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Development of Culturing Methods for Native Mayfly Taxa for Use in Laboratory Toxicity Testing

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DEVELOPMENT OF CULTURING METHODS FOR NATIVE MAYFLY TAXA FOR USE IN LABORATORY TOXICITY TESTING

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ABSTRACT

Salinity is increasing in freshwaters throughout the world due to anthropogenic impacts with the implication of the increases to biological communities only now being realized. Declining mayfly populations in Appalachian streams have generated increased interest in elevated dissolved solids in that region and their effect on benthic macroinvertebrates, particularly the sensitive Ephemeroptera taxa. Field and laboratory evaluations have indicated high sensitivity of mayflies to elevated dissolved solids. The research described herein is part of an ongoing effort to provide opportunity for toxicity testing with native mayflies in the laboratory. A successful endeavor would establish mayfly sensitivity to total dissolved solids and evaluate the relationship between the sensitivity of the native taxa and laboratory surrogates. The objective of this study was to investigate methods for rearing field collected, larval mayflies using three types of recirculating culturing systems. Five Ephemeroptera families were collected from streams in central West Virginia. Success of the rearing method was measured by emergence and mortality rates for each individual mayfly taxa. Mayflies are known to reproduce both sexually and asexually, and establishing a parthenogenetic population would allow for a more sustainable laboratory culture and facilitate the use of the taxa in toxicity testing. The ability of field collected mayflies to exhibit parthenogenesis was also examined with emergent adults from the rearing study. Flow in the culturing units was found to be a determining factor of emergence success. The most successful culturing unit based on mayfly emergence was the aquatic plant culturing system (Hexagon) which also generated the highest flow. Of the families evaluated, Baetidae was the only family to have exhibited parthenogenesis. Eggs from the genus Baetis had a hatch rate of 46 percent with incubation ranging from 11-29 days after egg collection. Eggs from the genus Pseudocloeon hatched between 17-24 days and had an average hatch rate of less than 1 percent. Ongoing research has focused on continued improvements in rearing and

emergence techniques for native taxa, better understanding of optimal incubation conditions for egg hatch, and increasing survival and longevity of newly hatched larval mayflies.

CHAPTER I

INTRODUCTION TO THE IMPACTS OF INCREASED SALINITY ON MAYFLIES

Global Salinity Issues & Its Effect on Benthic Macroinvertebrates

Salinity has greatly increased in global freshwater systems. Salinity is the total concentration of salts dissolved in water and can be expressed as total dissolved solids (TDS) or specific conductance (SC). The salinization of waters may be due to a number of anthropogenic activities including irrigation of agricultural areas (Williams 2001), mining discharges (Pond 2010), salting of roads (Kaushal et al. 2005), clearing of native vegetation (Black and Munn 2004), reverse osmosis effluent from water re-cycling plants (Kefford et al. 2012a) and industrial outfalls (Piscart et al. 2005). Elevated TDS levels can arise from the aforementioned human impacts but may also develop when any type of watershed disturbance takes place (Dow and Zampella 2000). Increased salinity concentrations can have great impacts on freshwater biota (Kefford et al. 2012a). Many studies have focused on the effects of elevated TDS on macroinvertebrates and have found that long-term salt contamination can alter community structure (Piscart et al. 2005; Cañedo-Argüelles et al. 2013; Kefford et al. 2012b). Particularly this is true for many of the most sensitive aquatic invertebrates represented in Ephemeroptera, Plecoptera, and Tricoptera (EPT) taxa (Kefford et al. 2011).

In general, benthic macroinvertebrate assemblages are good indicators of localized stream health. The dominating presence of tolerant taxa and absence of sensitive taxa can indicate poor water quality, habitat degradation, and overall community stress (Barbour et al. 1999). The order Ephemeroptera (mayflies) are known for being sensitive to changes in water quality and habitat due to disturbance (Bauernfeind and Moog 2000; Beketov 2004). Hassell et al. 2006 found less growth among larval mayflies (nymphs) that were exposed to higher salinities leading to the belief that more energy was invested in osmoregulatory functions rather

than growth. The study also states that inference in growth processes in mayfly populations appears to result in smaller and less fecund adults which could eventually lead to a reduction of individuals within the population.

TDS Toxicity to Macroinvertebrates in Appalachia

On a local scale, there has been considerable focus on elevated TDS levels occurring in Appalachia due to mining operations. Studies have shown that waters with elevated TDS levels exhibit stream SC from 100 $\mu S/cm^{-1}$ to 3,700 $\mu S/cm^{-1}$ mainly due to leached ions from mining practices (e.g., SO_4^{2-}, Mg^{2+}, Ca^{2+}, and HCO_3^-) resulting in mayfly populations to be reduced or extirpated from the region (Fritz et al. 2010; Pond 2010; Merricks et al. 2007). In 2011 the USEPA published *A Field-Based Aquatic Life Benchmark for Conductivity in Central Appalachian Streams* that suggests a SC benchmark of 300 $\mu S/cm^{-1}$ for the protection of aquatic organisms (USEPA 2011). This document used existing field data and linked elevated dissolved solids to mining operations in the Appalachian region to the extirpation of macroinvertebrates, particularly mayflies. The benchmark SC value the USEPA found to cause impairment is lower than those from other studies that range from 800-1000 $\mu S/cm^{-1}$(Hart et al. 1991; Horrigan et al. 2005).

The most common method to measure the salinity tolerance of an organism is an acute laboratory toxicity evaluation which derives an LC_{50} value, or the salinity concentration at which 50% of individuals die over a 48-96 h period (USEPA 2002). Toxicity testing helps to develop a causal link between salinity and mortality but it does not take into account additional changes to the natural environment that may occur with increased salinity (Kefford et al. 2004). King et al. (2011) found that the urbanization of watersheds can lead to an increase or decline of native macroinvertebrate taxa. The physical and chemical characteristics of a stream can change when a disturbance takes place. In addition to increased TDS, streams may experience sedimentation,

increased water temperature, changes in hydrology, nutrient enrichment and other alterations with disturbance in the watershed (Black and Munn 2004, Dow and Zampella 2000). Most streams affected by mining are headwater streams which are strongly connected to the surrounding terrestrial environment (Pond 2010; Hartman et al. 2005). This connection between the headwater streams and the terrestrial environment make those streams particularly sensitive to disturbance (Gomi et al. 2002; Benda et al. 2005). An increase in channel erosion rates in streams draining mined catchments (Fox 2009) could lead to sedimentation on the substrate where the mayflies live and feed. Deforestation alone can cause a loss in genetic diversity (Alexander et al. 2011), as well as, loss of riparian vegetation used for mating, sedimentation, changes in velocity/hydrology, and changes in habitat structure; both on a local and whole-watershed scale (Black and Munn 2004). Due to these changes in stream processes, functions, and morphology which can complicate field studies, laboratory toxicity testing with the potentially affected organism may shed light on the effects that increased TDS has on macroinvertebrates after a disturbance.

Generally, running a toxicity test with mayflies is difficult because they are not cultured within a laboratory. Studies have been conducted with field collected individuals (Hassell et al. 2006; Dunlop et al. 2008; Kefford et al. 2007a) however, they are largely of mixed sizes/ages and unknown general health. One study, utilizing field collected *Isonychia,* reported significant variability in replicate testing due to stress from field conditions (Echols et al. 2009). One parthenogenetic mayfly *Neocloeon triangulifer* (formerly *Centroptilum triangulifer*) in the Baetidae family has been used in toxicity evaluations due to its ease in culturing and its sensitivity to pollutants (Funk et al. 2006; Kim et al. 2012; Conley et al. 2009). The USEPA has furthered the use of *Neocloeon triangulifer* by developing a standard laboratory culturing and testing protocol (Struewing et al. 2014; Weaver et al. 2014). Although it is a lotic mayfly

species, it favors slow moving waters (Funk et al. 2006) which facilitates culturing but may not be representative of high-gradient Appalachian streams. One recent study which exposed *Neocloeon triangulifer* to elevated chloride levels derived an SC EC20 value for 672 μS cm^{-1} (Johnson et al. 2015). Significant mortality was not found in 1513 μS cm^{-1} and lower SC concentrations in the 20-day tests. Application of the findings of this recent study to effects in the mining region is complicated by two aspects of research design. The elevated ions in this study did not represent those indicative of the Appalachian mining region; and the control treatment in these experiments had conductivity levels in the range reported to result in mayfly extirpation in the field.

Rationale of Study Efforts

Previous field studies have indicated that increased dissolved solids in Appalachian streams has led to the loss of mayfly taxa due to mining operations in that region. Given the complex dynamics that can occur during a disturbance, it is hard to accept that the disappearance of mayflies in Appalachia is directly and solely a result of increased TDS in streams. Conducting toxicity testing using laboratory reared larval mayflies and salinities found in mining influenced streams would help to establish a direct relationship between TDS and mayfly sensitivity. This is important for both the protection and restoration of aquatic resources. In regards to toxicity testing surrogate organisms, the USEPA has standard freshwater test organisms that are commonly used in Whole Effluent Toxicity (WET) testing (e.g. *Ceriodaphnia dubia*, *Daphnia magna*, *Pimephales promelas*, etc). These organisms are relatively easy to culture, require little effort to use in tests, and do not show much variability between individuals in test results (USEPA 2002; Echols et al. 2009). Laboratory cultured organisms are better to use in toxicity evaluations because more confidence can be placed in their fitness as compared to field collected specimens. Although these organisms have been used extensively to establish NPDES permit

limits, they do not necessarily represent the more sensitive taxa like mayflies, especially regarding elevated TDS levels (Rosenberg and Resh 1996). There is a great deal of seasonal variability in collecting native mayfly larvae for use in toxicity evaluations indicated by macroinvertebrate mesocosm tests conducted in our laboratory. Other studies have also noted that the availability of field collected mayflies as test organisms is not persistent (Diamond et al. 1992). Echols et al. 2005 reported variability in mayfly (*Isonychia bicolor*) sensitivity when exposed to a NaCl solution and explained that the organisms that showed the most sensitivity had already been stressed at time of collection due to seasonal changes.

The objectives of this study focus on developing methods for rearing native, field collected mayflies in a laboratory culture. Whereas other toxicity evaluations have used *Neocloeon triangulifer* (Johnson et al. 2015; Struewing et al. 2014), an easily cultured lotic mayfly, this species may not be indicative of high-gradient Appalachian streams. Future endeavors plan to conduct toxicity evaluations on individuals from the culture, preferably newly hatched nymphs, using a reconstituted water with elevated TDS representative of mining influenced Appalachian streams.

Life History of Mayflies and Parthenogenesis

Mayflies are among the most primitive insects and have two adult winged instars after emergence from their larval stage. The first winged instar is called the *subimago* which is sexually immature in female mayflies. It is this instar, the subimago that is unique to mayflies. All other insects molt directly into a sexually mature winged instar or pupate before emerging into winged adults. The subimago stage can be identified by the dull gray or opaque coloring of the wings. The subimago stage can last for a few hours or a couple of days depending on the species. The final molt of mayflies is the *imago,* the sexually mature adult identified by transparent wings. The imago stage is strictly for reproduction and dispersal purposes. Adult

mayflies do not feed, only having vestigial mouthparts (Peckarsky et al. 1990). Mayflies are hemimetabolous and are well-known for having short-lived adult life stages which usually last from a couple of hours to a few days. Mayflies mate in swarms dominated by males. Females fly through the swarm and are grasped by males, copulation takes place amid flight, and the females oviposit their eggs in the water soon after mating occurs. Some mayflies are also known to reproduce parthogenetically. Eggs tend to be sticky and may have specialized anchoring devices to attach to the stream substrate. Mayfly nymphs may molt from 12 to 45 times depending on the species and the temperature of the water and generally overwinter as nymphs (Peckarsky et al. 1990).

In the aquatic larval phase, mayflies are primarily grazers and collector-gatherers feeding on periphyton and detritus occurring on substrate in streams. Some nymphs like Isonychiidae are filter feeders that use small hair-like projections on their forelegs to trap detritus and algae drifting through the water column. Many mayflies naturally exist in fast-moving, cold water to provide a high dissolved oxygen environment for the gills of the nymph to utilize. Some mayflies, such as *Hexagenia* (the burrowing mayfly) or *Siphlonurus*, have specifically adapted gills to allow them to live in low-oxygen conditions (Peckarsky et al. 1990).

One of the most difficult aspects in the development of culturing methods to facilitate a stable mayfly population in the laboratory is the housing in which the mayflies are kept. Several factors must be met to sustain a healthy population. The culturing units in which the mayfly nymphs are placed must mimic their natural habitat in terms of flow, temperature, light intensity, and a sufficient food source.

Ideally, a laboratory reared mayfly population would be made up of parthenogenetic taxa which would eliminate the need to facilitate copulation between male and female to obtain viable offspring. The first reference of parthenogenesis in a mayfly taxon was by Morgan (1911) for

Ameletus ludens Needham. In his study, he consistently noticed that male imagos were missing from field collections. Parthenogenesis was then demonstrated in *A. ludens* by Clemens (1922). Two Heptageniidae species were found to be parthenogenetic by McCafferty and Huff (1974) and Mingo (1978). *Cloeon triangulifer* (presently known as *Neocloeon triangulifer*) was found to be obligatory parthenogenetic (all female population conceived from maternal germ cells) by Gibbs in 1977. 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, Pennsylvania) to exhibit parthenogenesis in one of its various forms (obligatory or facultative). In the absence of parthenogenesis, Funk et al. 2010 manually induced mating between male and female mayflies by aligning their genitalia for seven Baetidae species from seven separate bisexual populations collected from the same stream.

Objectives

The main goal of this study is to develop methods for rearing field collected native mayflies to provide consistently available healthy organisms for use in toxicity evaluations. The specific objectives for this study are to:

- Investigate rearing chamber success for individual mayfly taxa,
- Investigate reproduction among mayfly taxa and the ability to exhibit parthenogenesis,
- Investigate dietary needs and preferences of mayfly taxa, and
- To rear newly hatched nymphs for use in toxicity testing.

Rearing chamber success was evaluated by the rate of emergence of the individual mayflies that were placed within the chambers. The rearing chamber was believed to be successful if at least 50% of those individual juveniles placed in the chamber were reared to adult

emergence. The rearing chamber that exhibited the most success will be used in the future to culture individual larval mayfly taxa.

Success of reproduction was evaluated by hatch rate of mayfly eggs collected from laboratory reared specimens that underwent experiments with both sexual and asexual reproduction via parthenogenesis. 120 test specimens are needed to run a standard toxicity test. Hatch rates are dissimilar for individual mayfly taxa and are dependent of the fecundity of the female at the time of egg collection. If the hatch rate was high enough and yielded enough eggs for a toxicity test, then those taxa were considered to have successful reproductive output and could further be used in culturing efforts.

The diet of mayflies was investigated by growing diatoms in the laboratory and facilitating their colonization on the substrate in the rearing chambers. Success was evaluated by observing how well the diatoms grew in the culture media provided and with the culturing methods made available in the laboratory. Evaluation of success was also subjective and measured by observing the feeding habits of the larval mayflies within the culturing chambers. By observing the feeding habits, we were able to distinguish which diatoms were preferred by the mayflies.

Success of rearing newly hatched nymphs was measured by the length of time between hatch and death. A standard chronic toxicity test can be conducted up to 10 days. If newly hatched nymphs were reared from the time of hatch to 10 days, the rearing was considered to be successful. Future long-term laboratory culturing would need to rear the nymphs from hatch to adulthood to provide a healthy and sustainable culture.

CHAPTER II

DEVELOPMENT OF MAYFLY LABORATORY REARING METHODS

Background

Salinity, indicated by total dissolved solids (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 the resource increases (Cañedo-Argüelles et al. 2013). The potential impacts of salinization on freshwater biological communities has only recently been realized despite the fact that it is widespread and results from numerous human impacts (Cañedo-Argüelles et al. 2014; Kefford et al. 2011; Kefford et al. 2012a). This may, in part, be due to the fact that increased salinity occurs with other types of disturbance, and is often used to indicate disturbance (Dow and Zampella 2000), making separation of the effects difficult. Several researchers have investigated lethal effects of salinity concentrations (Dunlop et al. 2008), but sub-lethal effects, on individuals and communities, are not well understood (Cañedo-Argüelles et al. 2014). This is true regarding aquatic communities in general (Cañedo-Argüelles et al. 2014) and for the sensitive Ephemeroptera, Plecoptera, and Trichoptera (EPT) orders specifically (Kefford et al. 2011).

It is well-known that mayflies (Order Ephemeroptera) are among the most sensitive macroinvertebrate taxa to many types of disturbance and water quality impairment (Baurenfeind and Moog 2000; Beketov 2004) including increased salinity (Dunlop et al. 2008; Kefford et al. 2011; Kefford et al. 2012a). Levels of dissolved solids resulting in mayfly impairment have been demonstrated in both laboratory (Hassell et al. 2006; Johnson et al. 2015; Kefford et al. 2003; Horrigan et al. 2011) and field studies (Pond 2010; Kefford et al. 2005; Kefford et al. 2012a; Schäfer et al. 2011) with general agreement between the field and laboratory toxicity levels (Kefford et al. 2004; Horrigan et al. 2007). Additionally, comparisons have been made

between salinity sensitivity in invertebrates from disparate geographical regions (Dunlop et al. 2008; Kefford et al. 2012a).

In the Appalachian region of the eastern United States, field studies have shown that mayflies have been reduced or extirpated from some streams with the loss attributed to elevated dissolved solids, primarily resulting from the mining industry (Fritz et al. 2010; Pond 2010; Merricks et al. 2007). Mining practices in this region have been under considerable scrutiny recently, with specific attention focused on TDS that runoff into surrounding streams. Some studies have shown increased stream SC from 100 $\mu S/cm^{-1}$ to 3,700 $\mu S/cm^{-1}$ 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. 2007). In 2011 the United States Environmental Protection Agency (USEPA) used existing conductivity and field data to devise a SC benchmark linking extirpation of macroinvertebrates in Appalachian streams to elevated TDS due to mining operations. The USEPA document "*A Field-Based Aquatic Life Benchmark for Conductivity in Central Appalachian Streams"* (2011) suggests a conductivity benchmark of 300 $\mu S/cm^{-1}$ for the protection of aquatic organisms with mayflies being specifically identified as among the most sensitive taxa to TDS (USEPA 2011). This threshold, corresponding to extirpation in the Appalachian watersheds, is lower than that of those identified as resulting in impairment in other studies (Hart et al. 1992; Horrigan et al. 2005) and lower than the control or reference condition in salinity used in some studies (Cañedo-Argüelles et al. 2014; Johnson et al. 2015).

Data, recorded as TDS levels, linking macroinvertebrate community impairment to salinity reported in the Appalachian region are sparse. Generally mayfly testing is difficult because they are not easily cultured in the laboratory. Studies conducted with field collected individuals are generally of mixed sizes/ages and unknown overall health. One study, utilizing field collected *Isonychia,* reported significant variability in replicate testing due to stress from

field conditions (Echols et al. 2009). Previous work in our laboratory has identified the variability in field collected macroinvertebrate communities as a substantial impediment to mesocosm studies. Other studies have identified lack of consistent organism availability as an impediment to mayfly toxicity testing (Diamond et al. 1992). One parthenogenetic mayfly *Neocloeon triangulifer* (formerly *Centroptilum triangulifer*) has recently been used in toxicity evaluations (Funk et al. 2006; Kim et al. 2012; Conley et al. 2009). Laboratory culturing and testing protocols have been developed for this mayfly (Struewing et al. 2014; Weaver et al. 2014). Although it is a lotic mayfly species, it favors slow moving waters (Funk et al. 2006) which facilitates culturing but may not be representative of organisms inhabiting high-gradient Appalachian streams.

Parthenogenesis was first observed by Morgan (1911) for *Ameletus ludens* Needham due to male imagos consistently missing from field collections. Later, parthenogenesis in *A. ludens* was demonstrated by Clemens (1922). McCafferty and Huff (1974) and Mingo (1978) were able to demonstrate parthenogenesis in two Heptageniidae species. Even more recently, Gibbs (1977) found evidence that *Cloeon triangulifer* (presently known as *Neocloeon triangulifer*) in the Baetidae family is obligatory parthenogenetic. In the absence of parthenogenesis, the method of facilitated copulation has been reported (Funk et al. 2010) which would also provide juveniles for testing.

Given the increasing salinity and need for additional research on sub-lethal effects, methodology is required for conducting chronic toxicity tests with mayfly taxa. Due to variability in salinity sensitivity between species and within species separated geographically, the development of methods allowing for the collection and rearing of different mayfly taxa is critical. Additionally, with younger organisms generally considered to be the most sensitive to salinity (Kefford et al. 2007b), the collection of newly hatched larvae is favored for providing the

most sensitive life stage for use in toxicity testing. The ability to rear native taxa for use in toxicity testing will allow for:

- Comparison of the sensitivity of multiple taxa to standard EPA test organisms,
- Provide opportunity to evaluate the sensitivity of mayfly taxa to multiple contaminants,
- Provide opportunity to compare the sensitivity of multiple taxa from disjunct geographical location,
- Provide for the development of a database of mayfly taxa sensitivity to standard reference toxicants so that general health of test organisms can be established and comparisons can be made between studies.

The specific goal of the research described herein was to develop culturing/rearing methods for native mayfly taxa to provide consistent test organisms for use in toxicity testing. As some mayflies are known to be parthenogenetic, these would be good candidates for rearing in the laboratory. Specific objectives of this study included determining the optimal rearing method for field collected larval mayflies to facilitate emergence, and investigation of the potential to obtain viable eggs through parthenogenesis or mating of the emergent adults.

Materials & Methods

Rearing Studies.

This study was conducted at facilities on the campus of Marshall University in Huntington, West Virginia. A variety of larval mayfly taxa were collected from relatively undisturbed streams in central West Virginia, and returned to the lab for the purpose of rearing to emergence using three different culturing techniques. The three rearing alternatives included stream mesocosms, a recirculating fish culturing system and a hexagonal aquatic plant culturing system as described below and shown in Figure 2.1.

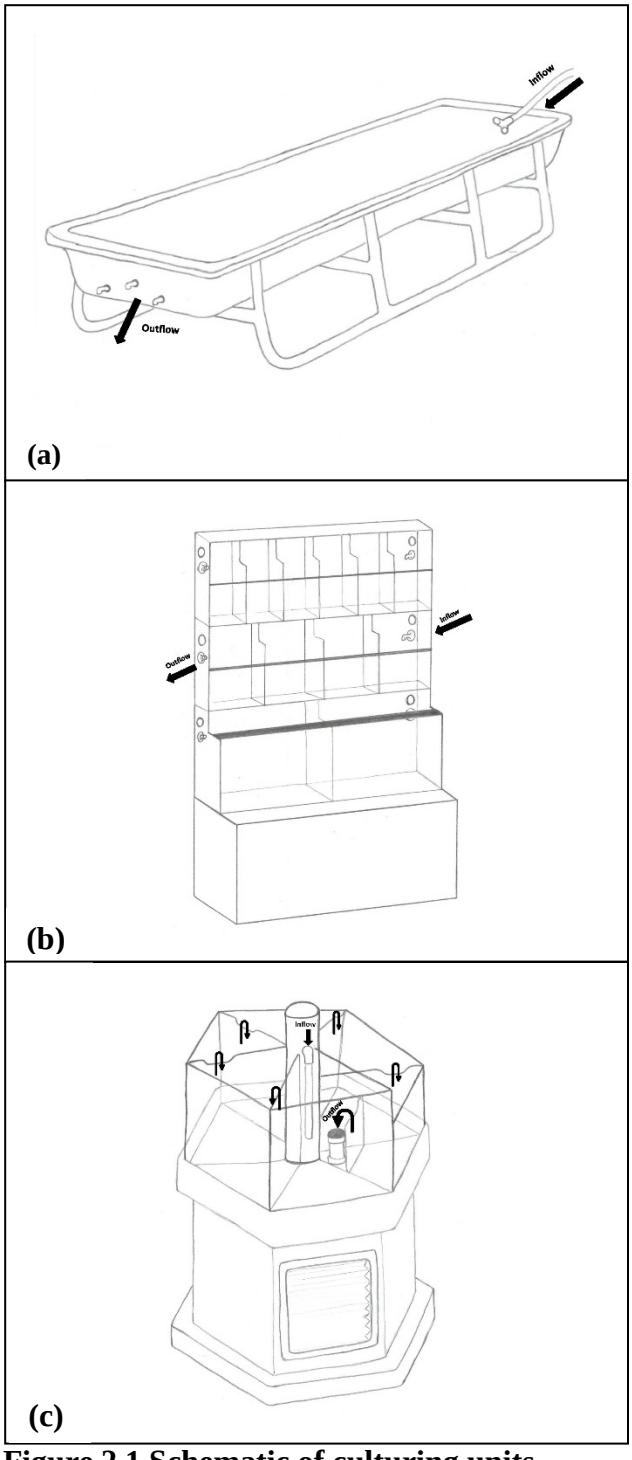

Figure 2.1 Schematic of culturing units
(a) Recirculating mesocosm stream culturing unit
(b) Re-circulating fish tank culturing unit (Only the second level was used)
(c) Hexagonal aquatic plant culturing system

Water chemistry from the three culturing scenarios is summarized in Table 2.1 and physical conditions in each unit are summarized in Table 2.2.

Table 2.1 Water quality for culturing units

Unit	Water Temperature (^{0}C)	pH (S.U.)	Dissolved Oxygen (mg/L)	Specific Conductance (µS/cm)
Stream #1	20.2 – 28.0	7.3 – 8.9	6.8 – 9.4	252.4 – 487.3
Stream #2	20.3 – 27.4	8.0 – 8.7	7.4 – 9.4	407.7 – 485.2
Hexagon	19.5 – 26.8	7.8 – 8.3	7.2 – 9.9	252.1 – 469.2
Fish Tank	20.3 – 30.4	8.0 – 8.7	6.2 – 10.0	402.7 – 486.4
Egg Incubation	21.2 – 24.0	8.1 – 8.2	8.7 – 8.9	320 - 331

Table 2.2 Physical conditions of culturing units

Unit	Flow (L/sec)	Depth (m)	Light Intensity (lux)
Stream #1	0.381	0.024 – 0.092	12
Stream #2	0.126	0.010 – 0.083	134
Hexagon	0.508	0.205; 0.245; 0.294	93
Fish Tank	0.025	0.205	92

Field collected individuals were randomly placed into one of the three culturing systems for evaluation of emergence success (Figure 2.1).

Culturing units were checked daily for emergence; winged mayflies could be found clinging to the shade cloth coverings. Upon collecting emerged adults, taxonomic identification was verified and they were utilized in subsequent reproductive experiments. Initial results of the ongoing study were used to adapt methodology in the ongoing research.

Stream Mesocosms.

Two artificial stream mesocosms were constructed from 8 ft horse troughs (Stream #1 & Stream #2) and fitted with a re-circulating pumping system providing flow (Figure 2.1a). The water used was dechlorinated Huntington, WV city tap water (Table 2.1). Streams were elevated

on one end to provide gradient and filled with natural gravel/cobble substrate. Water temperature was maintained at 20°C - 28°C by a dual chiller/heater unit operating on the 568 L tank connected to the streams. Flow rate in the streams ranged from 0.126 to 0.381 L/sec. Streams were exposed to natural light which was regulated with shade cloth which maintained light in the range of 12 to 134 lux and trapped emergent adults.

Fish Culture System.

A recirculating fish culture system (Fish Tank) was utilized in rearing evaluations (Figure 2.1b). This apparatus was essentially an upright series of chambers with multiple chambers on each of 3 levels. The second level of the tank series was used in these evaluations. The first and third levels were not used in the mayfly evaluations to maximize flow in the second level. Tank size on the second level was 1.268 x 0.292 x 0.215 m. Tanks on the same level were connected by perforated plexiglass and water circulated throughout the level. As with the mesocosms, the water used was dechlorinated Huntington, WV city tap water (Table 2.1) and temperature was maintained via connection to the Frigid Units, Inc.® dual chiller/heater unit housed in a 568-liter tank. The apparatus was exposed to natural light which was regulated with shade cloth to maintain light intensity in the range of 92 to 134 lux and trap emergent adults. Depth of the tanks housing mayflies was 0.212 m and water flow was 0.025 L/sec.

Plant Culturing System.

The final rearing method used was a hexagonal aquatic plant culturing system (Hexagon) which features a series of waterfalls and pools at different depths simulating a stream environment(Figure 2.1c). This unit is self-contained with a pump and holding tank. It was housed in the laboratory and a small chiller was used to keep the water at a near constant temperature. The water used was moderately hard reconstituted water (EPA Water) (USEPA 2002). Mosquito netting covered the Hexagon to trap emergent adult mayflies; light intensity

was 93 lux and was provided by fluorescent bulbs (16hr/8hr light/dark cycle). In early evaluations, mayflies were introduced into three different levels of the Hexagon to evaluate whether water depth had an effect on successful mayfly emergence. Optimal depth was utilized subsequently. Nitex© barriers placed between the levels of the Hexagon collected mayflies not successfully emerging.

Diatom Culturing.

Prior to the field collection of mayflies, gravel/cobble substrate in each of the culturing units was inoculated with *Navicula* sp. to provide a food source for introduced mayflies. Inoculation media was prepared by adding 20 mL of *Navicula* sp. starter culture in Alga-Gro® Freshwater (Source: Carolina Biological Supply Co., Burlington, NC), 13 mg sodium metasilicate, and 0.13 mL each of both Proline® F/2 Algae Food Part A and Proline® F/2 Algae Food Part B to 1 L of autoclaved EPA water. The solutions were aerated and allowed to grow for two weeks at 25^0C under a constant fluorescent light source of 805 lux. Both of the culture units, the 568-L tank supplying water to the stream mesocosm/fish tank system and the Hexagon, were each inoculated with 1 L of the suspended diatom culture.

Mayfly Collections.

Mayfly nymphs were collected from Ash Branch, a 2nd order tributary of Paint Creek in Kanawha County, WV; Shrewsberry Hollow, a 1st order tributary of Davis Creek in Kanawha County, WV; and Bells Creek, a 2nd to 3rd order tributary of Twentymile Creek in Fayette County, WV (Figure 2.2).

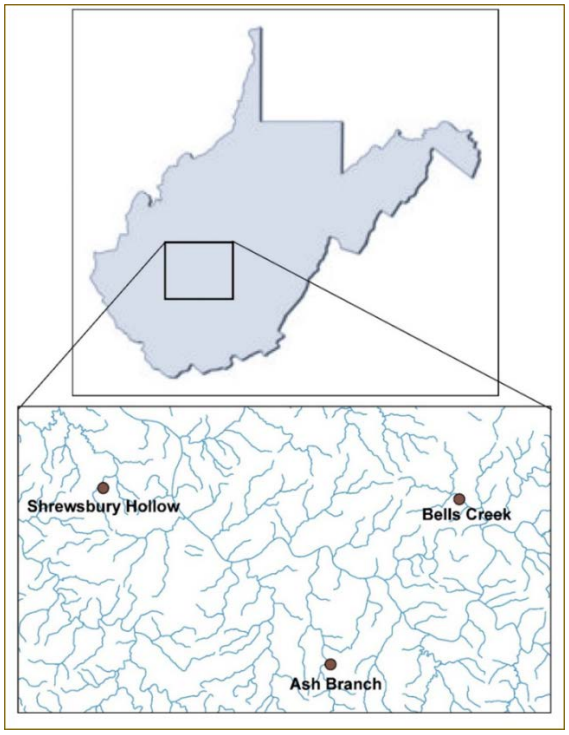

Figure 2.2 Mayfly collection sites in central West Virginia

The dominant land use for all three watersheds is forested (73.6% to >94.6%). mining (2.8% to 23.6%) and urban/residential (1.7% to 2.2%) comprise the remaining land uses (WVDEP 1997; WVDEP 2005; WVDEP 2008). The collection dates, number of individuals per family collected, and the units in which they were placed are summarized in Table 2.3.

Table 2.3 Larval mayfly collection information detailing collection site & date, number of individuals among families collected, and placement of mayflies in culturing units

Collection Date & Site	# of Individuals & Families Collected	Culture Unit Placement
June 15 Ash Branch	(23) Ephemerellidae; (7) Heptageniidae	Hexagon Level 1
June 17 Shrewsberry Branch	(4) Ephemerellidae; (37) Heptageniidae	Hexagon Level 2
June 25 Shrewsberry Branch	(6) Ephemerellidae; (44) Heptageniidae	Stream #1
	(48) Heptageniidae	Stream #2
July 11 Shrewsberry Branch	(1) Ephemerellidae; (18) Heptageniidae	Hexagon Level 3
	(18)Heptageniidae	Fish Tank
September 9 Bells Creek	(21) Baetidae; (44) Heptageniidae; (60) Isonychiidae	Stream #1
	(23) Baetidae; (2) Heptageniidae; (63) Isonychiidae	Hexagon Level 1
September 25 Bells Creek	(101) Heptageniidae; (44) Isonychiidae	Stream #1
	(46) Baetidae; (2) Baetiscidae	Hexagon Level 1
October 13 Bells Creek	(22) Baetidae; (148) Heptageniidae; (27) Isonychiidae	Stream #1
	(18) Baetidae; (3) Baetiscidae	Hexagon Level 1
October 20 Bells Creek	(3) Baetidae; (11) Baetiscidae; (112) Isonychiidae	Stream #1

The mayflies were collected with a rectangular kicknet (0.205m x 0.155m) and standard D-frame dipnets (0.330m x 0.330m) from the cobble substrate in riffle and run portions of the streams. The mayflies were placed in aerated, plastic screwtop containers with stream water and brought to the laboratory in coolers. At the time of collection, mayfly nymphs were identified to family level. The adult mayflies were then identified to genus level if reproductive success was achieved and the necessary lifestages were available. Mayflies were identified using *Aquatic Insects of North America* (Merritt et al. 2008). In order to identify the mayflies to species, multiple individuals from various life stages are needed which can be very difficult to obtain (Bauerenfeind and Moog 2000). For this reason, the taxonomy of the mayflies has been kept general and focus has been put on overall reproductive success. Representative individuals from multiple lifestages are retained during the course of the study. When a higher level of success is found in the methodology of this study, and representatives from multiple lifestages are obtained, individuals will be further identified to the lowest practical taxon.

Reproductive experiments.

Parthenogenesis and Collection of Eggs.

To investigate the possibility of parthenogenesis, females that were not used to attempt artificial mating were isolated from the time they were found in their subimago stage to their final molt at the imago stage. Upon transformation to imago, the females were placed in a watch glass (diameter: 0.05m) containing approximately 10 mL EPA water to allow female to oviposit. Females were offered partially submerged sticks which they could crawl on to get out of the water. If a female died without voluntarily depositing eggs, the eggs were taken by dissecting the abdomen. Eggs were maintained at 22^0C under ambient light conditions in the incubator which was fluorescent lights at 1180 lux on a 16/8-hr. light/dark cycle. Eggs were placed on a

shaker table at 45 rpm to provide aeration. The egg clutches were checked daily for hatch, fungus/bacteria, and dissipation.

When hatch occurred, the nymphs were placed in 100 mL beakers with EPA water and fed a *Navicula* sp. diatom mixture (both suspended in the water column and on mesh nylon squares).

Mating and collection of eggs.

Subimagos were placed in plastic boxes for the second emergence to final imago instar. Copulation was manually facilitated by holding the abdomens of a male and female together as reported by Funk et al. 2010. This manual copulation was attempted separately with the Ephemerellidae, Heptageniidae, and Baetidae species. Artificial fertilization was also attempted for Ephemerellidae and Baetidae by extracting the contents of the male's abdomen over eggs.

When hatch occurred, the nymphs were placed in 100 mL beakers with EPA water and fed a *Navicula* sp. diatom mixture (both suspended in the water column and on mesh nylon squares). The investigation of rearing techniques will be the subject of subsequent publications.

Results & Discussion

Flow rate was immediately found to be a crucial variable so much that the use of Stream #2 and the Fish Tank were discontinued prior to the collection of Baetidae and Isonychiidae due to low flow. Use of Stream #2 was discontinued to direct the entire flow of the mesocosm system to Stream #1, thereby increasing the flow. Flow in the Fish Tank apparatus was maximized, but still not sufficient to support the mayflies. This culture apparatus may be revisited for rearing of lentic taxa, but did not support survival of the lotic species as evidenced by the poor emergence numbers shown in Figure 2.3; zero emergence for Ephemerellidae and 5% for Heptageniidae.

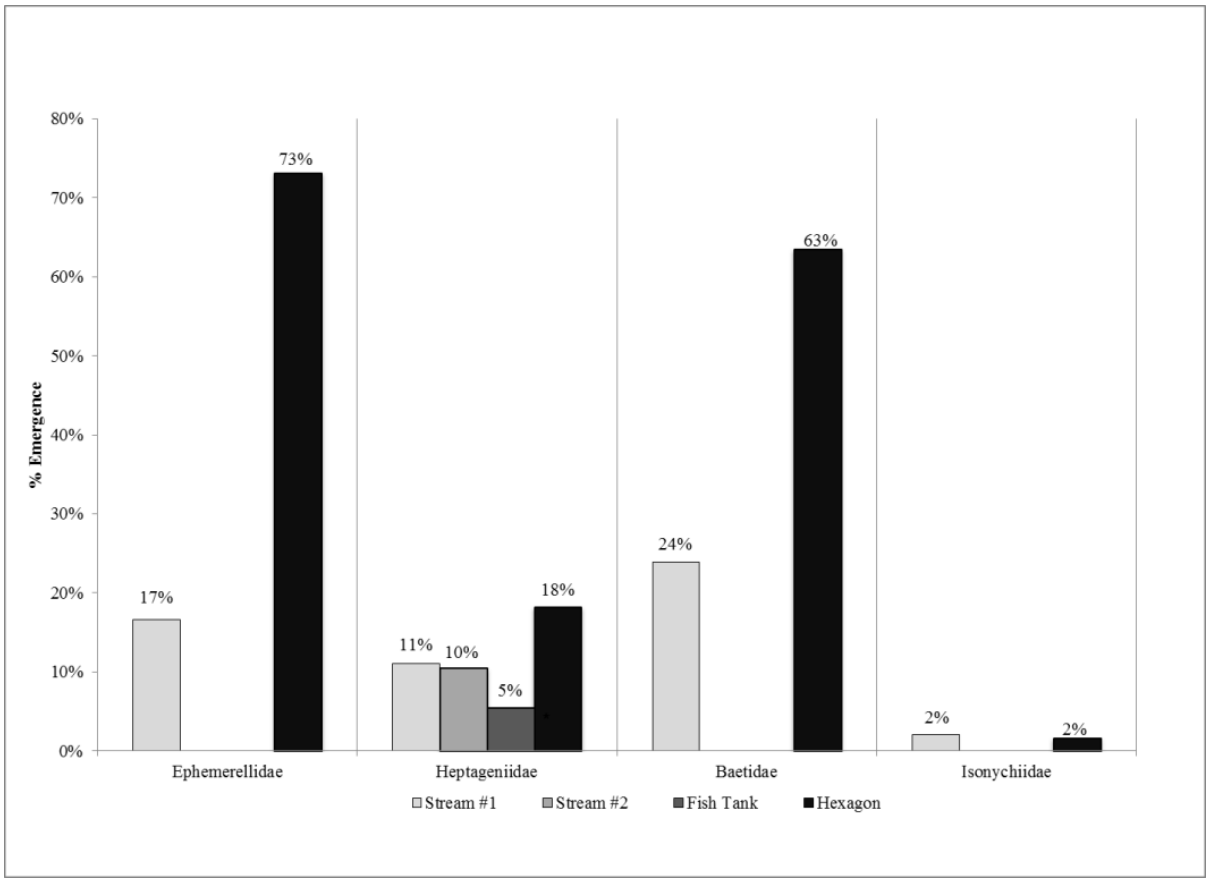

Figure 2.3 Emergence of mayfly families collected from June to December 2014

Emergence rates were greatest in the Hexagon for three of the four families tested: Baetidae, Ephemerellidae and Heptageniidae. This may be due to the unit having the highest flow rates of the three tested. Ephemerellidae had the highest emergence rates in the Hexagon with 73% emergence. Baetidae also emerged successfully in this unit with 63% emergence (Figure 2.3). Time to emergence was variable ranging from 3 to 24 days for Baetidae, 1 to 10 days for Ephemerellidae, and 5 to 16 days for Heptageniidae, with time to emergence likely a function of individuals maturity at the time of collection. Ephemerellidae collected in mid-June had the fastest and most successful emergence as would be expected when the collection date coincided with natural emergence period. Water temperature plays a crucial role in the development and emergence of mayflies (Merritt et al. 2008; Sweeney and Vannote 1978). Baetidae individuals, which also demonstrated a very high emergence rate, were primarily collected in fall which may also have coincided with an autumn emergence for those particular

taxa. There was not much success seen with the emergence of Isonychiidae, nor were any eggs obtained.

The two genera of Baetidae collected were *Pseudocloeon* sp. and *Baetis* sp. Emergence of Heptageniidae was not as successful as Ephemellidae and Baetidae in the Hexagon (Figure 2.3). Many Heptageniidae nymphs were found half emerged on the Nitex© barrier between the chambers with their exuvia still attached. Heptageniidae emerge from the water surface, as opposed to crawling out of the current. These mayflies may have trouble if the distance to the end of the tank is too short and the water current is too fast for them to shed their exuviae before becoming trapped between the chambers. Additional studies in the longer stream mesocosm are underway to better support the rearing of Heptageniidae in the laboratory.

Although easily collected, Isonychidae were not successfully reared in either the streams or the Hexagon. Isonychiidae had only2% emergence rate in the Hexagon and Stream #1(Figure 2.3). The inability to rear Isonychia is troublesome as this an easily collected organism and has been used in several toxicity studies. Additional investigations on the life history and emergence strategy of this organism are needed.

In general, the Hexagon provided a better rearing environment for Ephemerellidae and Baetidae than the other culture units. Heptagenidae appeared to live successfully in this unit, but was not successful in emerging due to its emergence strategy. The stream mesocosms exhibited limited success with Stream #1 demonstrating greater success than Stream #2, and also having a greater flow rate (Table 2.2). The Fish Tank demonstrated the least success in rearing the mayfly taxa and was discontinued from further testing with these lotic species. It may be suitable for rearing lentic species and may be tested thusly in future experiments.

The effects of water depth on emergence success were evaluated with the Heptageniidae and Ephemerellidae in the Hexagon. Mortality was recorded as the number of dead nymphs

found on the Nitex© barriers between levels. Level 3, with the greatest depth, had the highest mortality among both the Ephemerellidae and Heptageniidae (Figure 2.4).

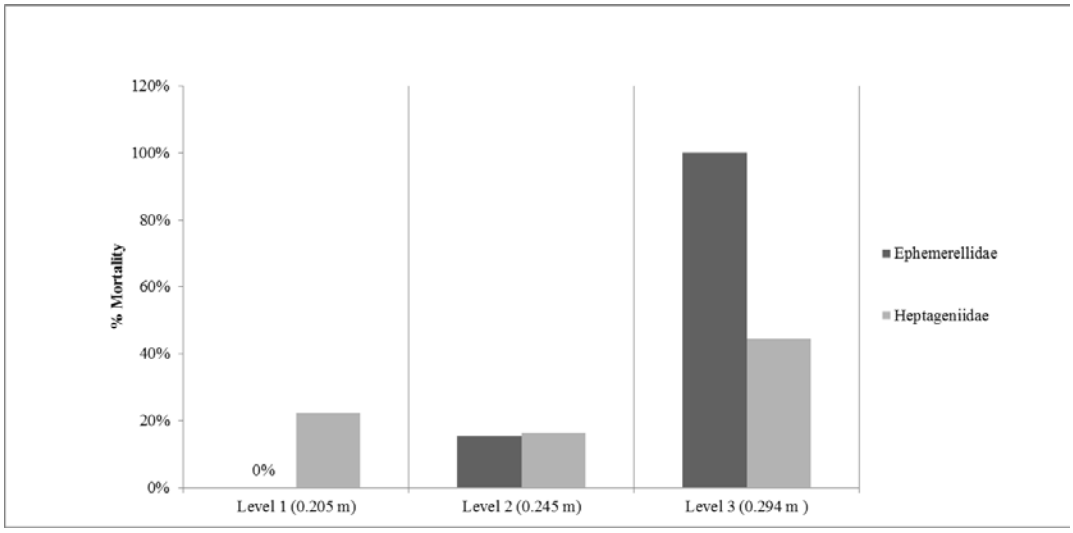

Figure 2.4 Hexagon depth comparisons for Ephemerellidae and Heptageniidae

Difference in sex ratios, specifically a higher number of females in the population, may indicate a parthenogenetic population (Funk et al. 2006; McCafferty and Huff 1974). No differences were seen in sex ratios for the taxa collected (Figure 2.5) indicating that the mayflies were likely not dependent on parthenogenetic reproductive strategies in the populations collected.

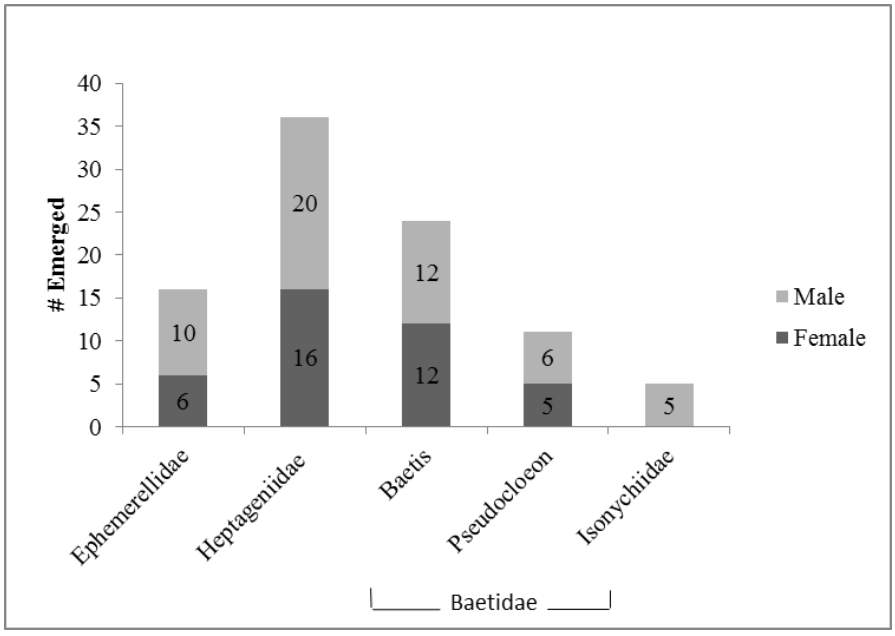

Figure 2.5 Emerged mayfly sex ratios

The ability to reproduce parthogenetically by *Pseudocloeon* sp. and *Baetis* sp. was demonstrated in our study by our successfully hatching offspring from females which had not mated. This supports that parthenogenesis is widespread within the family Baetidae reported by Funk et al. 2010. However, parthenogenesis was not likely obligate in either taxa as indicated by the sex ratios. In some mayfly species, hatching can occur anywhere from three to nine months, however, the period for Baetidae eclosion can be as short as 14 days (Merritt et al. 2008). Eggs from the genus *Baetis* had a hatch rate of 46% with incubation ranging from 11-29 days after egg collection. Eggs from the genus *Pseudocloeon* incubated between 17 and 24 days and had an average hatch rate of less than 1%. The eggs collected from all Heptageniidae and Ephemerellidae specimens did not have eclosion.

Further Research

This initial research has resulted in modifications to the rearing strategies for individual mayfly taxa. Research is ongoing with the following focus areas: continued improvements in rearing and emergence techniques for native taxa, better understanding of optimal incubation conditions for egg hatch, and increasing survival and longevity of newly hatched larval mayflies. Currently, nymphs that have hatched are not surviving to maturity. Our long-term goals are to successfully rear multiple native taxa for use in toxicity testing to determine the sensitivities of these native taxa to standard reference toxicants and TDS indicative of the regions mining influenced streams.

CHAPTER III

CONCLUSIONS

Summary

Overall, emergence success in any of the three culturing methods (stream mesocosms, fish culture system, and plant culturing system) was limited at the conclusion of this study. Flow was found to be a major component of how well larval mayflies adapted to the culturing units. The flow in the Fish Tank and Stream #2 was found to be too low to sustain a larval mayfly culture as indicated by the emergence success rates in each of the units. The Fish Tank was disconnected before collections at Bells Creek for Baetidae, Baetiscidae, and Isonychiidae; and it was not used again during this study. Stream # 1, Stream #2, and the Fish Tank were all housed in the greenhouse being fed by the same 568- L tank. The water that drained into the culturing units was gravity driven; it was not being pumped. Therefore to maximize flow to one stream, Stream #2 was disconnected.

Emergence success in Stream #1 and the Hexagon was variable and greatly dependent on the different taxa that were housed there. Emergence was greatest in the Hexagon for Baetidae, Ephemerellidae, and Heptageniidae. It is thought that the high flow rate in the Hexagon may be partly responsible for sustaining the mayflies till emergence. Ephemerellidae and Baetidae had the highest emergence from the Hexagon. Although these results showed the most success among the whole study, the collection and emergence of Ephemerellidae and Baetidae may have just coincided with their natural emergence times in native streams. Heptageniidae seemed to live in the Hexagon as nymphs but they were caught in the current when they came to the surface of the water to emerge. It is thought that the stream mesocosms may be a more suitable rearing method for Heptageniidae. Isonychiidae did not have much emergence from the Hexagon nor from the stream mesocosms. The inability to rear Isonychiidae is disconcerting because they are

an easily collected and identifiable organism and have already been used in toxicity studies. It was thought that if laboratory Isonychiidae were used in more toxicity evaluations, it would further establish their sensitivity to standard toxicants.

In regards to water depth, mortality among Heptageniidae and Ephemerellidae indicate depths between 0.205 m and 0.245 m to be optimal.

The reproduction studies concerning parthenogenesis and egg collection showed some degree of success. The ability to reproduce parthogenetically was demonstrated for two genera in family Baetidae. Two *Pseudocloeon* sp. individuals had an average hatch rate of less than one percent. *Baetis* sp. had a hatch rate of 46%. Reproduction studies simulating copulation by aligning male and female abdomens did not yield any egg hatch nor did those eggs that were artificially fertilized. Eggs collected from Heptageniidae and Ephemerellidae specimens never hatched. Although successive generations were accomplished for Baetis sp. and Pseudocloeon sp., the newly hatched nymphs were only kept alive for a maximum of four days.

Recommendations

In general, Baetidae genera show the most promise. Baetidae individuals showed emergence success in the Hexagon as well as in Stream #1. A factor that has not been mentioned thus far is the ability of a subimago individual to shed its exuvia to the imago lifestage. Baetidae individuals showed more success in this than Heptageniidae individuals did. Many times Heptageniidae would have their exuvia stuck on the tip of their wings which could affect their ability to fly and oviposit.

Among Baetidae genera collected, *Baetis* sp. demonstrated the most potential as a laboratory culture organism. These individuals emerged quickly, they are small, they have short lifespans, and their parthenogenetic eggs hatch within 11 days with a high hatch percentage. Females were also found to more readily oviposit their eggs whereas *Pseudocloeon* sp.

individuals were not. It was also found that the *Baetis* sp. female oviposited eggs and had a high hatch rate while *Pseudocloeon* sp. females were dissected to obtain eggs and they had a low hatch percentage. This suggests that females that readily oviposit their eggs have a greater likelihood of hatching and having a greater hatch percentage. In this study females were placed directly on the surface of water to oviposit but as a recommendation, females should be given the option to oviposit from the air or on the surface of the water. A study by Funk et al. 2010 used museum jars to allow females to oviposit their eggs above the water. As a part of this overall study, 118 mL (4 oz) larval fish jars were recently used to replicate the aforementioned study. Small Nitex© bridges were created for the mayfly to rest and oviposit eggs above the water (Figure 3.1).

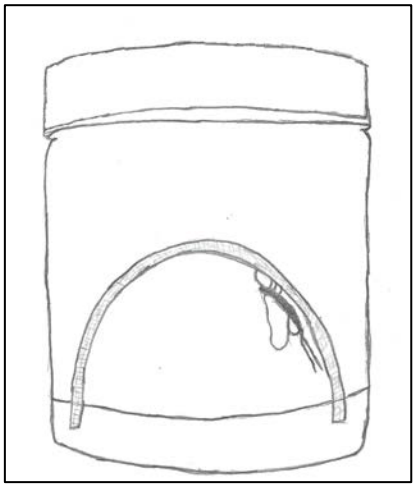

Figure 3.1 Oviposition jar. Larval fish jar with Nitex© bridge to allow female to deposit eggs

The Hexagon worked very well for Baetidae taxa. Baetidae nymphs were seen grazing on the diatoms growing on the rocks weeks after their collection. Heptageniidae may show more potential to emerge from the stream mesocosms if flow is increased. The low flow conditions of the Fish Tank may be suitable for a lentic or large river mayfly taxa. The nature of this study did not utilize any lentic taxa due to the focus on TDS impacts to headwaters streams and not larger rivers.

Many of the collection streams showed specific conductance in the 200-250 $\mu S/cm^{-1}$ range so units should be maintained at such to mimic reference streams in the central Appalachian region. Water temperatures should also represent natural conditions. Many mayflies are found in headwater streams that have very low temperatures. Increasing the

temperature in the culturing units may cause the mayflies to emerge faster than they would in their natural habitat (Merritt et al. 2008; Sweeney and Vannote 1978).

Research on the diet of mayflies is still necessary. Recently another diatom, other than *Navicula* sp., was fed to mayfly nymphs. *Achnanthidium minutissimum* was grown in Erlenmeyer flasks in an incubator and on ceramic tiles inside a biological safety cabinet and introduced to mayfly nymphs. Mayfly nymphs seemed to prefer *A. minutissimum* over *Navicula* sp. Efforts should also be made to further identify the dietary needs of newly hatched nymphs. Attempts to feed the nymphs solely diatoms have been rendered unsuccessful. It is thought that the nymphs may prefer bacteria or detritus which they would normally encounter in a natural environment.

By and large, the two factors that seem to inhibit the success of this study are flow and dietary requirements. More research needs to be put forth in these two areas as well as those previously mentioned to sustain a laboratory mayfly culture. The requirements of all mayfly taxa are different; finding a suitable test organism forces all of those needs to be met.

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APPENDIX A

MARSHALL UNIVERSITY
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Office of Research Integrity
Institutional Review Board

April 23, 2015

Kevin Rowsey
Graduate Research Assistant
Marshall University
Environmental Science
Morrow Library G31-E

Dear Mr. Rowsey:

This letter is in response to the submitted thesis abstract entitled *"Method development for rearing field collected mayflies to provide consistently available healthy organisms for use in toxicity evaluations."* 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