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Ephemeroptera Culturing Methods: Development of Culturing and Rearing Methodology and High Sulfate Simulated Mine Effluent Toxicity Test Using Field Collected Water

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EPHEMEROPTERA CULTURING METHODS: DEVELOPMENT OF CULTURING AND REARING METHODOLOGY AND HIGH SULFATE SIMULATED MINE EFFLUENT TOXICITY TEST USING FIELD COLLECTED WATER

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Approved by
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APPROVAL OF THESIS

We, the faculty supervising the work of Daniel Edward Brady, affirm that the thesis, *Ephemeroptera Culturing Methods: Development of Culturing and Rearing Methodology and High Sulfate Simulated Mine Effluent Toxicity Test Using Field Collected Water* 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.

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ABSTRACT

Ephemeroptera taxa are not frequently used in toxicity testing; however, some mayfly taxa may be more sensitive to aquatic pollutants than standard test organisms used to determine anthropogenic effects on aquatic ecosystems. Additionally, some standard test organisms are not native to the Appalachian region and may not be truly reflective of the effects on native organisms. With mayflies not being the typical test organism, there is not a great deal of literature on culturing methods for this organism. For a standard acute toxicity test, there must be 80% survival within the control organisms for the test to be considered viable. On that account, culture methods for rearing larval mayflies to emergence, collecting viable eggs, and rearing them to hatch have been developed in the Marshall University Lab. Further development of the methods in order to conduct native mayfly toxicity testing is dependent on a suitable food source being established for cultured mayfly nymphs. The objectives of this study were to investigate adequate feed treatments that will lead to a minimum of 80% survival in the first 48 hours for future acute toxicity testing, the optimal food source for chronic toxicity testing, and for long-term survival in laboratory culturing. Evaluations utilizing a variety of laboratory cultured diets given to individual nymphs in separate chambers were conducted. Mortality rate was used to narrow food types to the ones yielding the best results for further testing. Success is evidenced by 80% survival in the first 48 hours, growth and development of the nymphs, and long-term survival. Two of the four feeding treatments provided over 80% survival of newly hatched mayfly nymphs in 48 hours. One feeding treatment provided over 50% survival over 7 days. For long term survival, only 1 of the treatments, laboratory cultured *Navicula sp.* and leaf disks supported survival over the length of the 36-day study. Once an adequate food source was confirmed, toxicity testing was conducted using the optimal food source for ideal organism fitness. Furthermore, traditional toxicity testing uses reconstituted laboratory water as a base for the dilution series, which is not representative of natural conditions. Therefore, field collected water from Mash Fork, the same stream where mayflies were collected, was used as a base for the dilution series. A preliminary acute toxicity test was run on High Sulfate Simulated Mine Effluent, using the field collected water and the optimal food source. The results did not generate a statistically significant LC50; however, the 100% concentration (~2,400 µS/cm) resulted in ~50% mortality. Therefore, further rounds of testing should include a higher concentration or use organisms of ideal fitness and generate a statistically valid LC50.

CHAPTER 1

INTRODUCTION

Ephemeroptera

Ephemeroptera are an order of aquatic insects commonly referred to as mayflies. Even though, as their name would indicate, they can have prolific hatches in and around the month of May, they can hatch year-round depending on species and environmental factors such as, life history, streamflow, temperature, and water chemistry. Adult emergence is synchronous for the overwintering generation, most eggs from overwintering females are deposited during the first week of May (Sweeney and Vannote, 1984). About half of all Northern Hemisphere mayflies reproduce in June and July (Clifford, 1982). Therefore, approximately half of the remaining mayflies of the Northern Hemisphere can emerge the remainder of the year, depending on voltinism some species only have one emergence per year while others will have two or more.

Mayflies begin their lives as eggs, that are deposited into a waterbody by a female mayfly via oviposition. There are several different strategies that mayflies employ to deposit their eggs, which include becoming submerged, landing on objects over/near the water, or flying to the water and depositing eggs all at once or in several bunches. The eggs have different mechanisms that allow them to become stuck on objects within the waterbody such as detritus, substrate, or vegetation. Various attachment structures ensure the eggs adhere to submerged objects or the sub-stratum (Elliott and Humpesch, 1980). The eggs then hatch into nymphs anywhere from 10 days to several months depending on species and environmental factors. Although, there are some species of mayflies that are ovoviviparous and the nymphs can hatch within minutes; however, this is extremely rare and only been documented in the Baetidae family (Brittain, 1982). About 25% of all life cycles are summer cycles with a long period of egg dormancy in

winter (Clifford, 1982). The nymphs live in the waterbody for approximately 10 days to a couple of years, which is also species-dependent or driven by environmental factors. Nymph emergence is predominantly driven by temperature or degree days but can occur due to other environmental factors and/or stress from anthropogenic disturbances. For example, field and experiment data indicate that generation time (i.e. from egg deposition to adult emergence) for *C. triangulifer* can range from a minimum of about 30-35 days at 25-30°C to ~270 days at 10°C (Sweeney and Vannote, 1981). The nymphs emerge as sub-imagoes, the first life stage they have out of the waterbody; however, they are not physically able to reproduce. Therefore, they find a surface to dry their wings and prepare to go to the next instar, imago.

Figure 1.1. A sub-imago male Baetidae resting on a leaf after emergence. Image by Daniel Brady 2017.

Once the sub-imagoes molt into their final life stage, the imagoes then congregate in swarms where sexual reproduction can occur unless the female is parthenogenetic and sexual reproduction cannot occur. When the female's eggs have been fertilized by a male, or if the female is parthenogenetic, the eggs are deposited into the waterbody and the life cycle repeats.

The sub-imagoes or imagoes do not feed during their adult life, so when they have completed reproduction, or fail to do so, they are unable to gain any more nutrition and their lives are over.

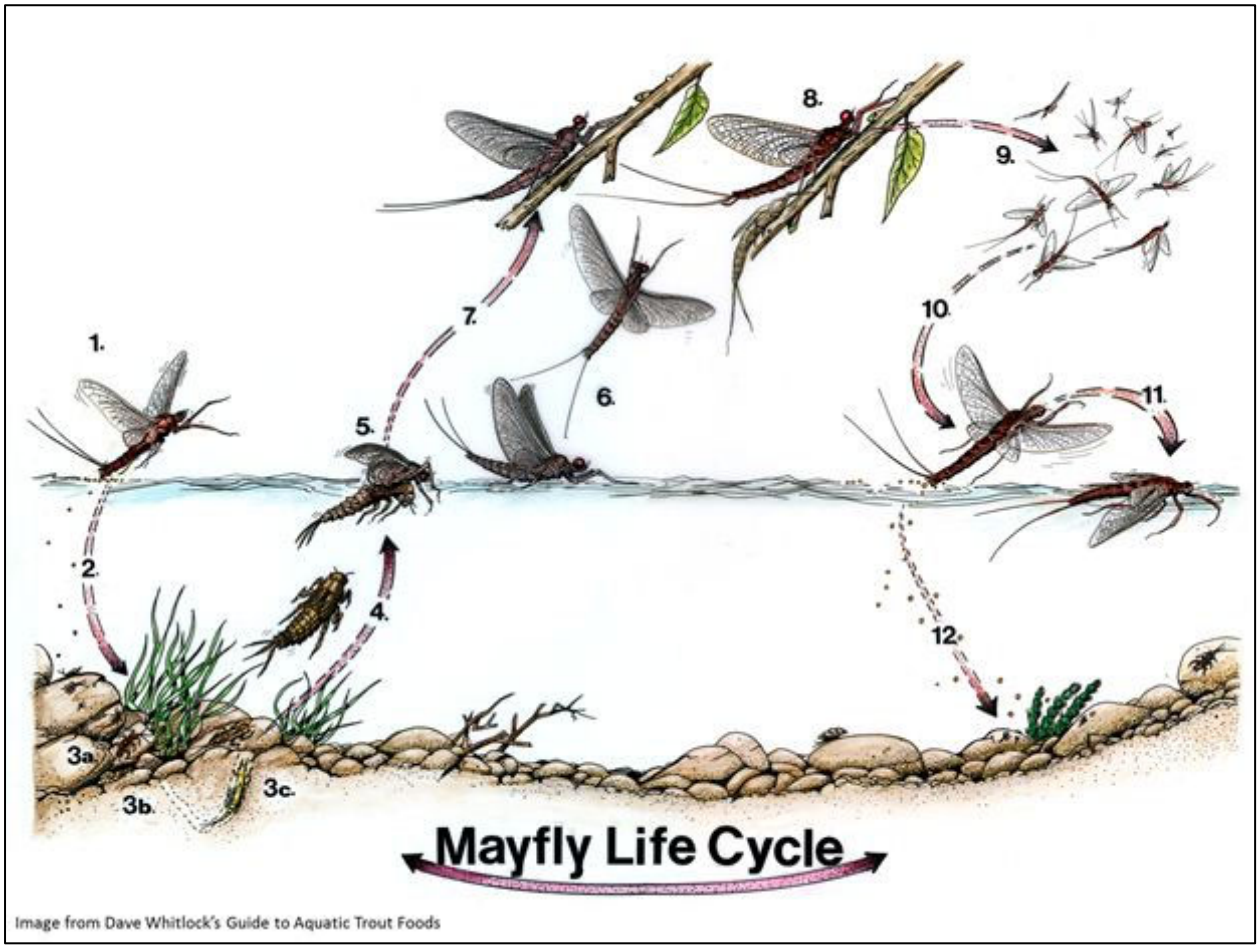

Figure 1.2. The Life Cycle of Mayflies. Image by Dave Whitlock 1982.

About 60% of all mayfly life cycles were reported as univoltine, 30% multivoltine, 4% semivoltine and 3% variable (Clifford, 1982). There tends to be a direct correlation with the size of mayflies and there voltinism, in which smaller species to tend to be multivoltine, medium sized species to be univoltine, and larger species to be semivoltine. Although, semivoltine life cycles, with generations lasting up to the three years, are uncommon in mayflies and are often but not exclusively associated with large size (Sartori and Brittain, 2015). Some species, such as those in the family Baetidae can employ all three types depending on environmental factors.

Baetidae have the ability to switch from multivoltine to univoltine, or even semivoltine in the northern mountains depending on temperature or food availability (Sand and Brittain, 2009).

Macroinvertebrate assemblages are good indicators of localized conditions, because many benthic macroinvertebrates have limited migration patterns or a sessile mode of life, they are particularly well-suited for assessing site-specific impacts (Barbour et al., 1999). The orders of Ephemeroptera, Plecoptera, and Trichoptera, commonly referred to as EPT Taxa, are regarded as some of the most sensitive benthic macroinvertebrates. These benthic macroinvertebrates are widely used in field studies to evaluate the environmental effects of point and nonpoint source pollution (Barbour et al., 1999). Ephemeroptera have been regarded as the most sensitive order of aquatic invertebrates (Echols et al., 2009). Therefore, mayfly taxa are widely accepted as bioindicators for water quality (Bauernfeind and Moog, 2000). However, the significance of mayflies in indicating the ecological integrity of running waters has been widely neglected previously (Karr, 1991), when compared to other more traditionally used aquatic biotic communities such as fish. Because most have a life span of about a year and many remain in the same short section of stream during most of their lives, they are particularly well suited for assessments of short-term, local disturbances within a watershed; fish often move throughout a stream system, enabling them to seek refuge from such disturbances (Paybins et al., 2000). They are often neglected in field or laboratory testing for other aquatic organisms due to the established methods in place for more commonly used organisms and the difficulty in rearing mayflies. Standard test organisms and established test guidelines exist, but the USEPA-recommended species may not be the most sensitive organisms to anthropogenic inputs (Echols et al., 2009). Studies have shown that species of mayflies are more sensitive to elevated toxicants than the more traditionally used cladoceran organisms such as *Ceriodaphnia dubia* or *Daphnia*

magna (Echols et al., 2009; Struewing et al., 2014). *Isonychia bicolor* were more sensitive to the coal processing effluent than *C. dubia* with conductivity lowest observable effects concentration (LOEC) values for mayfly survivorship ranging from 1,508 to 4,101 µS/cm, while LOEC values for *C. dubia* reproduction ranged from 2,132 to 4,240 µS/cm (Echols et al., 2009). The development of a standardized toxicity test using mayflies may be more beneficial for assessing potential adverse effects of point and non-point source discharges on aquatic organisms (Kennedy et al., 2004).

Desirable Test Organisms

Mayflies are desirable test organisms due to them being bioindicators, having high fecundity, known tolerance values, various functional feeding groups (FFG), some species being parthenogenetic, and having easily observable sub-lethal endpoints for toxicity testing. However, lack of culturing methods and variable health of field collected organisms often prevent them from being used in laboratory studies. There have been extensive studies on the effects of mayflies in the field but there is a significant data gap of laboratory studies on mayflies especially those that use field collected organisms.

There are numerous peer reviewed studies that have calculated tolerance values for Ephemeroptera taxa (Barbour et al., 1999; Hilsenhoff, 1988; Gerritsen et al., 2000). This allows for the tolerance of specific families, genera, and species to be easily quantified from field data. One issue with these studies is that in order to become universally accepted, they have to group tolerances into relatively large geographical areas and/or higher taxa (e.g. family or order) which might not be truly indicative of native species in a specific area. Although higher taxonomic units have occasionally been proposed either for rapid assessment of water quality (Alba-Tercedor, 1988; Hilsenhoff, 1988; Gerritsen et al., 2000), the loss of biological information is a

serious drawback, even if the method itself is adequate for the purpose stated (Bauernfeind and Moog, 2000). The universally used tolerance values in biological assessments by the United States Environmental Protection Agency (USEPA) or the West Virginia Department of Environmental Protection (WVDEP) for the families of Baetidae and Heptageniidae is 4 which is relatively high compared to other EPT Taxa. However, the Barbour et al. tolerance values for Baetidae species vary from 1.1 to 9.3 and for Heptageniidae species vary from 0.0 to 7.4 indicating a wide range of tolerance within the families. This exposes the downfall of grouping mayfly taxa into family tolerance values, it is not necessarily fully representative of the multitude of genera or species that comprise the family.

Mayflies have several different FFGs; the majority of mayfly nymphs are herbivores that feed on detritus and periphyton, they are considered to be either collectors or scrapers. Among the collectors, several genera are filter collectors, with setae on the mouthparts (e.g., Leptophlebiidae) or fore legs acting as filters (e.g., Isonychiidae, Oligoneuriidae); by using their gills to produce a current of water through their burrows, several Ephemeridae and Polymitarcyidae may also be regarded as filter collectors, at least for part of their food supply, but they may also leave their burrows at night and graze on periphyton (Sartori and Brittain, 2015). Many mayflies are considered fine particulate organic matter (FPOM) detritivores. The other major feeding group within the mayflies, the scrapers, feed on the periphyton (Satori and Brittain, 2015). For example, compared with stoneflies, the shredding habit is uncommon in mayflies; however, a species of *Paraleptophlebia* has been shown to shred leaves, but it depends on the fine organic matter produce by shredding along with colonizing microorganisms to successfully complete development (Dieterisch et al., 1997). The FFGs are well represented in the Appalachian region; therefore, native species can be used in a broad range of testing

scenarios or given various diets for laboratory culturing. This also presents the need for feeding treatments used in toxicity testing and/or laboratory culturing to be diverse for different types of mayflies due to the diverse variety of FFGs within Ephemeroptera.

Some species of mayflies are parthenogenetic, which means they are able to reproduce asexually. Mayflies being parthenogenetic has several benefits for organism culturing and toxicity testing. There are two different types of parthenogenesis, facultative and obligate. Facultative parthenogenesis, when a female can produce offspring sexually or asexually, combines the short-term advantages of doubling reproductive output with the long-term advantages of genetic variation associated with sexual reproduction (Funk et al., 2010). Obligate parthenogenesis is thought to be rare in mayflies but Funk et al. 2006 found 7 of 50 species (14%) in a small stream catchment (White Clay Creek, Chester County, PA) to exhibit parthenogenesis in one of its various forms (obligatory or facultative). There have been studies on species of mayflies that are parthenogenetic; however, none that have been studied extensively are prevalent in WV, especially species that are native to southern WV. *Ameletus ludens*, was proven to be parthenogenetic (Clemens, 1922) and is native to WV but it would be at the extreme southern portion of its range; therefore, is probably not that abundant or truly indicative of more abundant native species. *Neocloeon triangulifer* (Gibbs, 1977) is parthenogenetic and ovoviviparous but are not native to the state of WV. *Acentrella* and *Epeorus* are two genera of mayflies that are parthenogenetic and were found to be very common in southern WV during field collection. It is very beneficial to not have to rely on sexual reproduction in laboratory culturing due to the necessity of synchronized emergence and copulation that are difficult to reproduce in the laboratory.

Parthenogenetic organisms are not only beneficial in laboratory culturing; they are also ideal for use in toxicity testing. Parthenogenetic organisms in general are desirable for use in toxicity testing because being clonal eliminates genetic variability as a confounding factor (Soucek and Dickinson, 2015). However, being parthenogenetic does present potential issues. Unfertilized (parthenogenetic) eggs were found to develop more slowly than fertilized ones, and fewer of them hatched (Degrange, 1960; Humpesch and Elliott, 1980). Therefore, facultative parthenogenetic organisms seem to be the ideal candidate for laboratory culture due to asexual or sexual reproduction possibilities.

The more subtle sub-lethal effects of pollutant stress which should be examined as indicators of possible longer-term impacts on populations are difficult to quantify; these sub-lethal effects can take many forms such as physiological and biochemical alterations, behavioral changes, ecological changes, and pathological changes (Sindermann, 1980). Mayflies go through several life stages, or instars, as nymphs and when they go through one of these transformative events, or molt, they shed their exuvia. This allows for a sub-lethal endpoint during toxicity testing that is easily observed and measurable via the number of exuvia shed. Overall estimates of the number of nymphal instars are between 15 and 25 (Fink, 1980). Studies have suggested that body or head length could be a more appropriate quantification of growth; however, this requires high powered microscopes, would stress the organisms, and complicates testing procedures. Therefore, counting the number of exuvia shed is a more passive and may be a more cost-effective means of measuring growth.

Culturing and Rearing Methodology

Even though mayflies are a highly desirable test organism, they are often overlooked for more traditional test organisms with established methodology. Current protocols for freshwater

invertebrate toxicity testing almost exclusively utilize cladocerans, amphipods or chironomids rather than the more typical aquatic insect taxa found in lotic systems (Weaver et al., 2015). Therefore, little research has been done on laboratory culturing methodology, which exhibits the need for research to help establish stock populations of mayflies which would be ideal for testing due to the high costs and temporal variability associated with field collection. *Acentrella* (Baetidae) and *Epeorus* (Heptageniidae) are two common mayfly species in WV streams, they are also considered to be pollution/disturbance sensitive species. *Neocloeon triangulifer* is a mayfly taxon recently used in toxicity testing, which is not native to WV; therefore, it may not be representative of the effects on aquatic organisms in WV streams. *N. triangulifer* does inhabit lotic habitats but it prefers lower velocity streams (Funk et al., 2006); therefore, it may not be representative of organisms that inhabit the high-gradient and velocity streams that are found in many WV streams.

A widely used feeding treatment for toxicity testing of aquatic organisms is a mixture of yeast, cereal leaves, and trout chow (YCT), it has proven to be an acceptable food source for standard test organisms. However, little research has been done to see if it is an optimal feeding treatment for mayflies, especially those of the Appalachian region. There have been some studies that have used alternative food sources for mayflies, a mixture of fish food flakes and YCT was used in study (Echols et al., 2009). There have been some studies that exhibit the need for some sort of substrate in the testing chamber for mayfly survival and development (Sweeney and Vannote, 1984; Echols et al., 2009), this is usually accomplished by inserting portions of field collected leaves in the chamber with the organism. The leaves not only provide a substrate for the nymphs to use, but they also become colonized with microbes that can provide a supplemental food source for the nymphs. The larvae do not utilize whole leaves directly as

food, but readily ingest fine particulate floc composed of shredded leaves, algae, and associated microbes (Sweeney and Vannote, 1984). Therefore, the portions of leaves that are used as a substrate and/or food source in testing should be allowed to be colonized for a period of time before use. In previous mayfly testing, leaves were leached in stream water for several days and allowed to become colonized in the dark with microbial populations (i.e. bacteria, fungi, protozoa) prior to use as food (Sweeney and Vannote, 1984).

There are other environmental factors that affect larval growth and development such as temperature. Temperature can affect larval growth in natural populations directly by its influence on rates of feeding, assimilation and respiration, food conversion efficiencies, enzymatic kinetics and endocrine processes (Vannote and Sweeney, 1980; Sweeney et al., 2018) or indirectly by altering the quantity (e.g. density and/or productivity of periphyton algae) and quality (e.g. microbial population associated with detritus) of available food material (Cummins and Klug, 1979). Larvae grew, matured and metamorphosed successfully at 25°C when fed hickory leaves, even though no leaf shredding by larvae was observed and chlorophyll samples indicated no detectable colonization by algae, microbes were the primary source of nutrition in these experiments because ATP levels on the dead leaves were relatively high (Sweeney and Vannote, 1984). The preferred food source can change in direct correlation with temperature and/or stage of development representing the need for a diverse mixture of feeding treatments to maximize fitness over the duration of chronic testing and/or laboratory culturing. This exhibits a need for the development of standardized optimal food source for use in Ephemeroptera toxicity testing. The effect of salinity on mortality in mayflies should be greater when they are unfed, relative to when they are fed, because mayflies should be under greater energetic stress when not fed (Kefford, 2018). An optimal food source would ensure ideal fitness of the organisms and would

limit the uncertainty of the elevated toxicant used in the test as being the limiting factor on survival and/or growth.

Anthropogenic Disturbances

Humans have impacted the environment for as long as they have been on earth, this escalated severely as technology advanced. In the 1700's the Industrial Revolution sparked exponential population growth. In order to keep up with the demand of an ever-increasing population, technology was developed to increase production and overall quality of life. This includes advances in food production, health/medicine, transportation, communication, technology, and energy production. However, this did not come without consequence, this increased the demand on natural resources of the earth. This resulted in unregulated and unsustainable farming practices, gas/oil extraction, mining, timbering, and urbanization. This obviously had detrimental effects on the earth and resulted in some serious consequences. This occurred until the creation of environmental regulations in the 1900's. The Federal Water Pollution Control Act of 1948 was the first major U.S. law to address water pollution (USEPA). Although it wasn't until the public in general began to become fully aware of the potential problems that were resulting from these practices when events like the Cuyahoga River catching on fire in 1969 began to make the national news. Growing public awareness and concern for controlling water pollution led to sweeping amendments in 1972, the law became commonly known as the Clean Water Act (USEPA). The passage of laws and resulting creation of regulatory agencies, with the intention of protecting our natural resources and the environment, started to try to prevent further environmental impacts and mitigate previous disturbances. This, coupled with increases in technology, was a step in the right direction but the damage that was done in the past was not easily rectifiable in many cases.

In West Virginia (WV) and the surrounding Appalachian region, farming was not very feasible due to topography; therefore, the most detrimental anthropogenic impacts were from natural resource extraction, harvesting, and/or production. WV has a long history of coal mining dating back to the early 1800's. In 1883 the completion of the original railroad lines boosted coal mining production exponentially due to the ease of exporting by train. By 1931 WV overtook Pennsylvania as the leading producer of bituminous coal. In 2016 WV was the second largest coal producer behind Wyoming. Therefore, WV has been the leading, or one of the leading, coal producers for over a century. About 7% of all coal mined in the Nation comes from an area of 5,000 mi^2 in the Appalachian Plateaus part of the Kanawha-New River Basin (Paybins et al., 2000). This has resulted in some severely adverse environmental impacts. Elevated concentrations of ions are being introduced into WV streams from active and abandoned coal mining sites. This is not only a problem in WV and the Appalachian region; areas such as China that have more recently began coal mining are starting to see the same anthropogenic issues. Acid mine drainage (AMD) problems in abandoned coal mines have become a worldwide environmental concern (Wang et al., 2020).

The geologically younger coal beds found in northern WV contain more sulfur than the geologically older coal beds found in southern WV (WVGES, 2022). Low sulfur was not mined as heavily as high sulfur coal in the past but advances in technology have made it more effective and profitable to mine low sulfur coal. As a result, production of the predominantly low sulfur coal nearly doubled from 1980 to 1998 in WV (Paybins et al., 2000). AMD results from the formation of sulfuric acid in the oxidation of iron sulfide minerals such as pyrite (Sams and Beer, 2000). When the coal is mined, iron pyrite in the remaining coal and adjacent rock formations is exposed to oxygen and water which results in the pyrite to oxidize to form ferrous sulfate and

sulfuric acid. The oxidation products are then leached into ground water and/or runoff from precipitation events which introduces it to the water table. The pyrite weathering process is a series of chemical reactions but also has a key microbiological component. This reaction can be greatly accelerated by a species of bacteria, *Thiobacillus ferroxidans* (Singer and Stumm, 1970). Sulfur-containing minerals and groundwater replenishment are the main sources of the mechanism of AMD formation, pyrite is the prerequisite, oxygen is the inducement, water is the carrier, and Fe^{3+} and microorganisms are the catalyst (Wang et al., 2020). The AMD chemical reactions produce elevated concentrations of the insoluble precipitate ferric hydroxide [$Fe(OH)_3$], dissolved sulfate ($SO4^{2-}$), and acid (H^+) (Sams and Beer, 2000). This results in a decrease in pH and increase in concentrations of volatile ions in the waterbody. The increased acidity can be neutralized in streams that have high buffering capacity, but streams with lower alkalinity will exhibit a more rapid decrease in pH that will be sustained for a longer period of time. Once the neutralization capacity is exceeded, however, acid begins to accumulate and the pH decreases (Sams and Beer, 2000).

In southern WV, increased acidity from mine drainage is not a determinate factor in impacts to aquatic ecosystems due the abundance of limestone and sandstone. Most water that drains from coal mines in the Kanawha–New River Basin is naturally neutral or alkaline rather than acidic (Paybins et al., 2000). However, the decrease in acidity does not mean that it has no negative impacts; the increased surface area of fractured rock results in increased weathering and mineralization resulting in an increase in total dissolved solids (TDS).

This may have an inimical effect on native aquatic organisms especially the more sensitive organisms such as EPT Taxa. These organisms each have a species-specific range of pH or ionic concentration that can be tolerated and if conditions are extended beyond that range,

then they will become stressed, decrease overall organism fitness, and could potentially become extirpated. This is further compounded by the lower pH facilitating other toxicants, such as heavy metals, to dissolve into the water increasing their concentration. Secondary reaction of the sulfuric acid with compounds in adjacent rocks or mine spoil can produce high concentrations of aluminum, manganese, zinc, and other constituents in mine drainage waters (Toler, 1982). When the water goes downstream or is introduced into other waterbodies the pH will eventually increase, resulting in the metals to precipitate and become bound to sediment which can have further adverse effects on native aquatic organisms that either live in the benthos or eat microorganisms that are bound to sediment. Although, it appears that heavy metals are not as detrimental to aquatic life as previously thought when compared to effects of the increase of soluble ions that are introduced into the aquatic ecosystem, therefore, they are potentially not a limiting factor in native aquatic organism survival.

Regulations like the Federal Surface Mining Control and Reclamation Act (SMCRA) of 1977 and the National Pollutant Discharge Elimination System (NPDES) of 1972, a part of the CWA, were enacted to combat the known effects of AMD and point source discharges, respectively, which at that time were thought to be mostly driven by acidity and metals. SMCRA was intended to mitigate the effects of non-point source pollution, especially from abandoned or inactive mines, and cumulative impacts. The NPDES was enacted for point source pollution, which regulated active mines discharging into receiving waters. Median concentrations of total iron and total manganese were lower in 1998 than during 1979–81 in 33 basins that had been mined both before and after SMCRA, but sulfate concentration and specific conductance were higher (Paybins et al., 2000). In one study, the coal mine processing impoundment from the Callahan Creek Watershed effluent had minimal trace metals present with concentrations below

water quality criteria or detection limits; the suspected factor influencing mayfly toxicity was likely ionic salts (Echols et al., 2009).

Salinization of Freshwater Ecosystems

The traditionally freshwater aquatic ecosystems of the world have become ever-increasingly high in concentrations of salinity due to various anthropogenic activities such as road treatment during the winter months, the accidental release of brine water during fracking activities, agriculture irrigation water which leaches soil ions, and effluent waste from water treatment facilities. The prominent sources of salts in the Central Appalachian and Western Allegheny Plateau regions, are weathering mine overburden and valley fills from large-scale surface mining, but they may also come from treatment of AMD, slurry impoundments, coal refuse fills, or deep mines (USEPA, 2011). If these changes in salinity and/or ions are of sufficient magnitude, salinity may have adverse effects on freshwater organisms, their populations, communities and ecosystem functions (Kefford, 2018). Ephemeroptera are among the oldest flying insect orders yet appear never to have evolved the ability to live in marine or inland saline waters (Kefford et al., 2016). Acute toxicity testing of 377 species from Australia, France, Israel and South Africa shows that Ephemeroptera is one of the most salinity-sensitive groups of stream macroinvertebrates (Kefford et al., 2012).

It has been hypothesized that Ephemeroptera have adopted an osmoregulatory strategy that while suited for pulling in ions in very dilute waters, puts them at a severe disadvantage when confronted with slight increases in salinity (Olson and Hawkins, 2017). Because they would normally lose salt in freshwater, their epithelium is selectively permeable to the uptake of certain ions and less permeable to larger ions and water (USEPA, 2011). Salinity, indicated by TDS and/or specific conductance (SC), is increasing in freshwaters throughout the world as a

result of human activities and will continue to increase as the demand on aquatic resources increases (Cañedo-Argüelles et al., 2014).

TDS is the quantification of the cumulative dissolved concentration of all inorganic and organic substances present in a liquid in any form. Increases in TDS in freshwater ecosystems originate from non-point sources such as runoff or leaching from contaminated soils, or point source discharges from industrial or sewage treatment plants. TDS toxicity is influenced by ionic content of the test solution especially that of mining effluents (Echols et al., 2009, Soucek and Dickinson, 2015). This exposes the need for further research on the effects of TDS on native aquatic organisms. This has implications for the establishment of water quality criteria and discharge limits in the coalfields of Virginia and West Virginia, particularly limits for TDS (Echols et al., 2009).

Increasing salinity puts additional stress on native organisms, as they employ osmoregulation to attempt to acclimate to the increased ionic concentrations. This is a zero-sum game, i.e. more energy for ion homeostasis means less energy for other functions including growth, reproduction, other maintenance and the building up of stores of energy (Kefford, 2018). This decreases organism fitness and can ultimately result in mortality. Mortality presumably occurs when ion homeostasis demands so much energy that other vital functions are compromised (Kefford, 2018).

Scheibener et al. (2017) observed greater transport of Na with increasing external Na^+ concentration in *Macaffertium* sp., so as external Na^+ increases *Maccaffertium* sp.'s turnover of Na is increasing. This could negatively affect the organism due to an increased energy demand. Increased Na^+ uptake should be accompanied by less energy stores, reduced growth rates and/or fecundity and upregulation of mechanisms to combat the localized increase in pH or Na

(Kefford, 2018). Although, other ions can have variable effects on organisms. Uptake of SO_4^{2-} in five mayfly species increased with increasing external SO_4^{2-}, but unlike Na^+, saturation of SO_4^{2-} was observed (Scheibener et al., 2017). The rate of increase in SO_4^{2-} uptake decreased with increasing external SO_4^{2-} concentration; however, the uptake rates of other major ions as their external concentration increases appear to not have been measured in Ephemeroptera and other benthic macroinvertebrates (Kefford, 2018).

Increased salinity can have a detrimental effect on freshwater ecosystems as native aquatic organisms do not have high tolerances for salinity and an increase in SC could result in conditions that are above their optimal range or in extreme cases above their tolerance range. Laboratory studies have shown that mayfly survival was negatively correlated with conductivity (Echols et al., 2009, Soucek and Dickinson, 2015). Clements and Kotalik (2016) found that 'seeded' experimental mesocosms with invertebrates from a low salinity site (60-70 µS/cm) and then applied various experimental salinity treatments; they observed that salinity of ~300 µS/cm caused declines in the abundance of baetid and heptageniid mayflies and total Ephemeroptera. Recent studies have identified this threshold of conductivity as the upper end of several organisms' optimal range; therefore, it has been proposed as a benchmark for conductivity in Appalachian streams. At the request of U.S. Environmental Protection Agency's (EPA) Office of Water and Regions, the EPA Office of Research and Development has developed an aquatic life benchmark for conductivity for the Appalachian Region; the benchmark is applicable to mixtures of ions dominated by salts of Ca^{2+}, Mg^{2+}, SO_4^{2-} and HCO_3^- at a circum-neutral to alkaline pH (USEPA, 2011). This resulted in a proposed benchmark of 300 µS/cm for Appalachian streams. Some studies have shown increased stream SC from 100 µS/cm to 3,700 µS/cm mainly due to

leached ions from mining practices (e.g., SO_4^{2-}, Mg^{2+}, Ca^{2+}, and HCO_3^-) in the watersheds (Fritz et al., 2010; Merricks et al., 2006).

The cumulative effects of increased conductivity, salinity, and TDS have demonstrated increased stress on aquatic organisms and in many cases have been found to be more of a limiting factor than increases in acidity and metal concentrations (Echols et al., 2009). An increase in certain ionic concentrations can increase the toxicity of metals, Ericksen et al.(1996) found that the addition of potassium chloride markedly increased copper toxicity. Kefford 2018 has hypothesized that: (1) the increased Na turnover observed in *Maccaffertium* sp. with increasing external Na^+ concentration occurs in other salt-sensitive mayflies; (2) that increased Na^+ (SO_4^{2-} and potentially other ions) uptake requires more energy; (3) either salt-sensitive mayflies cannot increase their total energy supply with increased energy needed or they cannot increase this supply sufficiently to meet osmoregulation demands without diverting some energy from other uses (Kefford, 2018). This increased expenditure of energy supplies can have detrimental effects on other essential biological functions. Therefore, organisms must adapt to the anthropogenic impacts, migrate from the area, or face extirpation.

Toxicity Testing

The USEPA has established protocol for use in acute and chronic toxicity testing. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations (USEPA, 2002). They are suited for determining the toxicity of specific toxicants contained in discharges or effluents. The data are used for NPDES permits development and to determine compliance with permit toxicity limits and can be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50 and appropriate dilution, application, and persistence factors (USEPA, 2002).

Effluent acute toxicity tests use a multi-concentration, or a definitive test, consisting of at least one control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that is lethal to 50% of the test organisms (LC50) within a prescribed time frame (24-96 h), or the highest effluent concentration in which survival is not statistically significantly different from the control (USEPA, 2002). If the NPDES permit on a point source discharge has a whole effluent toxicity (WET) limit for acute toxicity for the receiving water concentration (RWC), the RWC should be used as the 50% concentration in the dilution series to ensure the likelihood of a dose-response relationship.

The USEPA Office of Surface Mining reported in 1995 that AMD was the single greatest threat to water quality in the Appalachian Mountain region of the USA. This has traditionally been associated with decreases in pH and increases in concentrations of heavy metals, which has resulted in regulations, such as SMCRA or NPDES, to mitigate the known effects. This has resulted in a disproportionate improvement of water quality. In 1998, median total manganese, specific conductance, sulfate, and pH were higher in 37 basins mined since 1980 than in 20 basins unmined since then; median total iron was lower in the mined basins, possibly reflecting aggressive treatment of permitted discharges (Paybins et al., 2000). Although AMD is most often the stressor correlated to poor stream health in coal-mining-impacted streams, point source discharges high in TDS and associated conductivity are gaining concern for their role in limiting benthic communities (Echols et al., 2009). Studies on two Southern WV streams showed, Peters Creek near Lockwood and Clear Fork at Whitesville, specific conductance was directly correlated with sulfate concentration (Paybins et al., 2000).

Armstead et al. 2013, studied the effects of elevated ionic concentrations on field collected benthic macroinvertebrates and created a recipe for the creation of a simulated high sulfate mine effluent to use in toxicity testing that would replicate receiving waters of mining impacted streams in Southern WV. This resulted in the following high sulfate simulated mine effluent recipe: calcium sulfate (0.86 g/L), magnesium sulfate (0.68 g/L), potassium chloride (0.02 g/L), sodium bicarbonate (0.32 g/L), and sodium chloride (0.02 g/L) (Armstead et al., 2013). The simulated mine effluent has been used in the Creek Geeks laboratory in several toxicity tests on various species.

Traditional toxicity tests use reconstituted water as a base for the dilution series. Reverse osmosis (RO) water is used as a diluent to achieve the necessary concentration, if necessary; a commonly used water filtration device is the MILLIPORE Super-Q® System. Reconstituted water (EPA water) and diluted EPA water are commonly used as control water in toxicity tests that use RO water as a base. There are five types of EPA water that range from very soft to very hard; moderately hard EPA water and diluted EPA water is used by the Creek Geeks laboratory to best replicate the hardness of waters generally found in WV. The concentration of reagents used and resulting water chemistry is shown in the following table:

Table 1.1. Preparation of Synthetic Water Using Reagent Grade Chemicals (USEPA, 2002)

Control Water	**Reagent Added (mg/L)**				**~ Water Chemistry**		
	$NaHCO_3$	$CaSO4+2H_2O$	$MgSO_4$	KCL	pH*	Hardness**	Alkalinity**
Moderately Hard EPA Water	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64

*~pH after 24 hours of aeration
**expressed as mg $CaCO_3$/L

Reconstituted water is not representative of actual receiving waters of mine effluent; therefore, field collected water would better replicate natural conditions. It was hypothesized that the dilution series used in future testing should use field collected water from the same stream that the mayflies, or other field collected organism, were collected would better replicate elevated ionic concentrations the organisms would encounter in their natural habitat and be a more accurate quantification of the lethal or sub-lethal effects.

There are three types of toxicity tests that are widely used and considered comparable: static non-renewal, static renewal, and flow through. Static non-renewal tests are the most simple and cost-effective option but are potentially the least reflective of apparent toxicity and organism survival. Static renewal tests are more indicative of natural conditions than non-renewal tests but are less reflective than flow through tests. Flow through tests are the most reflective of natural conditions because they replicate the stream flow that organisms face in natural conditions and deliver consistent toxicant dosing but they are the most complex and expensive type of testing, which could limit the number of replicates that can be easily achieved.

The use of field collected organisms from the aquatic ecosystem where the actual disturbance is occurring would be the most truly reflective indication of toxicity on natural organisms. The use of test organisms taken from the receiving water has strong appeal and would seem to be the logical approach (USEPA, 2002). However, there are several potential issues that limit this from being commonly used in testing. The organisms that live in the water could already be acclimated to the disturbance or the sensitive organisms could already have become extirpated. The age and fitness of the organisms would be very difficult to gage and result in natural variability of results which would severely limit the QA/QC process of testing. Identifying the organisms to the lowest possible taxon could be very difficult and result in

additional stress to the organisms. The organisms would need to be monitored for a minimum of one week to observe fitness; therefore, the most sensitive early life stages would not be tested. Young organisms are often more sensitive to toxicants than are adults; the use of early life stages, such as first instars, is required for all tests (USEPA, 2002). This exposes the need for stock populations of laboratory cultured native organisms that would address these issues and not rely on the more traditionally used testing organisms that are potentially not depictive of the effects on native organisms. In a given test, all organisms should be approximately the same age and should be taken from the same source; it would enhance the value and comparability of the data is the same species in the same life stages were used throughout a monitoring program (USEPA, 2002).

The purpose of this research was to establish laboratory rearing and culturing methods for native Ephemeroptera taxa for use in toxicity testing. Improve the effectiveness of field collections and laboratory culturing, increase hatch success and long-term survival. Determine which readily available field collected species can be developed for use in laboratory culturing, improve/establish laboratory rearing and culturing techniques, optimize organism fitness for use in toxicity testing and culturing, and establish stock populations (entire life cycle), to limit field collection and ensure organism age and fitness.

CHAPTER II

MATERIALS AND METHODS

Objective 1a. Field Collection

- Identify availability and factors limiting targeted species abundance and fitness for use in laboratory culturing and/or toxicity testing
- Determine which readily available field collected species can be developed for use in long-term laboratory culturing

Mash Fork, a direct tributary of Camp Creek, is located predominantly in Camp Creek State Forest and/or Park in Mercer County, WV. This makes it an ideal location for field collection due to minimal anthropogenic disturbances and land use practices, which also makes it an ideal candidate for a reference stream. The land use of the watershed is 0% commercial, <1%

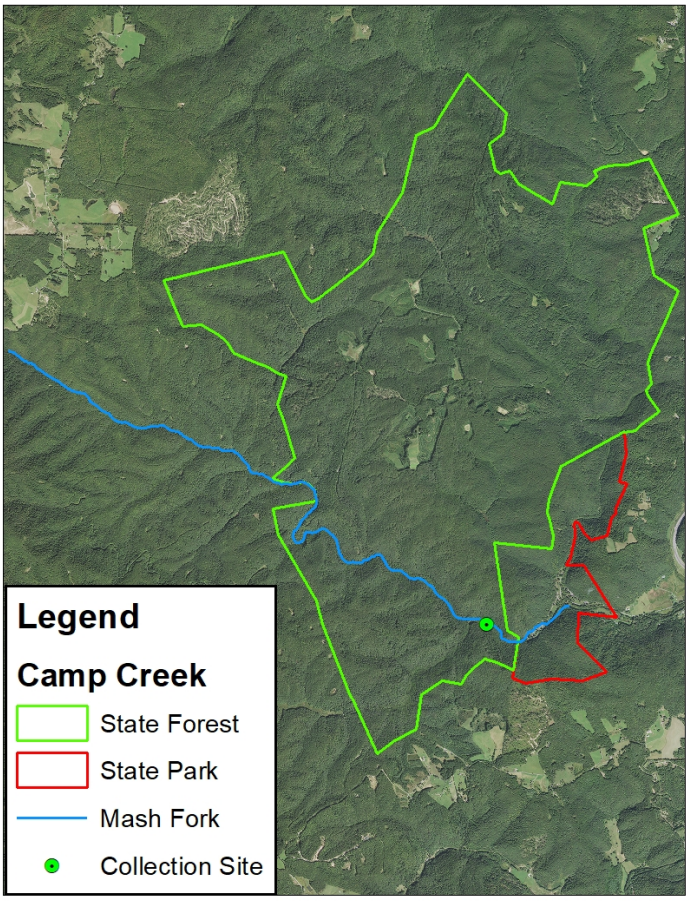

Figure 2.1. Mash Fork and Camp Creek State Forest/Park. Map by Daniel Brady 2022.

low intensity residential, and over 90% forested (NLCD, 2011). There are no active mines, dams, NPDES outlets, or Superfund sites in the entire watershed. There were gas wells and a 6" pipeline installed within the watershed, but none have been drilled/constructed since 2007. The target area of collection was above Mash Fork falls. There is a gate on the bridge below the falls which allows for foot traffic only from the public and minimal vehicular traffic from park personnel, local police, or West Virginia Department of Natural Resources staff. The minimal disturbance in this watershed allows for the organisms that are collected to remain relatively undisturbed, in great abundance, and of ideal fitness.

Mash Fork is a cold-water stream that is relatively shallow and fast moving due to topography, especially in the target area where collections occurred. The collection area is a fast-moving riffle that has a maximum depth of ~1-2'. Mayfly abundance and species richness

Figure 2.2. A Creek Geeks collection effort at Mash Fork. Image by Geneve Brady 2018.

is negatively correlated with water depth and positively correlated with velocity (Vilencia et al., 2018). It has an abundance of cobble and gravel substrate that provide ideal microhabitat, not only for benthic macroinvertebrates but also their preferred food sources such as diatoms and

algae for scrapers/grazers. The stream is surrounded by forested areas which provides ample amounts of detritus introduced to the stream during the fall or runoff events for shredders. The high velocity of the flow in the area is ideal for collector/gatherers or filter feeders. Based on their feeding strategies, most mayflies depend on certain microhabitats during their larval stages (Vilencia et al., 2018). Therefore, there are ideal conditions for all of the most common FFGs of mayflies in Mash Fork, especially in the collection area.

Objective 1b. Laboratory Culturing

- Improve/establish laboratory rearing and culturing techniques
- Optimize organism fitness for use in toxicity testing and culturing
- Establish stock populations (entire life cycle), to limit field collection and ensure organism fitness

Mayfly nymphs and water were collected from Mash Fork in Mercer County, West Virginia. Mash Fork water was filtered through a 54-micron sieve after collection to remove any potential predators or sediments, was bubbled to maintain DO during storage, and was re-filtered before any use in the culturing unit, toxicity testing or autoclaving occurred. Once identified to genus, the nymphs were counted and added to the hexagonal culturing unit (referred to as the hexagon). The hexagon is a six-chambered re-circulating hexagonal simulated stream connected to chilling unit to regulate temperature.

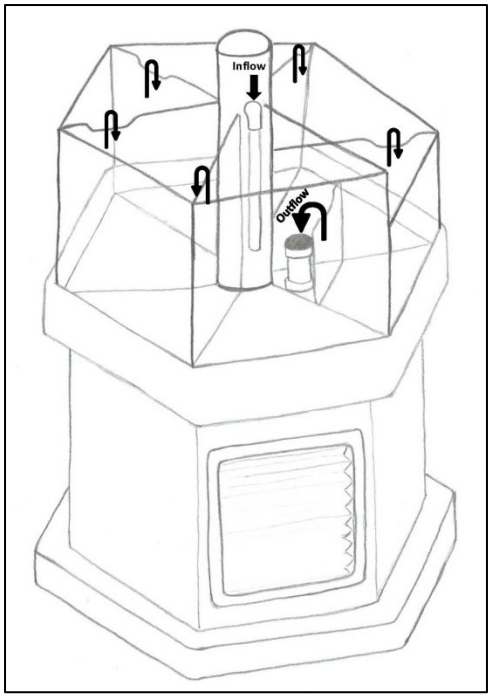

Figure 2.3. A diagram of the re-circulating hexagonal simulated stream (Hexagon). Image by Mandee Wilson 2015.

Water chemistry was monitored regularly to ensure optimal conditions, resulting in a DO of ~9-10 mg/L, temperature of ~19-21° C, and a pH of ~6-8 SU. Mash Fork water, a field collected diatom mixture from Carolina Biological Supply Company, and laboratory cultured diatoms were added to the hexagon as a food source for the nymphs. Field collected rocks and sticks were added to the hexagon to replicate natural conditions and as a substrate for diatoms and nymphs. A net enclosed the hexagon to facilitate easy capture of the nymphs that emerged as adults (sub-imago or imago), which were collected for testing as early as possible. If a male and female of the same species were collected within the same 24-hour period, they were both placed into a mating chamber to facilitate fertilization, the female's eggs were collected via oviposition, if possible, or via dissection. If no male of the same species was available on the same day and the species was parthenogenetic, the female's eggs were collected via oviposition, if possible, or

via dissection. The eggs were counted, clutches were assigned ID numbers called the clutch identifier which included the date of collection and a letter to designate the female organism that laid the eggs (e.g, #050118A) and logged into a database and then placed into watch glasses with autoclaved Mash Fork water. Water was autoclaved to ensure the absence of any unwanted organisms or bacteria.

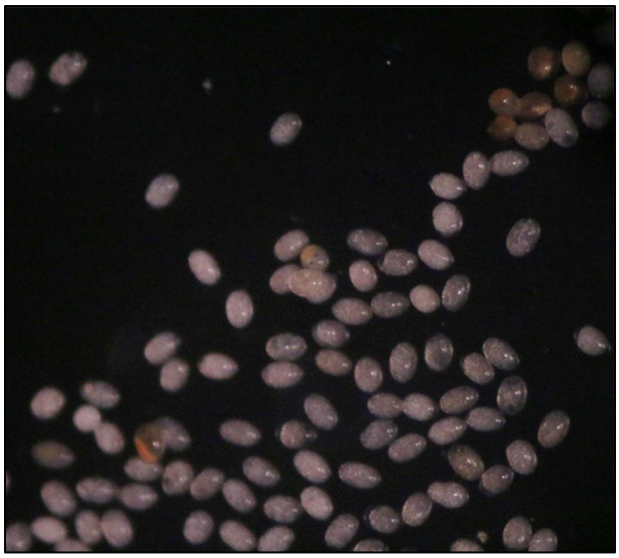

Figure 2.4. An egg clutch in a watch glass. Image by Geneve Brady 2017.

After egg collection, the mother was preserved in a 70% Ethanol solution and given the identical clutch identifier as her egg clutch for future identification of species, if possible. The eggs were then placed in the incubation unit set at 20° C and covered using a shade cloth. The watch glasses were placed on a shaker set at ~55 rpm to replicate water movement and on a 16/8 light/dark cycle to replicate natural lighting conditions. Water changes were conducted daily using autoclaved Mash Fork water and were monitored for hatched nymphs. Upon hatching, nymphs were used in the diet analysis and the toxicity test. The well plates were stored in the incubator during the remainder of the tests, when not undergoing daily maintenance, until total mortality occurred.

Figure 2.5. A freshly hatched Baetidae nymph, that would be ready for use in testing. Image by Geneve Brady 2017.

Objective 1c. Database Analysis

- Determine the effect(s) of temporal variability, FFG, and/or water chemistry on collection rate
- Determine the effect(s) of incubation time and/or initial egg count on hatch rate and total hatch

The Creek Geeks laboratory has kept collection and incubation data from Mash Fork since 2015. Collection data from other streams in WV was kept from 2014-2015. Portions of this dataset was from exploratory or minimal effort collections so that data was removed from the dataset during analyzation to prevent bias and skewing; collection data was removed if less than 10 organisms were collected. This data has been entered into a database that makes the data easily accessible for analysis. Collection data was recorded in the form of date of collection, season of collection, taxa of organism (identified to the lowest practical taxon), FFG of the

organism, number collected, date of placement and type of culturing unit, and water chemistry of the stream and/or culturing unit. Egg incubation data was recorded into a database that included clutch identifier, taxa of organism (identified to the lowest practical taxon), initial egg count, total hatch, percent hatch, incubation days, and hatch length. Incubation days is defined as the number of days an egg clutch had until the first nymph hatched. Hatch length is defined as the number of days an egg clutch had from first hatch to final hatch. These databases were analyzed to maximize collection and incubation efforts for future collection, rearing, testing, and culturing methods.

Objective 2. Diet Analysis

- Investigate feed treatments that will lead to a minimum of 80% survival in the first 2 days for acute toxicity testing and first 7 days for chronic toxicity testing
- Investigate feed treatments that will lead to optimal fitness for long-term survival and culturing
- Find a suitable food source for Baetidae *&* Heptageniidae nymphs
- Increase fitness and longevity of newly hatched nymphs

Upon hatching, nymphs were placed in a 12-well plate with 2-5 nymphs of the same egg clutch per well in 2 mL of autoclaved Mash Fork water. All nymphs were less than 24-hour old and all nymphs in a single testing chamber were from the same clutch. This ensured that all organisms in a single replicate were of comparable age and fitness. During the food test, each food source was assigned to a well plate with the nymphs assigned to the rows sequentially as they hatched which initially established the “rolling method” for nymph placement (which is now used universally in the Creek Geeks laboratory). The nymphs were assigned a feeding treatment using the rolling method. Each well was identified using the clutch identifier and an alpha-numeric identifier corresponding to placement in the well plate (e.g., A1, B2).

Laboratory cultured *Navicula* sp. and *Selenastrum* sp. were used as feeding treatments, they are a common species of diatoms and algae, respectively, that are found in nature. *Navicula* sp. was cultured using growth media obtained from Carolina Biological Supply Company. *Navicula* sp. starter culture Alga-Gro®, 13 mg Na_2SiO_3 and 0.13 mL of Proline® F/2 Algae Food Part A and 0.13 mL of Part B was added to 1 L of autoclaved EPA water. This was cultured for ~3 weeks in a growth chamber and then put into a centrifuge before use as a feeding treatment in the diet analysis. *Selenastrum* sp. was cultured using 0.13 mL of Proline® F/2 Algae Food Part A and 0.13 mL of Part B to 1 L of autoclaved EPA water resulting in an ~7.5 pH SU. It was then placed into the growth chamber for ~1 week before storage or use as a feeding treatment in the diet analysis.

YCT is a standard feeding treatment used in toxicity testing on standard test organisms such as cladocerans. YCT was prepared by adding 5 g of trout chow to 1 L of RO water and aerating for one week. On day six of aeration adding 5 g of dried cereal leaves and blending for ~5 minutes then placed in a refrigerator overnight. On day seven, the mixture is stopped aerating and distilled water is added to replace any evaporation that has occurred resulting in a total volume of 1 L. The mixture is allowed to settle for one hour in a refrigerator before adding a blended mixture of 5 g of yeast and 1 L of distilled water. The two mixtures are filtered through 60-μm Nitex® screen into a 4 L flask and thoroughly mixed before being put into 500 mL containers for storage or use as food sources. Fish food flakes can be used instead of trout chow in the mixture of YCT and were a feeding treatment used in previous toxicity testing (Echols et al., 2009). The fish food flake feeding treatment was made by blending 5 g of TetraVeggie® Spirulina Enhanced Flakes with 1 L of autoclaved EPA water before use as a feeding treatment. Sand and leaf disks were used as a representative of a natural substrate and were cultured with

diatoms and/or colonized with microbes. Sand was colonized with *Navicula* sp. for ~2 weeks, using the same method as the culturing of *Navicula* sp. previously stated before use as a feeding treatment. Leaf disks were colonized with microbes for ~1 week before use as a feeding treatment. The feeding treatments are further explained in the following table:

Table 2.1. Feeding Treatments Used in Diet Analysis

Feeding Treatment	Notation	Amount per well	Reasoning
Navicula sp.	N	5 µL	Natural food source
Selenastrum sp.	S	5 µL	Standard food source used in toxicity testing
YCT	Y	5 µL	Standard food source used in toxicity testing
Fish Food Mix	F	5 µL	Food source used in previous Ephemeroptera study
Navicula sp. & *Selenastrum* sp.	NS	5 µL of each	Provide food source for different developmental stages
Selenastrum sp. & YCT	SY	5 µL of each	Provide food source for different developmental stages
Navicula sp., *Selenastrum* sp. & YCT	NSY	5 µL of each	Provide food source for different developmental stages
Navicula sp. & YCT	NY	5 µL of each	Provide food source for different developmental stages
Leaf Disk (Field Collected)	LNC	1 disk	Provide a substrate & colonized with diatoms and microbes
Sand (Autoclaved)	SD	5 µL	Provide a substrate & colonized with diatoms and microbes

Once the nymphs were assigned a food source they were monitored for growth, development, and mortality on a daily basis using a dissecting microscope. A 50% water change

was conducted every other day to remove uneaten food sources and to maintain a clean environment for the nymphs. It was observed that nymphs can get stuck in old food sources which almost always resulted in mortality, although some could have gotten stuck in the food source after mortality had already occurred. Leaves were field collected from Mash Fork and were observed for any potential predators before use in testing. The leaves were cut into small circles called "leaf disks" and then submerged in water, a growth media was added to facilitate colonization of diatoms and microbes. They were allowed to colonize for approximately one week before use in testing. Leaf disks were then changed on a weekly basis throughout the length of the test. Growth was evaluated via the number of exuvia shed during the test, which indicated the nymph going to the next instar. Mortality was recorded during daily maintenance activities; an organism was deemed to have died if no movement was observed during the length of all daily maintenance activities and the organism was then removed to not interfere with the remaining live organisms.

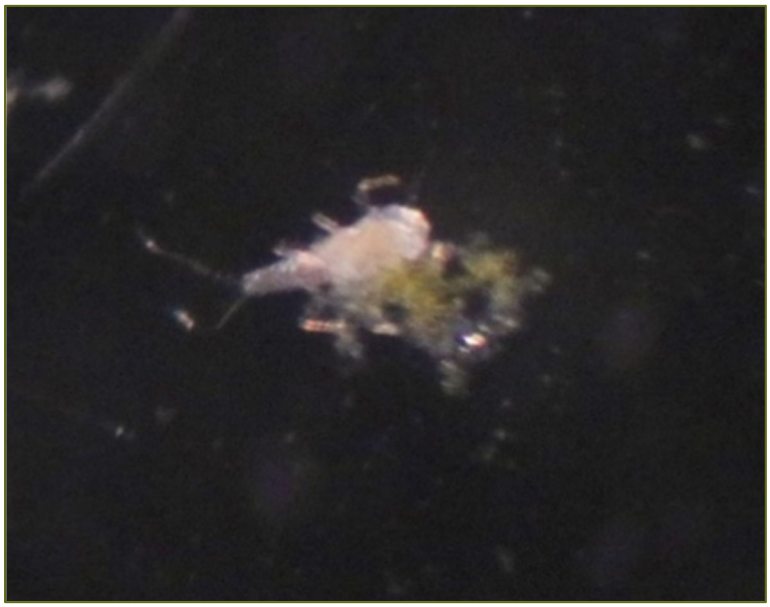

Figure 2.6. An *Acentrella* sp. nymph feeding on one of the feeding treatments used during the Diet Analysis. Image by Geneve Brady 2016.

Objective 3. Toxicity Testing

- Observe effects of High Sulfate Simulated Mine Effluent using field collected Mash Fork water as a base for dilution series
- Determine the LC50 or LOEC of High Sulfate Simulated Mine Effluent

Upon hatching, nymphs were placed in a 12-well plate with 3-6 nymphs of the same egg clutch per well in 2 mL of the treatment water that they were assigned. All nymphs were *Acentrella* sp., less than 24-hour old, and all nymphs in a single testing chamber were from the same clutch. This ensured that all organisms in a single replicate were of comparable age and fitness. The nymphs were assigned one of the seven different types of water using the aforementioned "rolling method". Field collected Mash Fork water, diluted USEPA moderately hard water, and USEPA moderately hard water were used as controls for the experiment, which had approximate conductivities (µS/cm) of 30, 100, and 300, respectively. High sulfate simulated mine effluent was used for the dilution series, which was a replication of the effluents from coal mining discharges found in southern WV. Four treatment groups were used from a dilution series of 25, 50, 75, and 100% concentrations which resulted in approximate conductivities (µS/cm) of 600, 1200, 1800, and 2400, respectively.

Figure 2.7. Daily maintenance activities performed during laboratory testing. Image by Geneve Brady 2018.

Once the nymphs were assigned to one of the three control waters or one of the four concentrations of simulated mine effluent, they were monitored for mortality daily. Due to this being a 48-hour acute test using the static non-renewal method, daily maintenance was not required beyond mortality observation. The nymphs were fed a mixture of *Navicula* sp., *Selenastrum* sp., and YCT because it has been previously demonstrated to be an effective feeding treatment to ensure a minimum of 80% survival in the acute diet analysis test for mayflies. Traditional feeding treatments such as YCT and/or fish flakes did not yield sufficient survival in mayflies in the diet analysis. The nymphs were not feed beyond the initial food source and the treatment water was not changed from the beginning of the 48-hour test.

CHAPTER III

RESULTS

Collection Analysis

In the trimmed collection database, 8,220 individuals were collected in 36 collection events from 2014-2018. The highest number of collection events was Summer at 15 and lowest was Winter with only 1. The highest total collected was in Spring with 3,835 total organisms. The highest average collected was Spring with 383.5. The total organisms by FFG resulted in 3,783 collectors, 757 collector/filterers, and 3,552 scrapers. The highest average FFG collected was Spring/collectors at 200.1.

Table 3.1. Summary of trimmed Collection Database from 2014-2018

Season	Collection Rate			Functional Feeding Group					
	Events	Total	Avg.	Collector		Collector/Filterer		Scraper	
				Total	Avg.	Total	Avg.	Total	Avg.
Fall	10	1977	197.7	472	47.2	590	59.0	915	91.5
Spring	10	3835	383.5	2001	200.1	3	0.3	1831	183.1
Summer	15	2155	143.7	1310	87.3	164	10.9	653	43.5
Winter	1	253	253.0	0	0.00	0	0.00	153	153.0

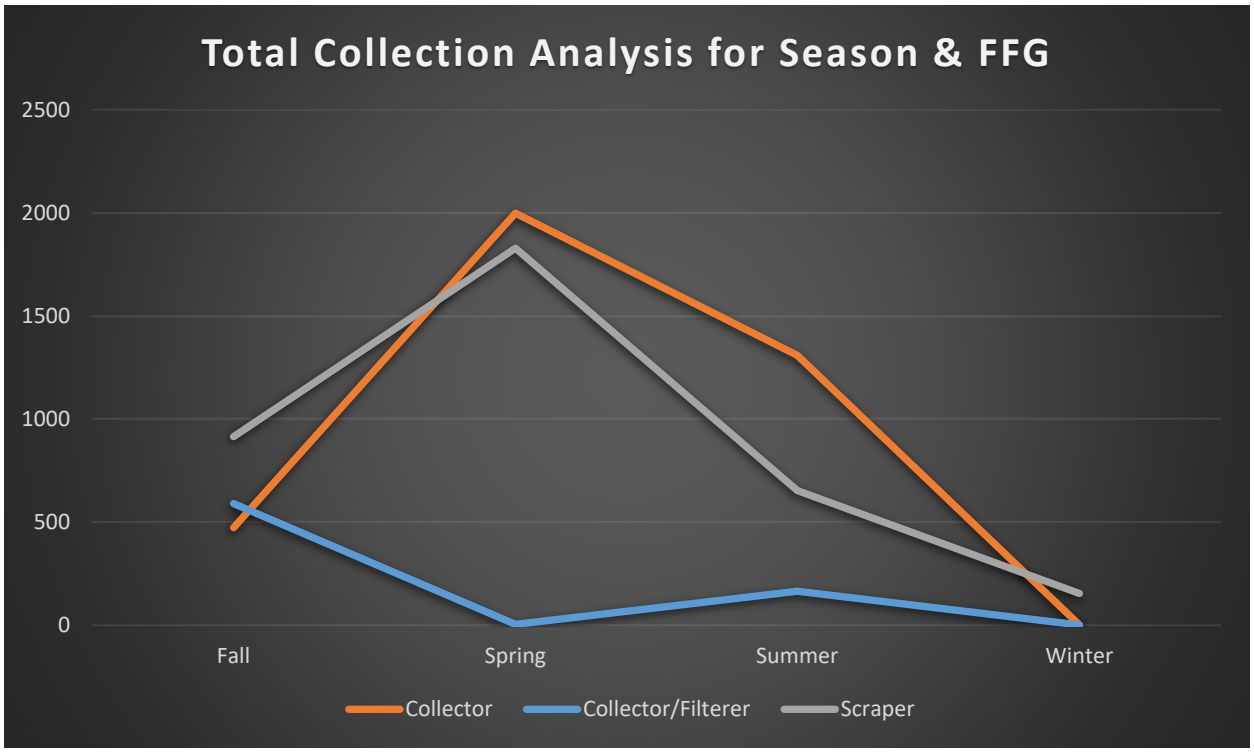

Figure 3.1. Analysis on Temporal Variability and FFG effects on total collection rate.

Incubation Analysis

A one-way ANOVA showed no statistical difference ($F=1.64$, $p=0.187$) between initial egg count and percent hatch. A one-way ANOVA showed no statistical difference ($F=0.72$, $p=0.733$) between incubation days and percent hatch. Even though there was no significant statistical difference on incubation days and percent hatch, a linear regression showed an overall negative correlation between them (see Fig 3.2).

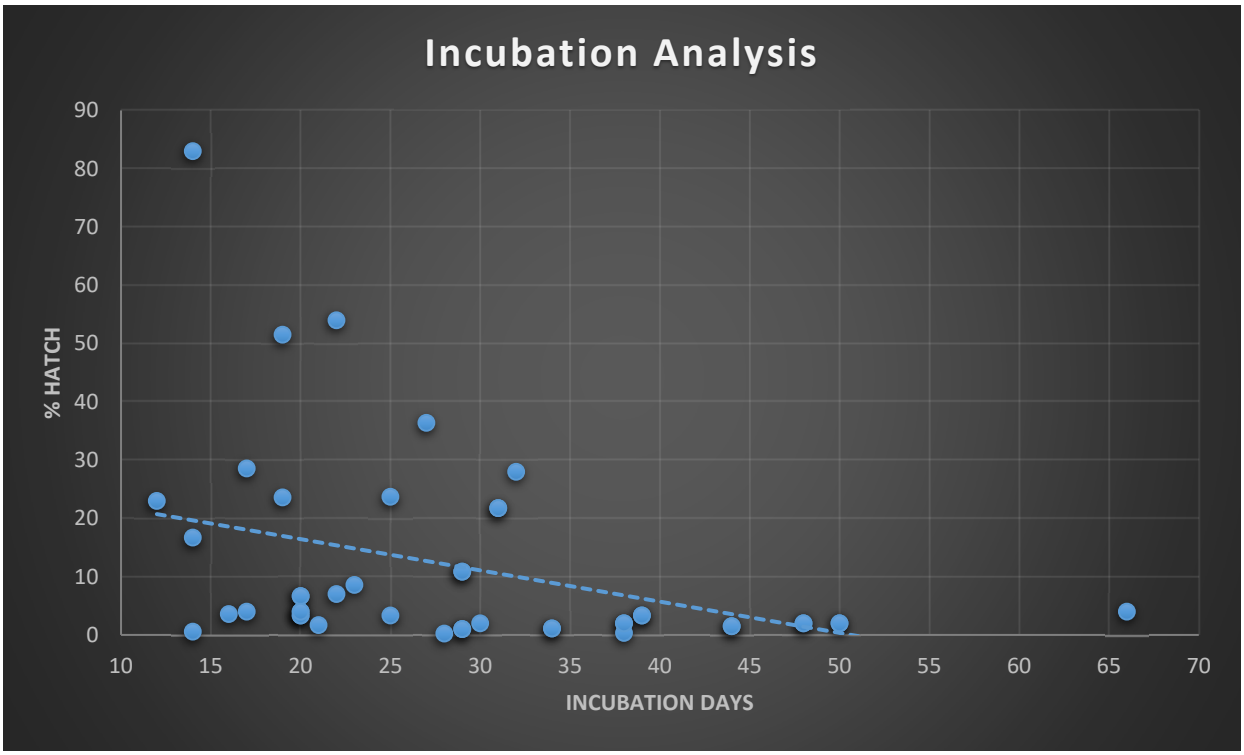

Figure 3.2. Analysis of Incubation Days effect on Percent Hatch.

Diet Analysis

Prior to statistical analyses, data from the Baetidae Diet Analysis were arc-sin transformed to meet normality and homogeneity assumption. A two-way ANOVA showed a significant difference ($F=3.69$, $p=0.026$) between clutch identifier and *Baetidae* sp. survival within the first 2 days of hatching, although it is unknown currently what factor is significant within clutch identifier. At 4 days post-hatch there was a significance difference between both clutch identifier ($F=3.56$, $p=0.029$) and food source ($F=2.19$, $p=0.027$) on organism survival, with food source being slightly more significant. A highly significant difference ($F=6.28$, $p=0.000$) was found between food source and organism survival at day 7 post-hatch, showing that food source has a significant effect on survival. A Fisher's LSD Multiple-Comparison test showed a significant difference between NL and NSYL and all other food sources, including

each other, on long-term survival with NSYL (mean value=11.062 days) having the best survival followed by NL (mean value=7.188 days).

Table 3.2. Statistical Results of Baetidae Diet Analysis

Statistical Test	Variable	Survival (Days)	F or H value	p value
t-w ANOVA	clutch ID	2	3.69	0.026
t-w ANOVA	food source	2	1.63	0.115
t-w ANOVA	clutch ID	4	3.56	0.029
t-w ANOVA	food source	4	2.19	0.027
t-w ANOVA	clutch ID	7	2.69	0.069
t-w ANOVA	food source	7	6.28	0.000
t-w ANOVA	clutch ID	36	3.07	0.048
t-w ANOVA	food source	36	6.89	0.000

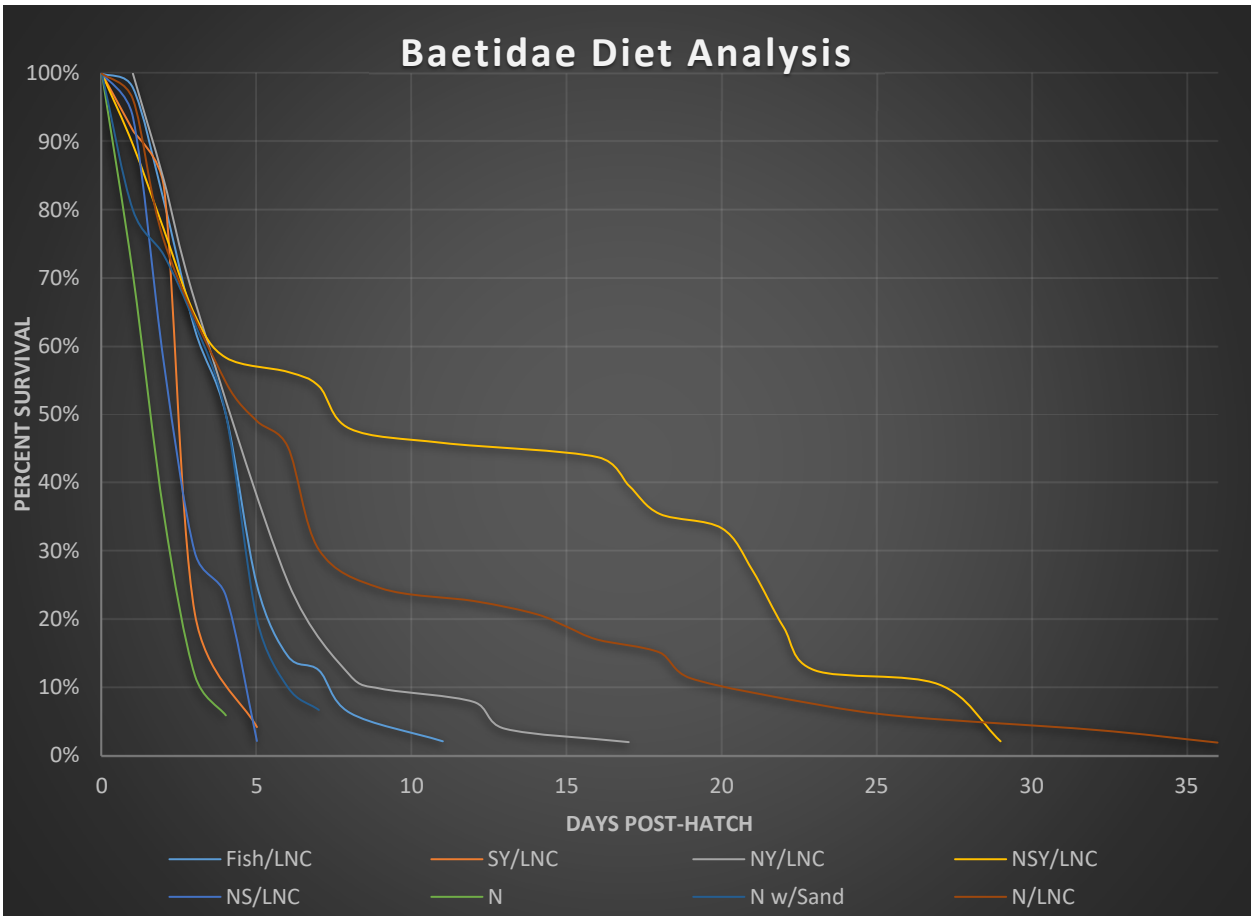

Figure 3.3. Baetidae Diet Analysis of Food Source on Percent Survival.

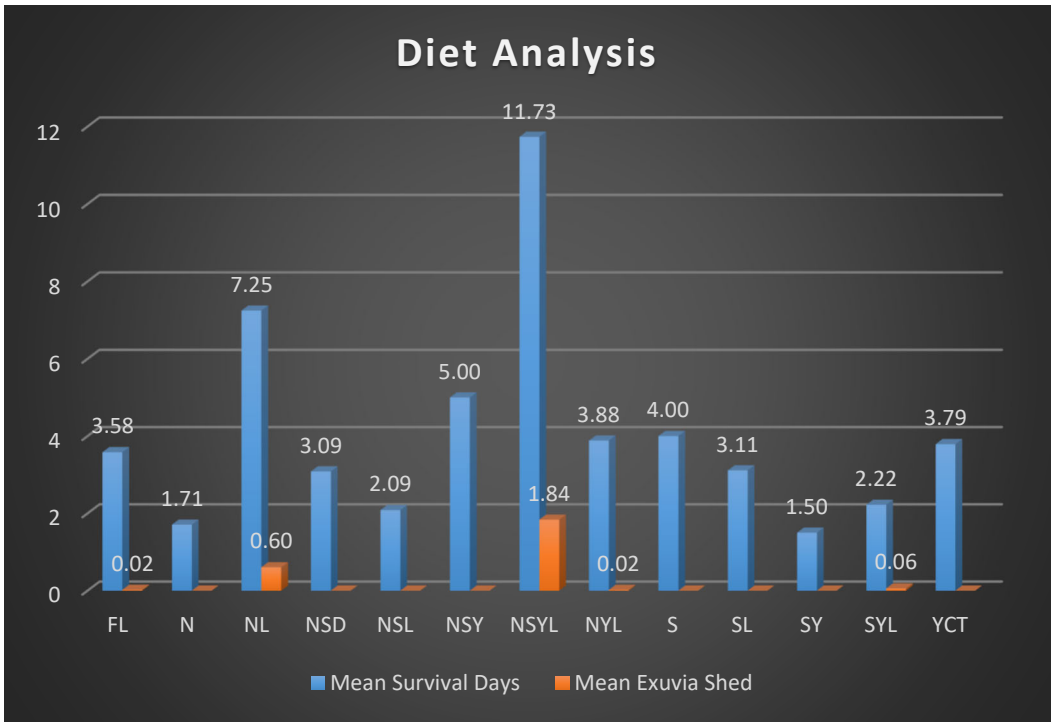

Figure 3.4. Analysis of Feeding Treatment for Baetidae and Heptageniidae on Survival (MSD) and Growth (MES).

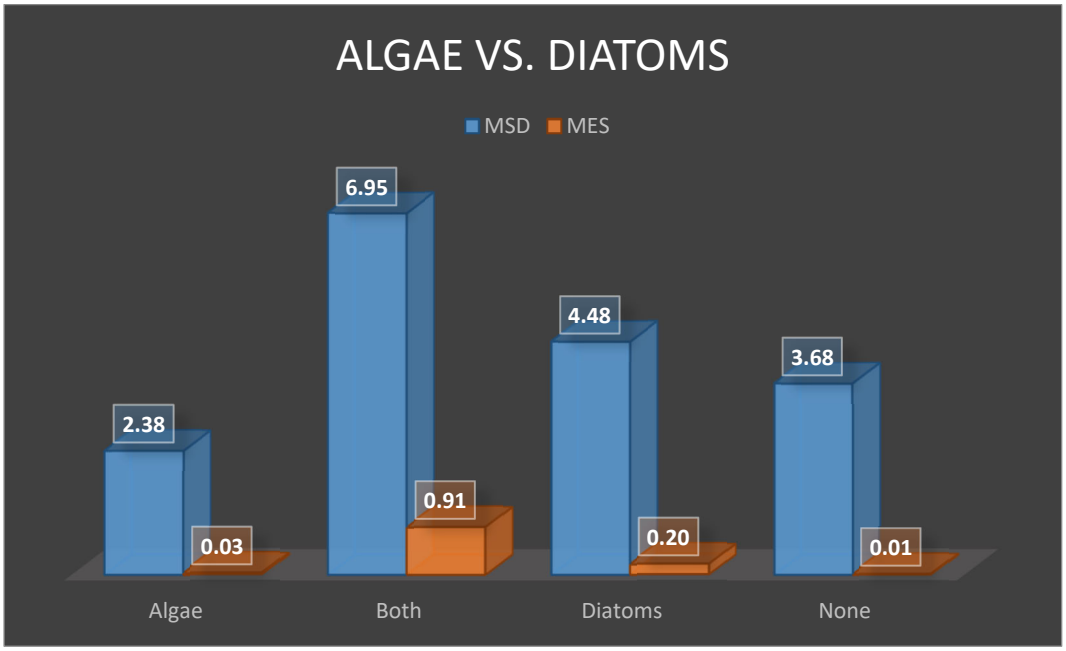

Figure 3.5. Analysis of Feeding Treatments that included Algae, Diatoms, a Mixture or None for Baetidae and Heptageniidae on Survival and Growth.

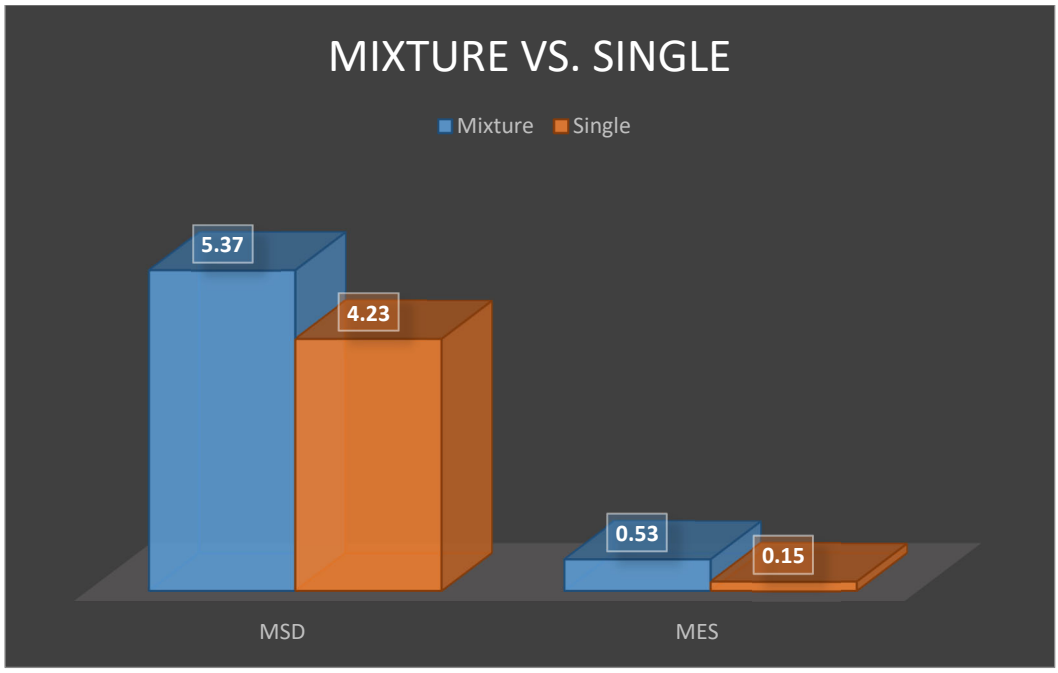

Figure 3.6. Analysis of Mixtures or Single Feeding Treatments for Baetidae and Heptageniidae on Survival and Growth.

A one-way ANOVA showed a significant difference (F=15.63, p=0.001). between the feeding treatment *Navicula* sp. and family on survival.

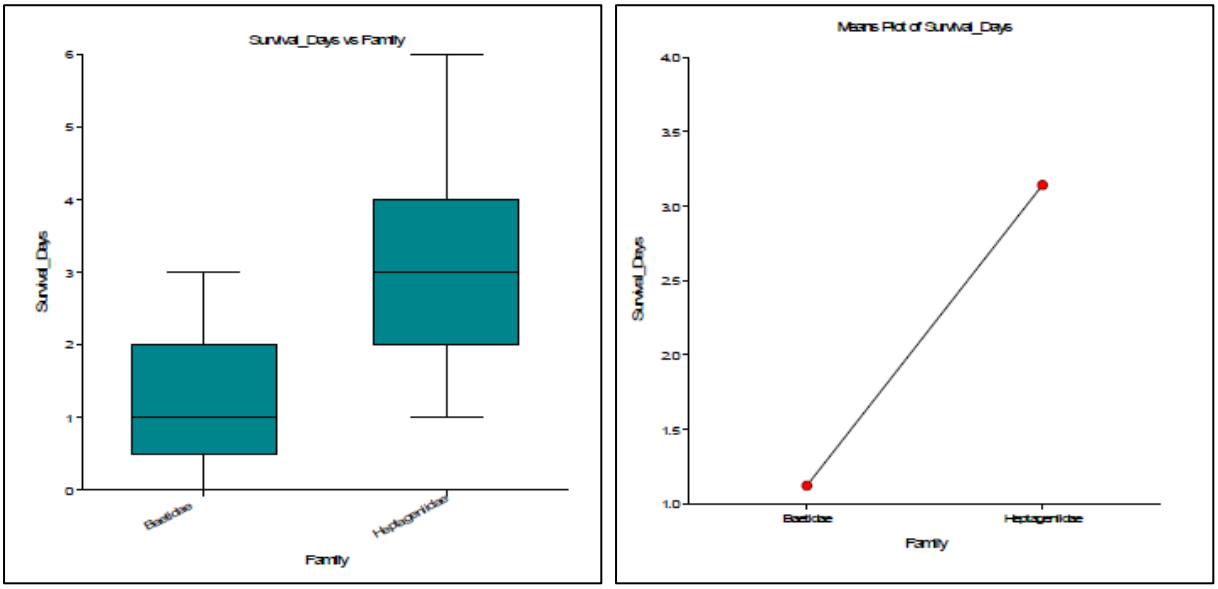

Figure 3.7. Analysis of *Navicula* sp. on Baetidae and Heptageniidae Survival.

A one-way ANOVA showed a significant difference (F=18.39, p=0.000) between the feeding treatments that included a leaf disk (FL, NL, NSL, NSYL, NYL, and SYL) on *Baetidae* sp. survival. A Fisher's LSD Multiple-Comparison showed a difference on the feeding treatments NL and NSYL on the other feeding treatments, including each other.

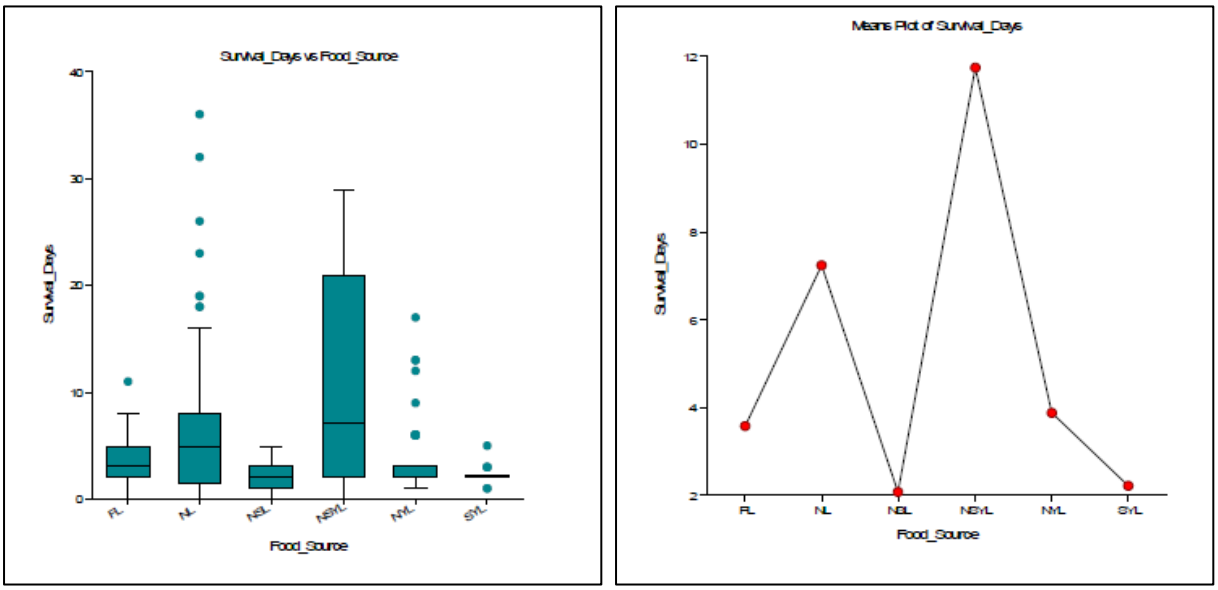

Figure 3.8. Analysis of Feeding Treatments that included a Leaf Disk on *Baetidae* sp. Survival.

A one-way ANOVA showed a significant difference (F=23.67, p=0.000) between the feeding treatments that included YCT and leaf disks on *Baetidae* sp. survival.

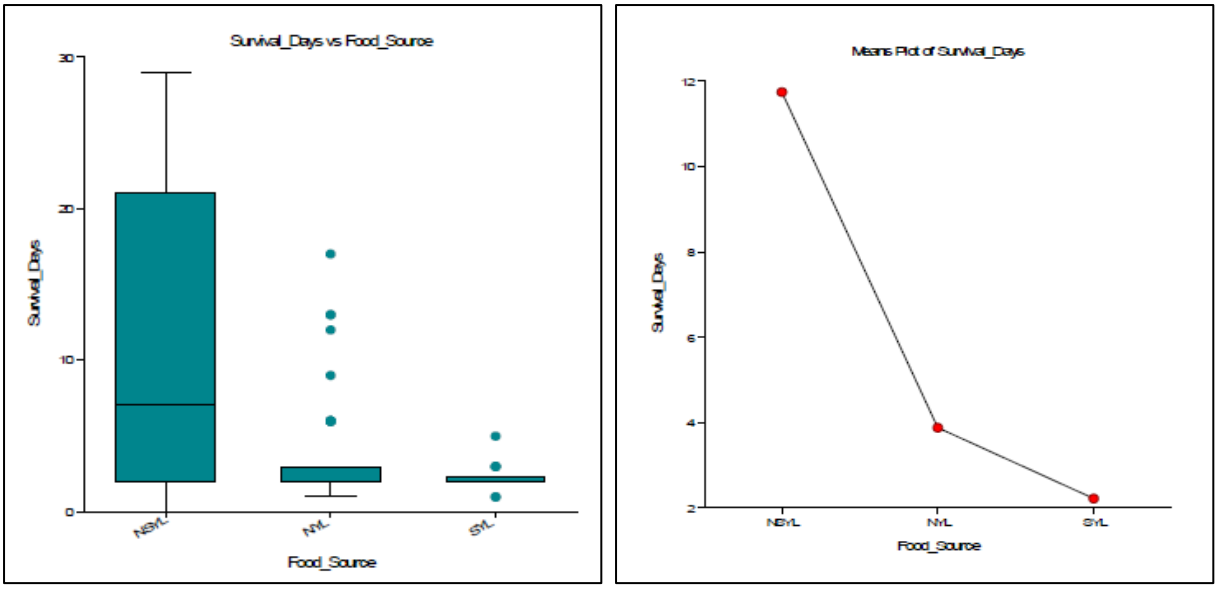

Figure 3.9. Analysis of Feeding Treatments that included YCT and a Leaf Disk on *Baetidae* sp. Survival.

Toxicity Testing

The results of the acute toxicity test did not generate a statistically significant LC50; however, the 100% high sulfate simulated mine effluent concentration (~2,400 µS/cm) resulted in ~50% mortality (50.85% survival in 12 replicates). A Parametric-Multiple Comparison showed a NOEL on the 50% and LOEL on the 75% concentrations when compared to the Mash Fork control water; when compared to the EPA and Dil. EPA water, they showed a NOEL on the 75% and LOEL on the 100% concentrations.

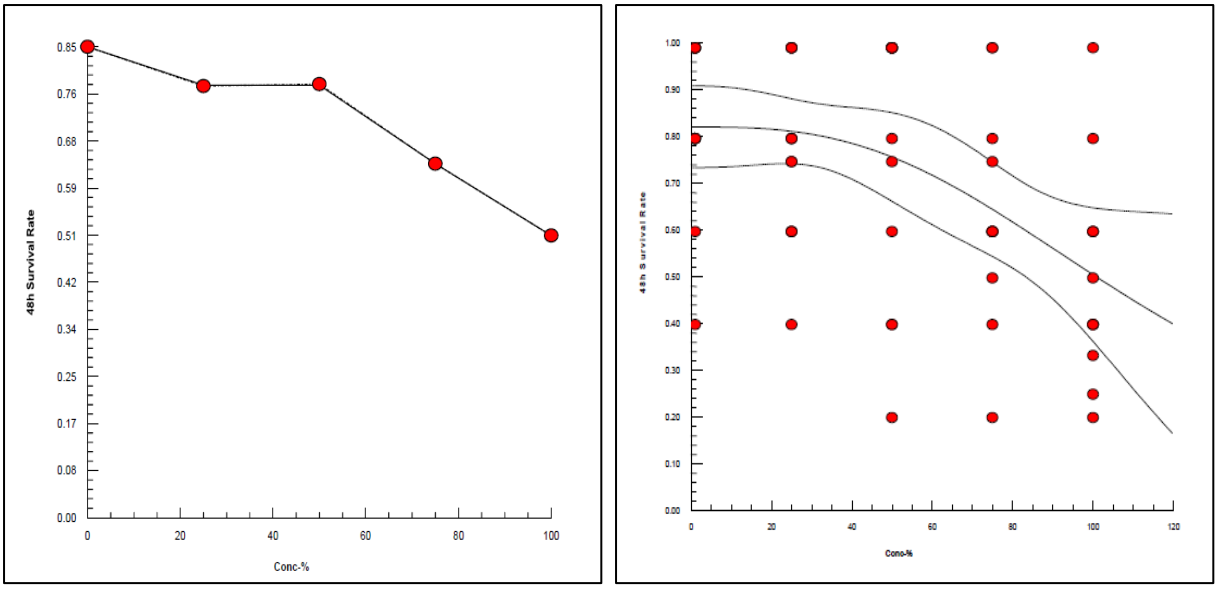

Figure 3.10. Analysis of Mash Fork water and High Sulfate Simulated Mine Effluent on *Acentrella* sp. Acute Survival.

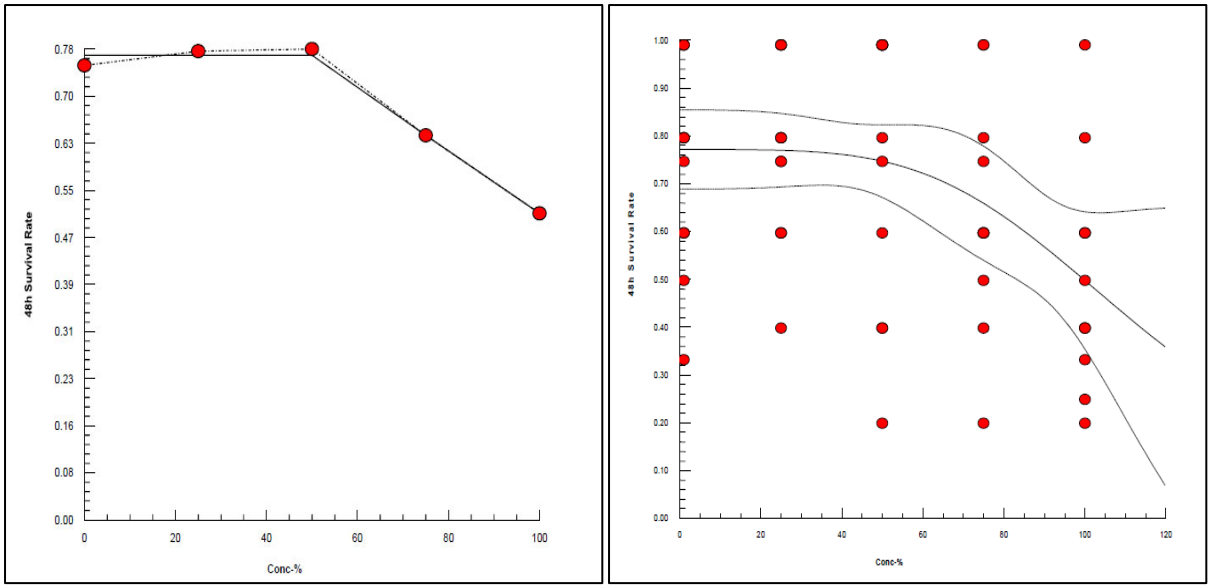

Figure 3.11. Analysis on Diluted EPA water and High Sulfate Simulated Mine Effluent on *Acentrella* sp. Acute Survival.

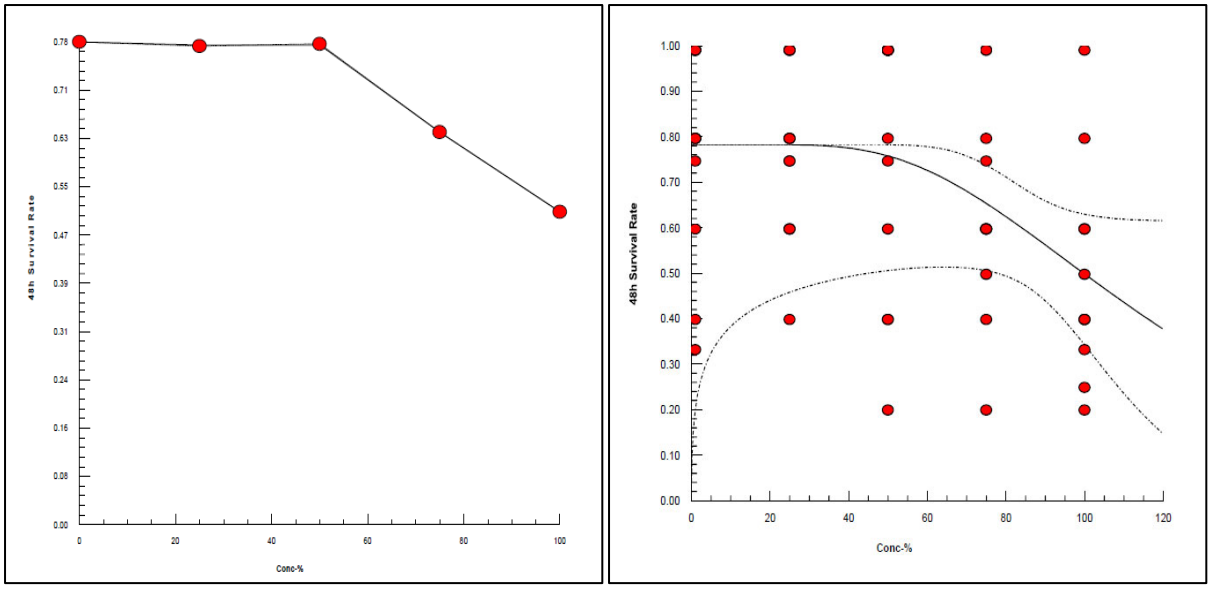

Figure 3.12. Analysis on EPA water and High Sulfate Simulated Mine Effluent on *Acentrella* sp. Acute Survival.

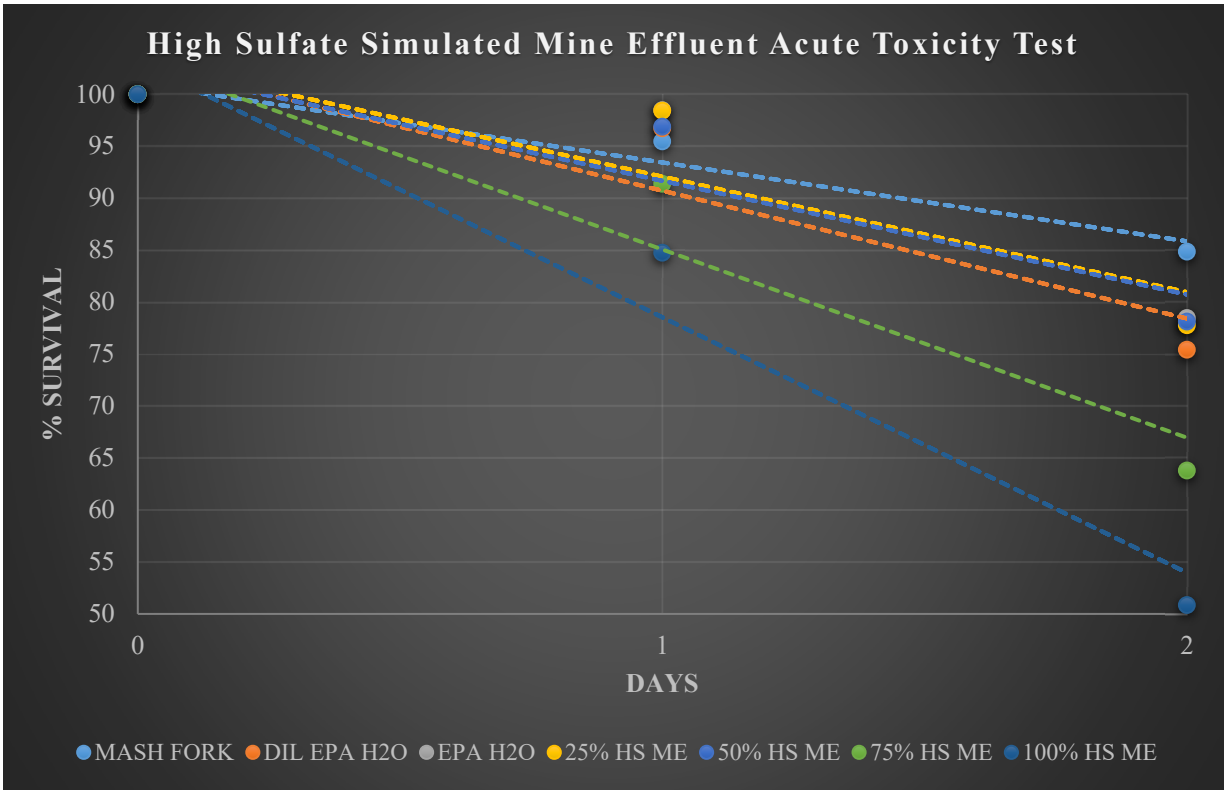

Figure 3.13. Analysis on all Treatment Groups and High Sulfate Simulated Mine Effluent on *Acentrella* sp. Acute Survival.

CHAPTER IV

DISCUSSION

Collection Analysis

The Creek Geeks laboratory has kept collection data during mayfly research from 2014-2018. The trimmed dataset with outliers removed, resulted in 36 collection attempts with an average collection rate of ~245 organisms per collection event. The average number of organisms collected in the summer was much lower than in spring, when diatom abundance and streamflow are relatively low. This could also be attributed to an overall lower relative abundance of mayflies due to the emergence of the spring cohort in multivoltine or univoltine species. The highest collection rate was observed in spring, most likely due to being prior to or during the spring emergence and the highest abundance of diatoms.

Some valid conclusions can be made from this analysis, collection efforts in general can use environmental factors such as stream flow or temperature to maximize efforts. Collection efforts that are focused on a target species can use voltine status, FFG, and size of the species to hypothesize when relative abundance would be higher or lower and take advantage of those times or avoid them if necessary.

Incubation Analysis

The incubation analysis did not show any statistically valid differences between initial egg count and incubation days on either percent hatch or total hatch. This was potentially skewed by several relatively large egg clutches (i.e. 500 to > 1,000) having minimal hatch total and rates. This was mostly seen in the larger mayflies such as *Hexagenia* sp. that only had minimal hatch throughout testing attempts. The average percent hatch of the entire dataset was 14.0%, *Epeorus* sp. had 8.6% and *Hexagenia* sp. had 5.0%; if you remove the *Hexagenia* sp. from the dataset it

increased to 19.2%. The smaller Baetid species had relatively higher percent hatch with *Acentrella* sp. at 19.3% and *Baetis* sp. at 34.9%.

Differences in percent hatch could potentially be linked to biological mechanisms such as r/K strategies. The smaller species tend to have lower fecundity, which allows them to put more energy into each individual egg. The larger species, generally have higher fecundity, would put less energy into each individual egg, which may mean each individual egg would have less energy used to develop it and be generally less likely to hatch. However, this is not a direct comparison as the *Hexagenia* sp. did not emerge in the laboratory; they were field collected, put into a mating chamber and the eggs were collected. The remaining genera were hatched and emerged in the laboratory culturing unit. Furthermore, *Hexagenia* sp. are the only genera used in this comparison that are not parthenogenetic; therefore, it is unsure if all clutches of eggs were thoroughly fertilized.

Diet Analysis

Due to the significant difference found within the clutch identifier during the first 2 days post-hatch, further research is needed to determine the variable(s) that is most significant for organism survival during the first 48 hours. The variable(s) encompassed in the clutch identifier include species-specific traits, health or holding time of mother, temporal variability, and unknown site-specific factors. Clutch identifier is an alpha-numeric classification assigned to each individual egg clutch. It includes the date of egg collection and a letter assigned to make it unique from other egg clutches that were obtained on the same date (i.e. #041616 A). The significance difference observed between clutch identifier and survival in the first 2-4 days, would indicate that there may be a disparity between the health of the female organisms whose egg clutches were used in testing. This exacerbates the need for the establishment of laboratory

populations of organisms with ideal fitness. If organisms could be cultured with high fecundity, then a single egg clutch could be assigned to an individual test, which would eliminate this confounding factor.

It has been hypothesized that as the nymphs grow and develop, especially when going to the next instar, that their dietary requirements change, which would limit some of the feeding treatments effectiveness for that particular life stage. This was supported by the food study, which showed that the optimal feeding treatment at day 2 was not the optimal feeding treatment at day 7 and a mixed feeding treatment resulted in the longest survival of the test. Therefore, a diverse mixture of several food types (i.e. algae, bacteria, diatoms, microbes) appears to be more effective for long-term survival as the nymphs developed over time. Feeding the nymphs both, the combination of *Navicula* sp. and leaf disk and the combination of *Navicula* sp., *Selenastrum* sp., YCT and leaf disk had a significant positive effect on *Baetidae* sp. survival at day 7 post-hatch, however the organisms did not survive to emergence, demonstrating the need for further research of optimal culturing requirements.

Once culturing methods are established to be able to achieve the proper survival rate; acute and chronic toxicity testing can begin with the nymphs. Being able to culture the organisms in the laboratory will increase confidence in organism fitness. This would provide organisms for year-round testing because field collection is costly and is greatly affected by weather and temporal variability.

Toxicity Testing

It should be noted that, this study was conducted with organisms of less-than-ideal fitness; therefore, if culturing methods could be established then further rounds of testing would involve organisms with ideal fitness that would generate scientifically and statistically valid

mayfly sensitivity values. Therefore, this test should be considered as preliminary testing to establish methods for further rounds of testing. Even though the results of the test did not generate a statistically valid LC50, the 100% concentration resulted in ~50% mortality. Therefore, future rounds of testing should increase the concentration of the 100% to result in ~3,200 µS/cm and include a 12.5% treatment to the dilution series to observe the effects of higher sulfate concentration on Ephemeroptera to better replicate actual conditions of anthropogenic disturbances. This could result in the generation of an LC50 and/or LOEC. The increased significance shown in the Parametric-Multiple Comparison, would seem to indicate that the field collected Mash Fork control water resulted in a greater dose-dependent response than the synthetic control waters of EPA and Dil. EPA. Therefore, the preliminary toxicity testing would indicate that the hypothesis that field collected water used in the dilution series could promote optimal fitness of testing organisms. Although, this will need to be confirmed in further rounds of testing.

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Office of Research Integrity

March 14, 2022

Daniel Brady
1606 Mountain Road
Charleston, WV 25303

Dear Daniel:

This letter is in response to the submitted thesis abstract entitled *"Ephemeroptera Culturing Methods: Development of Culturing and Rearing Methodology and High Sulfate Simulated Mine Effluent Toxicity Test Using Field Collected Water."* 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,

Bruce F. Day, ThD, CIP
Director