | 1 | 0.15 | 0.704507 |
| Temp, Low_Temp | | 0.05 | 0.831394 |
| **Hatch Length** | | | |
| Temperature | 1 | 1.72 | 0.219338 |
| Low_Temp | 1 | 0.05 | 0.821777 |
| Temp, Low_Temp | 1 | 0.01 | 0.940076 |

**Table 1 Two-Way ANOVA On Incubation Duration and Low Temperature Incubation**

A two-way ANOVA was used to compare temperature treatments and incubation duration (days between initial egg collection to first hatch) hatch percent and hatch length (days between first hatched nymph to last). No significant difference (P=0.05) was found between hatch percent and hatch length and the temperature treatments (Table 1).

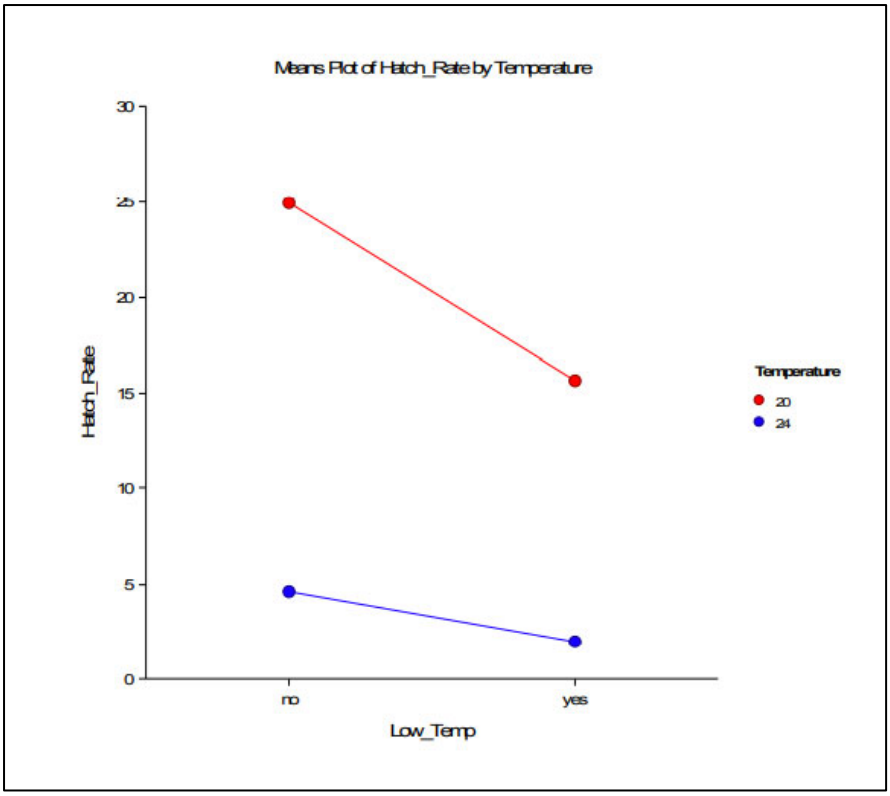


**Figure 12a Two-Way ANOVA on Hatch Percent & Temperature Treatments**

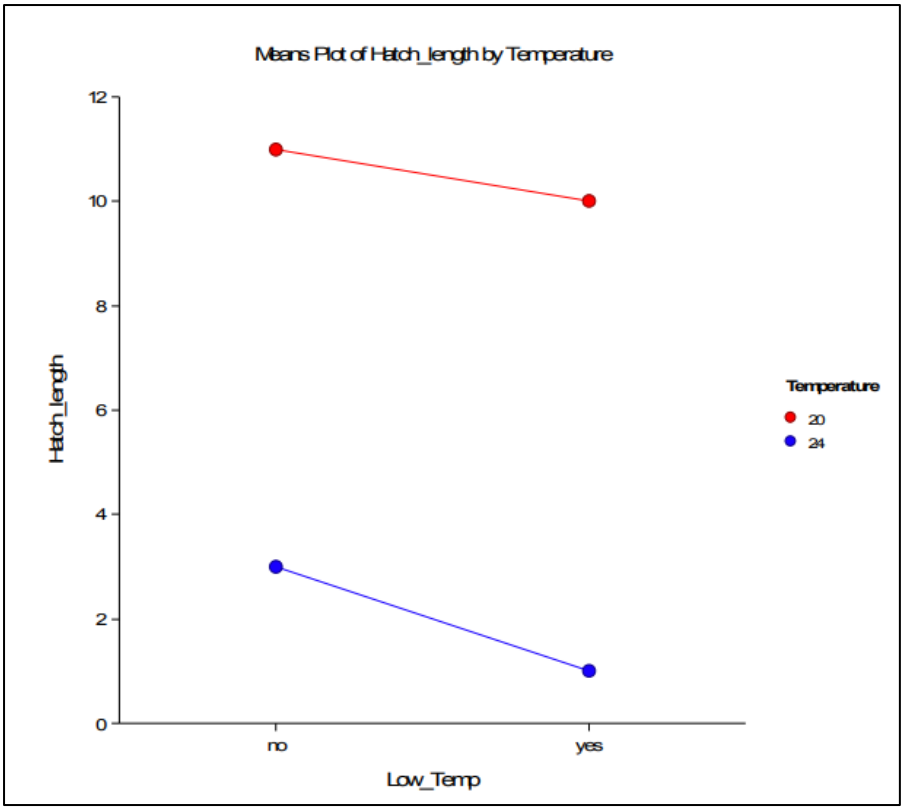


**Figure 12b Two-Way ANOVA on Hatch Length (Days) & Temperature Treatments**

The above graphs (Figures 12a & 12b) compare the effects of the various temperature treatments on egg development. Figure 12a compares the effects of control treatments 1 & 3 (NO), temperature manipulated treatments 2 & 4 (YES), 20°C, and 24°C hatch percent. Hatch length was not different between constant temperature treatments and those with initial low temperature storage (figure 12b). All graphs showed no significant difference between the various temperature treatments and egg development.

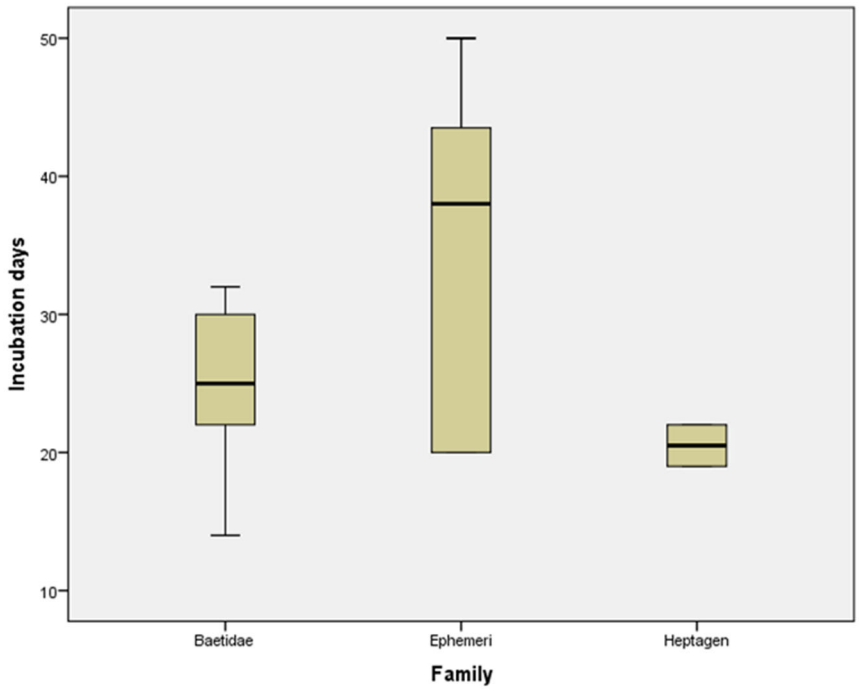


**Figure 13a Incubation Length Compared Between Mayfly Families**

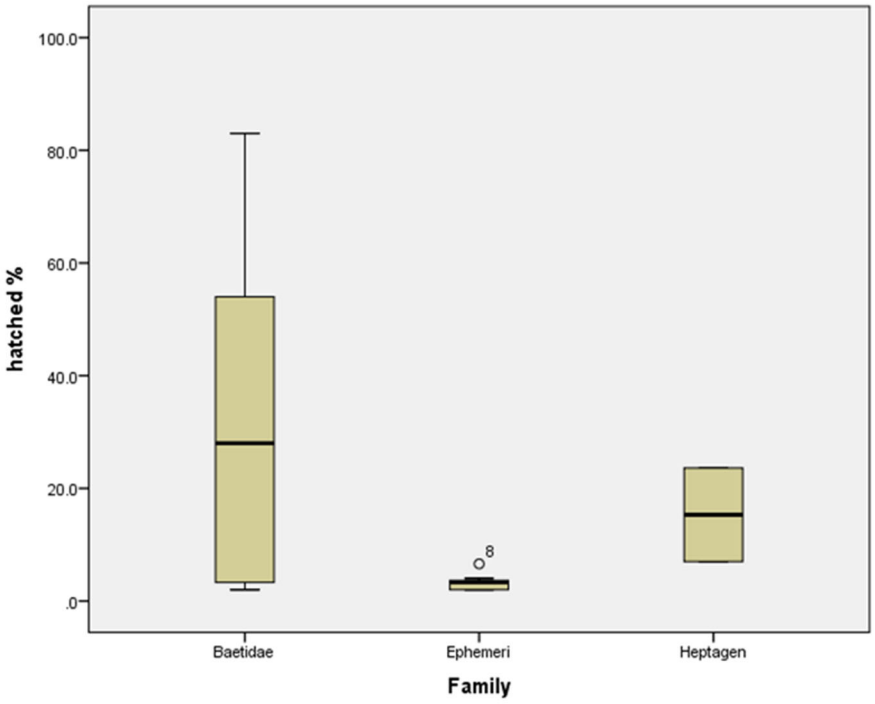


**Figure 13b Hatch Percent Compared Between Mayfly Families**

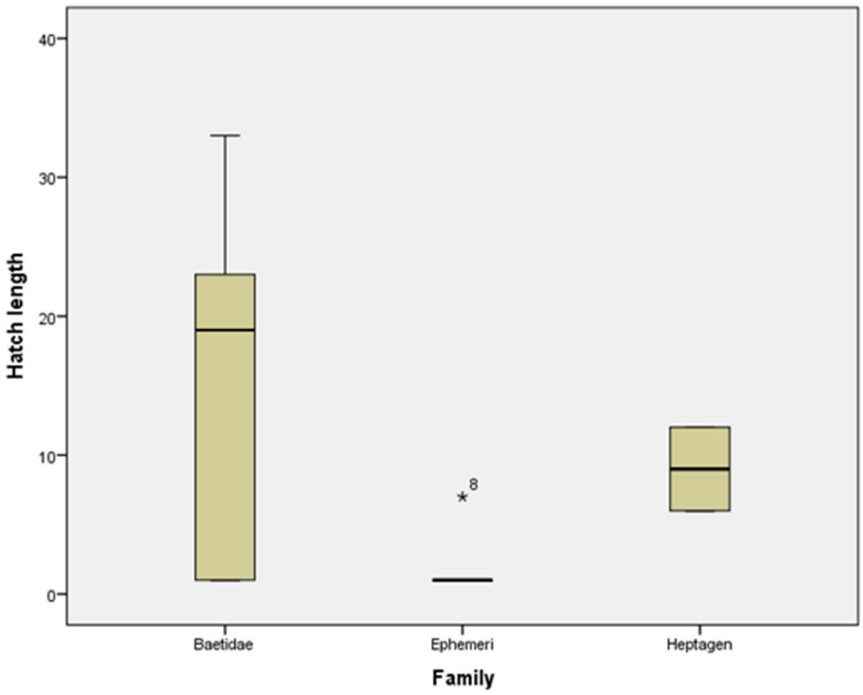


**Figure 13c Hatch Length (Days) Compared Between Mayflies Families**

Incubation length, percent hatch and hatch length were variable between the three families used in testing. Ephemeridae had the highest variability incubation length (Figure13a), the lowest overall hatch rate (Figure 13b), and the shortest hatch length (Figure 13c).

**Toxicity Test Results**

Regression analysis was used to compare conductivity versus egg incubation duration, hatch percent and hatch length.

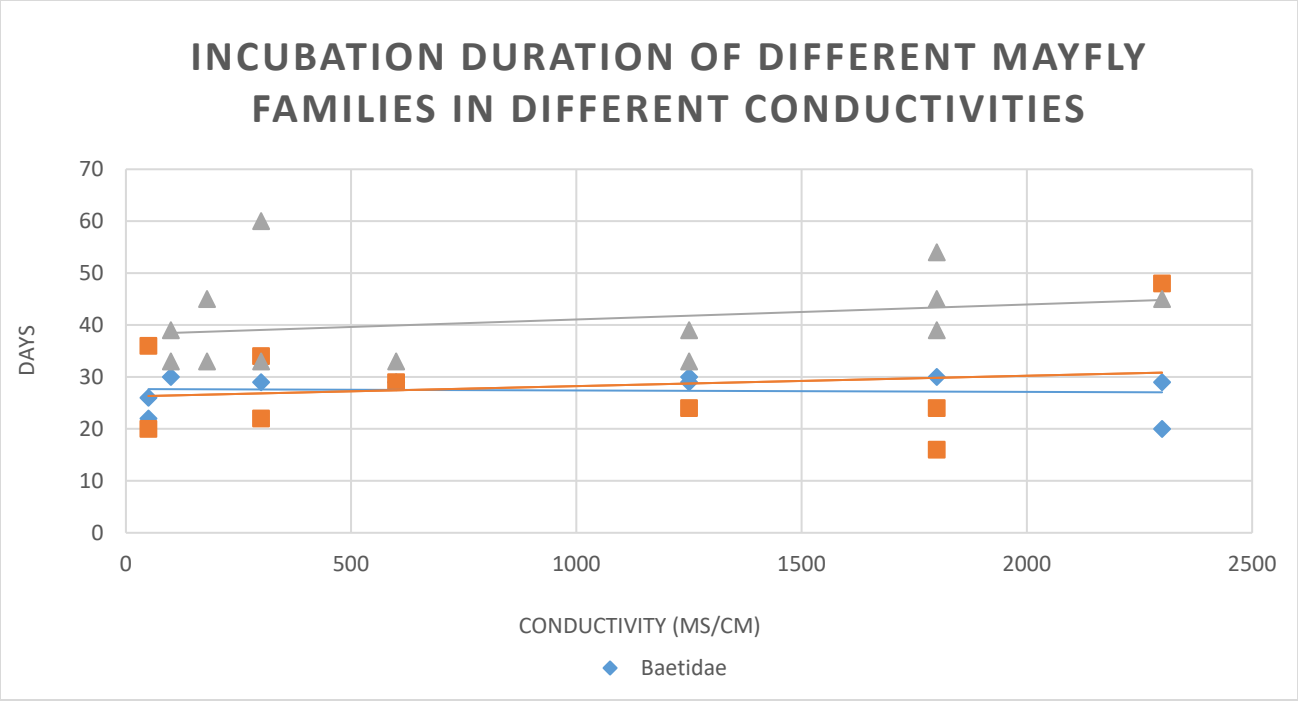


**Figure 14 Relationship Between Conductivity and Incubation Period (Days) for the Families Tested**

As shown in Figure 14 conductivity did not influence incubation duration ($p<0.05$) ($R^2=1.0$) for any of the mayfly families tested (Baetidae $R^2=0.004872$, Heptageniidae $R^2=0.030466$, Ephemeridae $R^2=0.0706$).

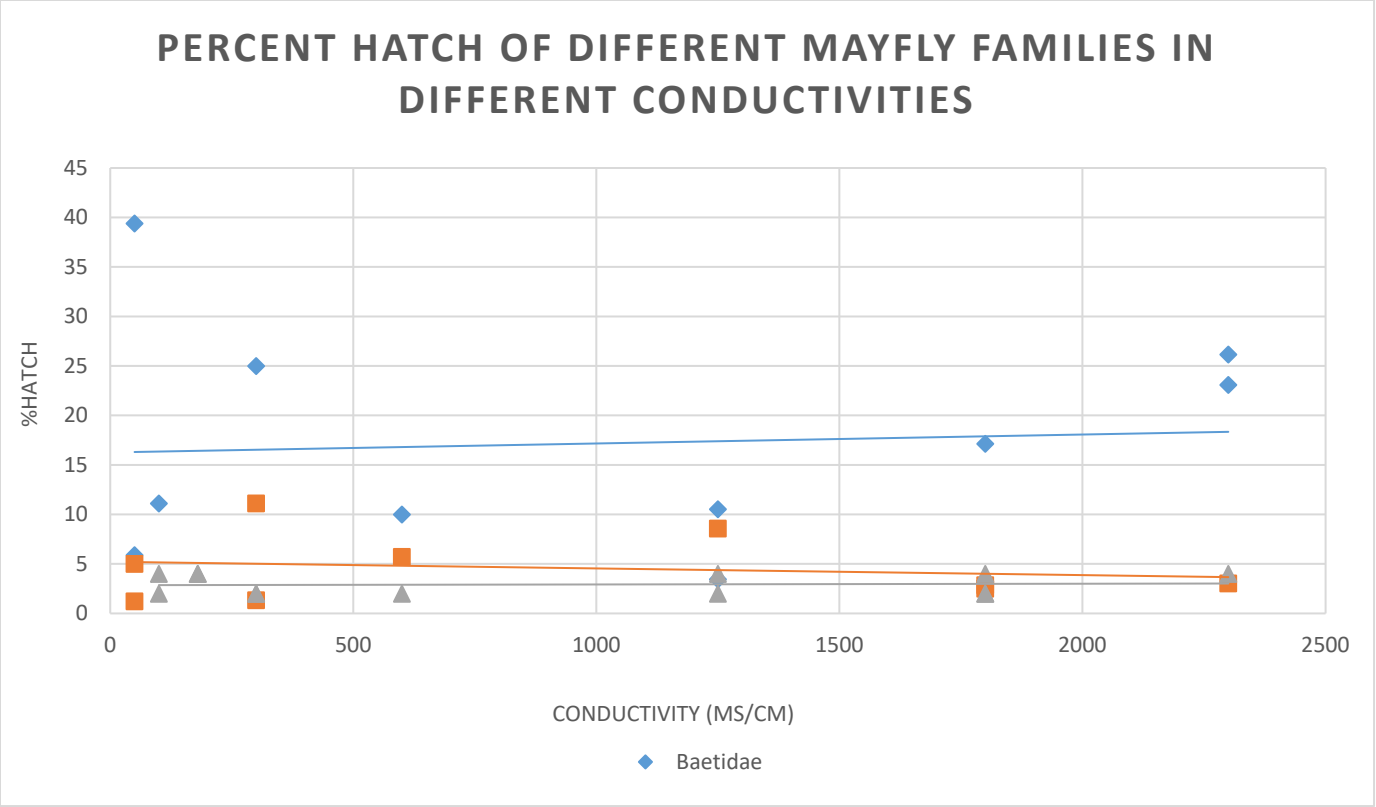


**Figure 15 Relationship Between Percent Hatch and Conductivity for the Families Tested**

Hatch success for mayflies with eggs incubated in waters with increasing conductivity (Figure 15) similarly indicated no significant relationship ($p<0.05$) ($R^2=1.0$) between percent hatch and conductivity (Baetidae $R^2=0.064$, Heptageniidae $R^2=0.112$, Ephemeridae $R^2=0.00336$).

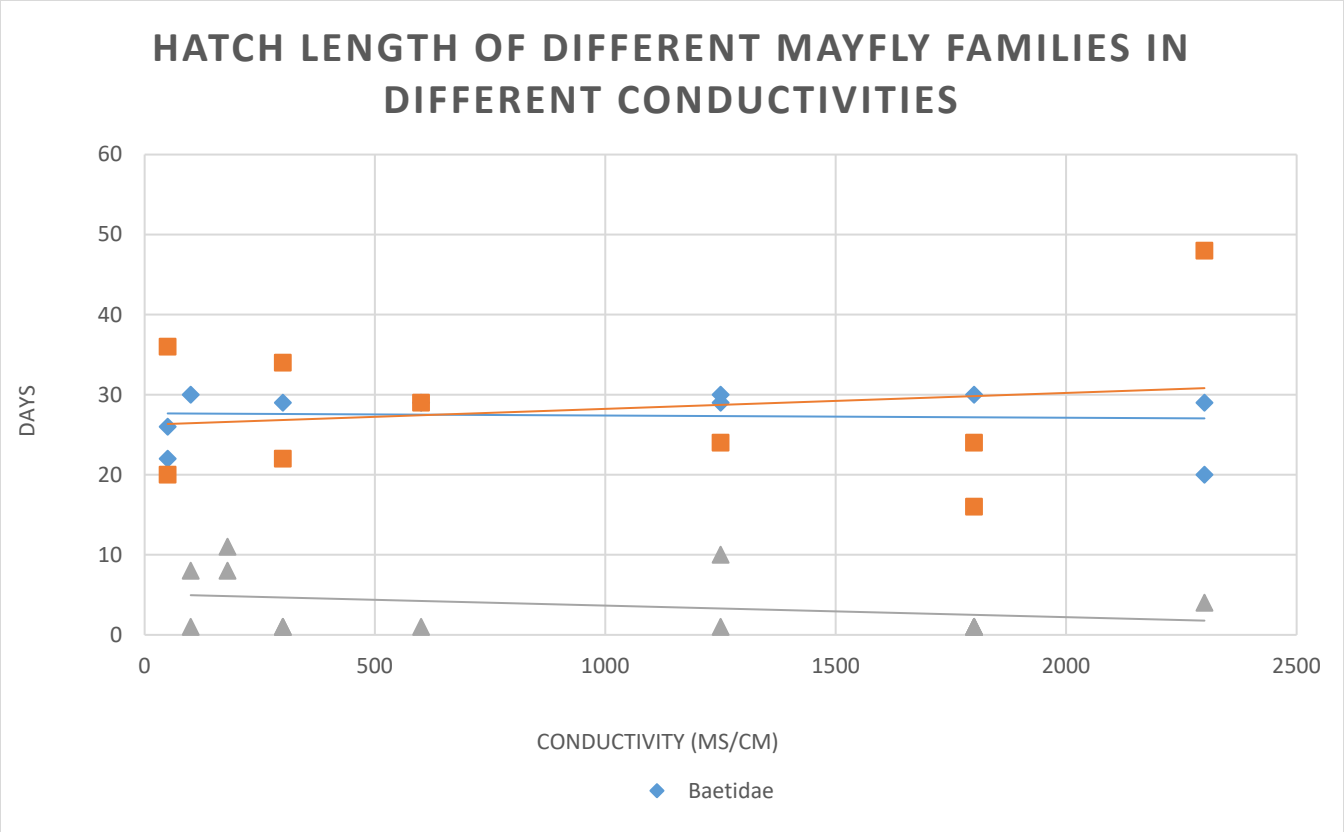


**Figure 16 Relationship Between Hatch Length (Days) and Conductivity for the Families Tested**

Hatch length was also not influenced by conductivity (Figure 16) ($p<0.05$) ($R^2=1.0$) of any of the mayfly families tested (Baetidae $R^2=0.0276$, Heptageniidae $R^2=0.1014$, Ephemeridae $R^2=0.0860$).

**Acute Nymph Testing Results**

The EPA's Whole Effluent Toxicity (WET) spreadsheet was used to generate LC50s. LC50 values are calculated using only linear interpolation.

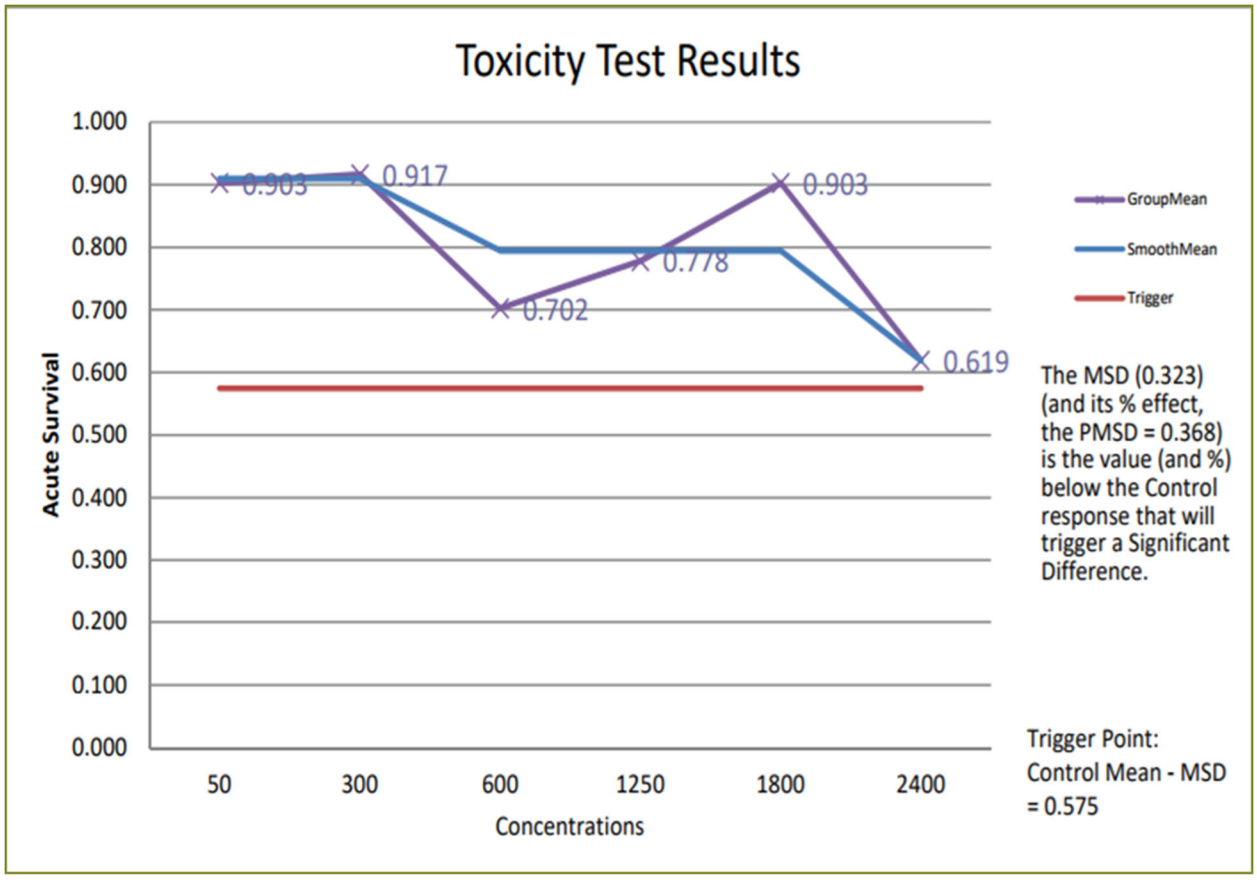


**Figure 17 Heptageniidae Acute Toxicity Test Results**

No LC50 was generated for newly hatched Heptageniidae (*Epeorus* sp.) nymphs at simulated mining effluent conductivity concentration as high as 2400 µS/cm (Figure 17).

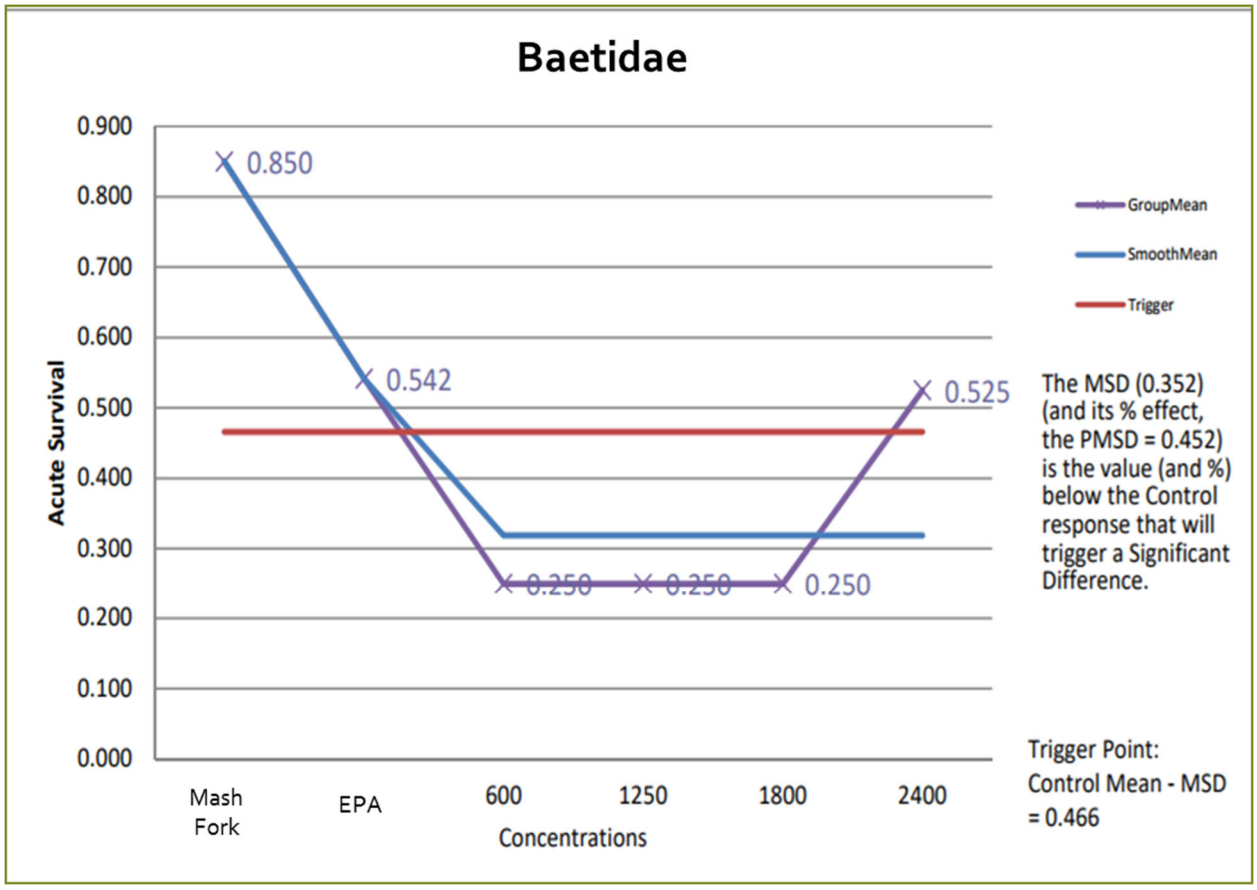


**Figure 18 Baetidae Acute Toxicity Testing Results**

Acute toxicity testing conducted with newly hatched Baetidae (*Acentrella* sp. & *Baetis* sp.) nymphs indicated organisms placed in natural waters controls survived better than those placed in reconstituted water controls. Survival was reduced in moderately hard EPA water and elevated ionic concentrations as compared to the natural water control (Figure 18) with no dose response apparent.

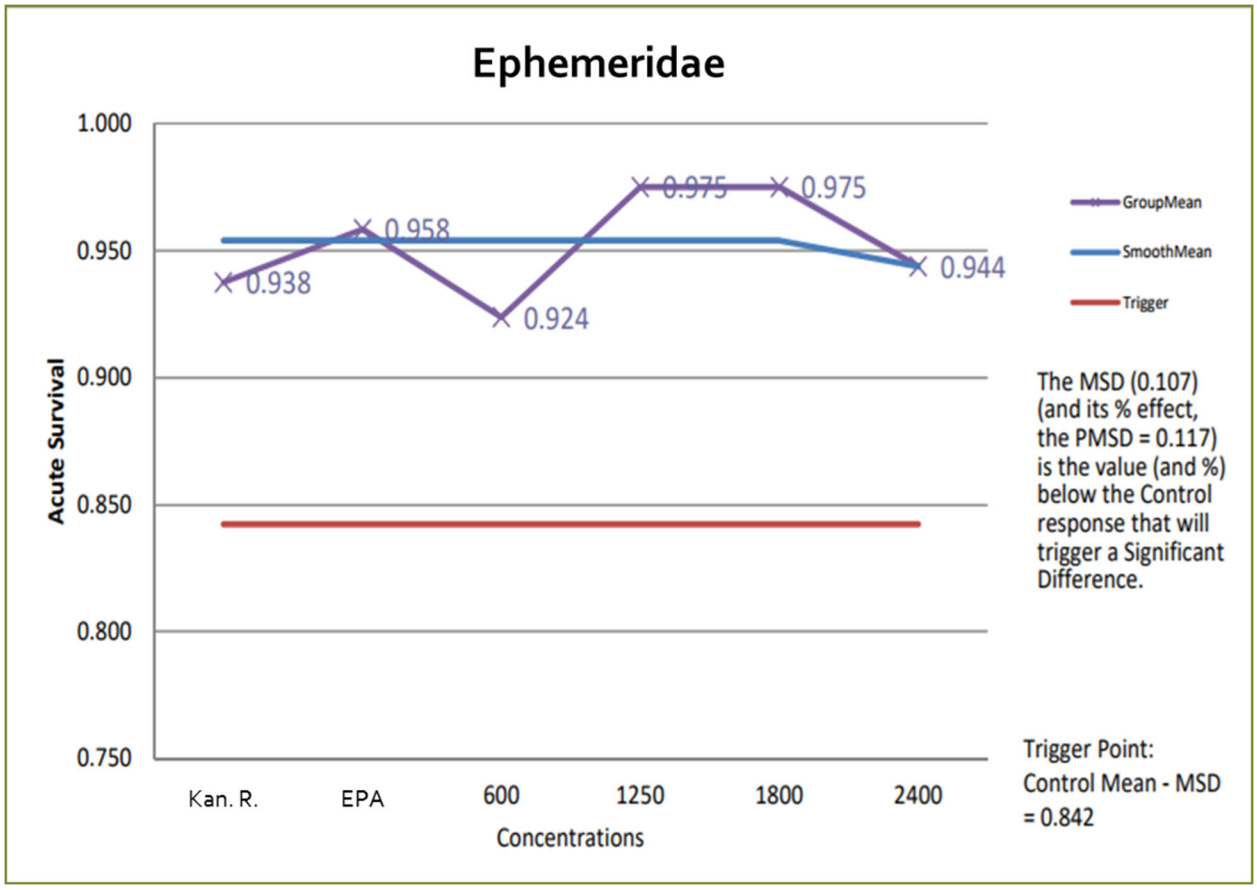


**Figure 19 Ephemeridae (*Hexagenia* sp.) Acute Toxicity Testing Results**

Acute toxicity testing conducted on newly hatched Ephemeridae (*Hexagenia* sp.) nymphs indicated no significant difference between natural control waters and reconstituted water controls, and no response of the organisms to the elevated conductivity in the simulated mine effluent (Figure 19).

**Chronic Ephemeridae (*Hexgenia* sp.) Testing Results**

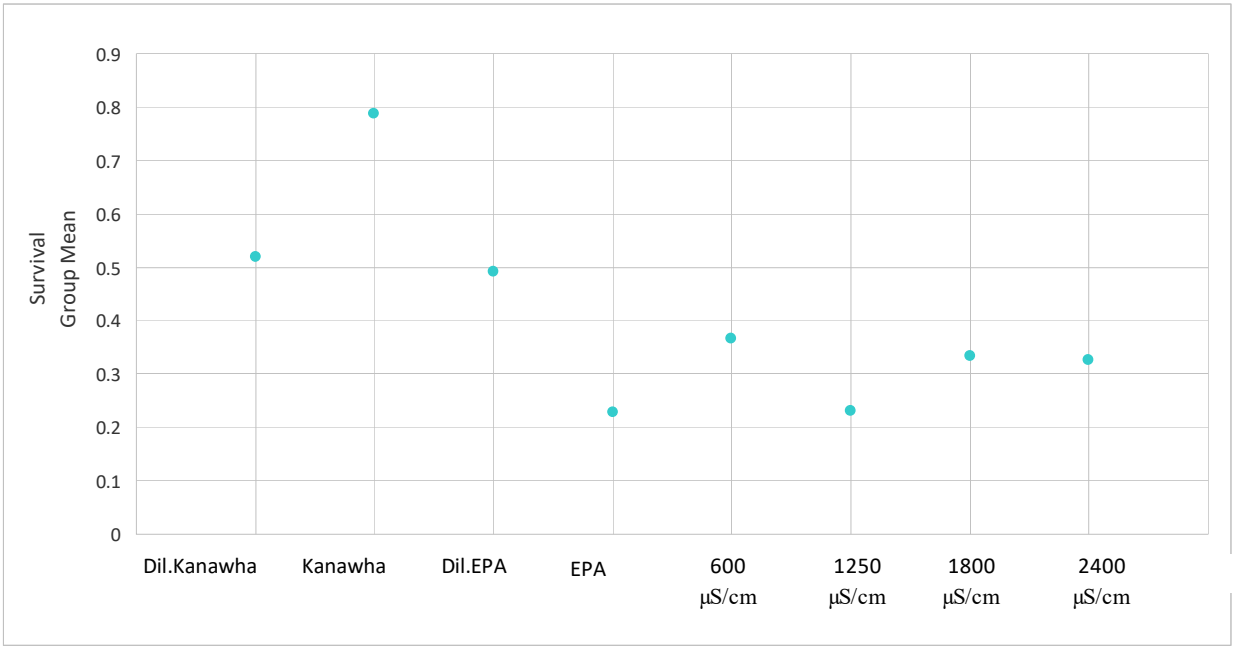


**Figure 20 *Hexagenia* sp. Chronic Survival**

Chronic *Hexagenia* sp. with four controls and an elevated conductivity dilution series indicated that the group means in the controls are higher than in the synthetic waters (Figure 20).

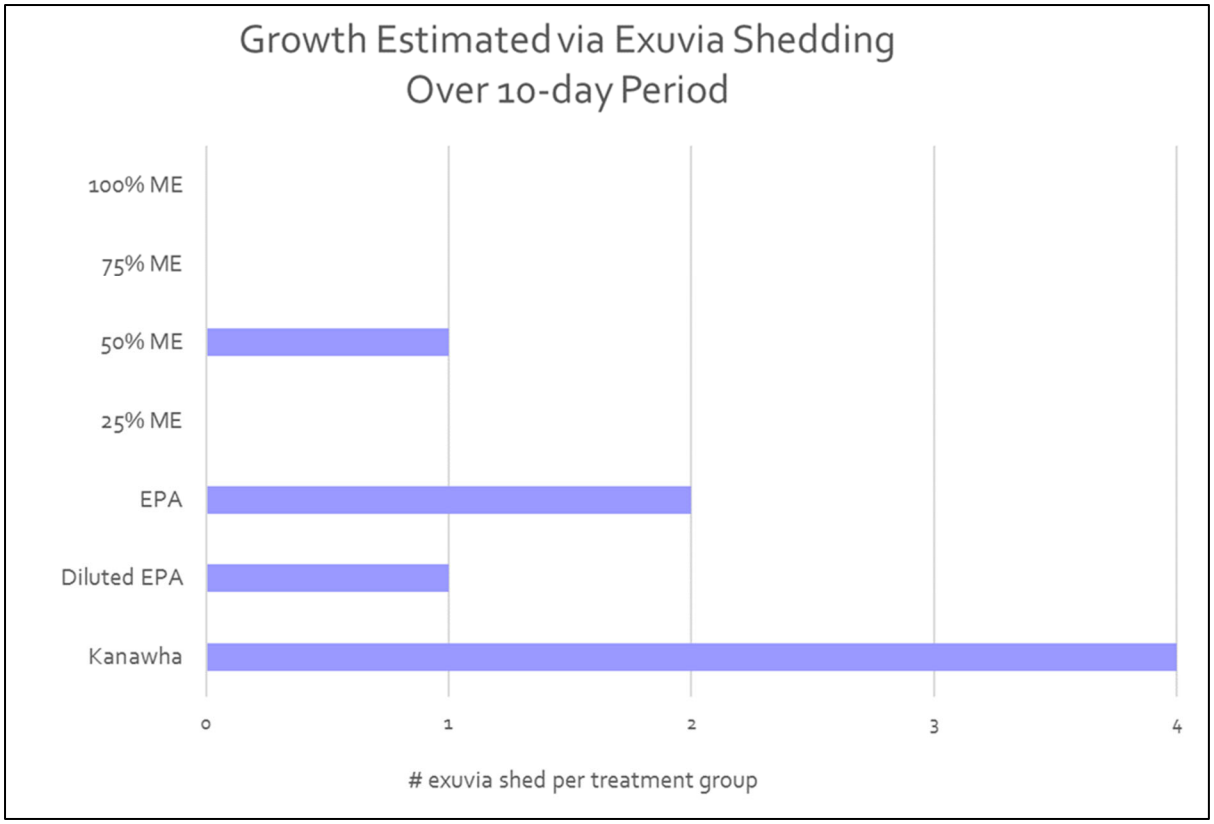


**Figure 21 *Hexagenia* sp. Growth Results**

Although testing conducted was preliminary, there was more growth (exuvia shed) in the controls compared to the other treatment groups, with the most growth in the Kanawha River control (Figure 21).

# CHAPTER IV

# DISCUSSION

## Temperature Test

With there being no significant difference in the Two-way ANOVA between the temperature treatments and egg development (incubation duration, hatch length and hatch %), temperature manipulation can be utilized to ensure a steady supply of mayflies for toxicity testing in the laboratory. A previous study done on the effect of temperature fluctuations on embryonic development also found that the percentage of eggs that hatched at each fluctuation temperature cycle ranged from 0 to 49% and values were like those obtained for eggs reared under constant temperature conditions (Humpesch, 1982). It appears that the effect of temperature on the rate of change in the hatching time and the rate of development is approximately similar for both constant and fluctuating temperatures (Humpesch, 1982). Further testing could be conducted on the nymphs that hatch from the different temperature treatments to evaluate organism fitness.

## Toxicity Test

The factors driving variability in the high sulfate egg toxicity test remain substantial and are still under investigation; therefore, the results of this toxicity test will be considered preliminary as organism fitness cannot be verified. Nevertheless, a past study done on life history strategies of benthic macroinvertebrates has shown that mayflies have a lower hatching success (under 50%) in comparison to other benthic macroinvertebrates (Brittain, 1990).

In the acute nymph toxicity testing, the significant difference between the natural water controls and the reconstituted water controls demonstrates some natural conditions are optimal for genus-specific survival which is not currently met in our rearing regimen. Although, testing

shows no relationship between conductivity and toxicity at the range of conductivities tested, future testing could be conducted to compare sensitivities between Baetidae, Heptageniidae and Ephemeridae populations from various aquatic systems.

In the chronic Ephemeridae (*Hexagenia* sp.) test the mechanisms of greater development and survival in the natural water have not been confirmed but are under further investigation. When compared to synthetic water controls, the NOEC (~2,400 µs/cm) for survival of the mayflies in the EPA water was the highest concentration treatment group which exhibited no observed effect on any level of the treatment group compared to the EPA water control group which may indicate the test organism's higher tolerance to elevated conductivity. Further toxicity testing could be done to compare sensitivities between *Hexagenia* sp. populations from various riverine systems.

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March 14, 2022

Geneve Edwards
1606 Mountain Road
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Dear Geneve:

This letter is in response to the submitted thesis abstract entitled *"Comparative Analysis of Toxicity of Simulated High Sulfate Mine Effluent to Sensitive Life Stages of Native Ephemeroptera Taxa."* 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 Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. 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,

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Director