ABSTRACT

GREINER, RENAE DIANE. Detritus Shredding Aquatic Insect Contributions to Freshwater Mussel Nutrition. (Under the direction of Dr. Jay F. Levine.)

Shredding insects play an important role in the breakdown of leaf litter that falls into streams. These consumers convert a significant portion of leaf litter into fine particulate organic matter (FPOM) and dissolved organic matter (DOM) through various feeding modes. The products of this particle size reduction process provide important nutrients and energy for other biota downstream. To elucidate this process in a controlled setting, we conducted laboratory studies with three genera of shredding insects (*Pteronarcys*, *Tipula*, and *Pycnopsyche*) to assess differences in the rate and dynamics of particulate and dissolved organic matter processing. At four time points over a period of three weeks, water samples were collected from tanks containing the separate insect genera and an insect-free control. These samples were analyzed by three methods. We used flow cytometry to count the number of particles in the sample, absorbance spectroscopy to measure concentration of light-absorbed DOM and determine relative molecular weight of these molecules, and fluorescence spectroscopy to study chemical composition changes over time. *Tipula* caused a significant increase in FPOM and light-absorbing DOM concentrations in ambient water over that in other insect and control treatments. Spectral slopes of the absorbance data showed that while all treatments exhibited apparent microbial degradation, *Tipula* treatments also contained larger molecular weight DOM molecules. *Tipula's* unique gut anatomy and associated bacteria may be responsible for generating this increased molecular weight DOM. Fluorencence results indicated increased amounts of both humic material and microbial-derived protein in the *Tipula* treatments. These insects may provide a more important ecological function in the river ecosystem than previously thought. *Tipula's* unique organic

matter processing likely provides important nutrients, energy, and trophic pathways to support the health of other aquatic species and the river ecosystem.

Studies were conducted to explore the potential function shredding insects could provide in enhancing the diets of juvenile freshwater mussels in captivity. Freshwater mussel biologists have long recognized the critical role of fish in the reproduction of most freshwater mussels, but other sympatric species may contribute to their survival. Freshwater mussels are filter feeders, gathering their food from the FPOM in streams, and algae are considered an important component of their diet. Typically mussels reared in research facilities are fed a diet of commercially available algae, but these animals exhibit slow growth and low survival. Recent studies have found bacteria are an important component to freshwater mussel diet as well. We examined aquatic insects as potential contributors to mussel diet in the wild to aid in captive feeding methods. Shredding aquatic insects are significant contributors to the FPOM suspended in the water column. The added protein and other nutrients contributed by shredding insects may be important to mussel health. We tested the hypothesis that the presence of shredding insects would increase juvenile freshwater mussel growth. We found increased mussel growth in the presence of *Tipula* for two lampsiline mussel species (subfamily Lampsilinae). The increased growth demonstrated by *Villosa delumbis* and *Lampsilis fasciola* in the presence of *Tipula* indicates that it somehow contributes to an increase in food quality or abundance for these mussel species. Future research is needed to further enhance our understanding of the role and mechanisms of shredding insects in mussel nutrition.

Detritus Shredding Aquatic Insect Contributions to Freshwater Mussel Nutrition

by
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DEDICATION

To my Grandparents. My life would not be as fulfilling and free as it is now without the strength and endless love I received from the both of you. I am proud to be part of your family and will never forget the beautiful time we spent together. I miss you.

BIOGRAPHY

Renae Greiner was born in rural western Pennsylvania, where she spent her childhood and teenage years on the small family farm. Her passion for learning about nature, plants, and animals has been with her for as long as she can remember. After graduating from Karns City Area High School, she attended Allegheny College in Meadville, Pennsylvania, to obtain her Bachelor of Science degree, majoring in biology and physics and studying music on the piano for her minor. She spent the next five years seeking an interesting and engaging career until she stumbled upon Dr. Jay Levine's laboratory. Working closely with a staff member, Chris Eads, she fell in love with freshwater biology and ecology, and developed a deep interest in questions of her own. She is intensely appreciative to Chris and Jay for allowing her the freedom to study these questions for her Master's thesis.

ACKNOWLEDGMENTS

Thanks to Chris Eads for introducing me to the beautiful world that is freshwater ecology. Through his constant teaching and encouragement, I have grown to appreciate and embrace all the fascinating intricacies that take place every day in freshwater ecosystems. Thanks to Dr. Jay Levine for combing through my rough writing and helping me effectively write what it is I'm trying to say. Thanks to my whole committee for their constant support and encouragement, and to Dr. Arthur Bogan for his continued commitment and contributions (despite having to declare him an "unofficial" committee member). Thanks to Dr. Chris Osburn's students for processing my giant stack of samples without complaint. Thanks to the many folks who helped me collect craneflies, even when I didn't know how or where to find them. Thanks to the North Carolina Department of Transportation for funding this study and providing extensions to help me finish what I started. Thanks to Tammy Ball who managed to keep her patience with me after I had changed my mind 5 times about committee members. Finally, thanks to my mom for her endless support even when she has no idea what it is I'm trying so hard to do.

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INTRODUCTION

Freshwater mussels help clean our streams and rivers, the source of many people's drinking water, by filtering the water column (McMohan and Bogan 2001). Their filter-feeding removes particulates and dissolved compounds floating in the water (McMohan and Bogan 2001), some of which are harmful toxins (Foster and Bates 1978, Naimo 1995). Mussels can accumulate and metabolize these compounds in their tissues, functionally removing them from the ecosystem (Foster and Bates 1978, Naimo 1995). Mussels deposit nutrients and processed organic material in the sediment when feeding, and aerate bottom substrates with their burrowing behaviors (Howard and Cuffey 2006). In this manner, freshwater mussels are important contributors to both aquatic ecosystems and human health.

Despite their importance, freshwater mussels (Unionidae) are among the most endangered animals on the planet (Williams *et al.* 1993). North America has the greatest diversity of mussel species, but seventy percent of its 300 freshwater mussel species are imperiled (Williams *et al.* 1993, Neves *et al.* 1997). The urgency of conservation efforts for freshwater mussels cannot be overstated.

Freshwater mussels live in the top layer of stream sediments and require stable substrate and dissolved oxygen (among other factors) for suitable habitat (McMohan and Bogan 2001). Sedimentation, stream impoundment, and erosion of streams degrade mussel habitat and are among the top contributors to their decline (Bogan 1993, Williams *et al.* 1993). These problems can cause habitat fragmentation, which exacerbates population declines by further isolating mussel populations (Neves *et al.* 1997). For example, only about half of the remaining mussel species in the mainstem Tennessee River are reproducing

populations due to the numerous anthropogenic impairments of that system (Neves *et al.* 1997).

To offset some of these effects and associated population declines, conservationists have been augmenting mussel beds with individuals of remaining species or reintroducing populations of historic species (Layzer and Scott 2006). Mitigation for stream impairment sometimes includes translocation of mussels from impacted areas to areas with more stable habitat, even though these efforts have shown low success rates (Cope and Waller 1995). Augmentation and reintroduction methods require sufficient numbers of individuals for specific species of mussels, which are usually obtained through propagation and culture of mussels in a laboratory or hatchery setting (Neves 2004).

Propagation and culture of freshwater mussels is a complex process. The life history of most unionids includes an obligate parasitic stage during which larvae attach to a specific species of fish (McMohan and Bogan 2001). The attachment lasts approximately 2-3 weeks, and transforms the larvae into young juveniles, which are usually no larger than a grain of sand. These mussels are raised in captivity until they reach the desired size for release into the wild. The vulnerable first 4-6 years of their life is their fastest period of growth (McMohan and Bogan 2001).

Some unionids have been successfully propagated in the laboratory, but rearing freshwater mussels in captivity has proven to be a challenge for many species (Henley *et al.* 2001). Young juvenile mussels grown in facilities that lack a natural water source exhibit slow growth and low survival (Henley *et al.* 2001). Propagation would be more efficient and successful if we could increase mussel survival and growth in the laboratory.

Growth of juvenile mussels reared on natural water sources is much faster than those mussels that are kept in the laboratory (Henley *et al.* 2001). Most laboratories that propagate freshwater mussels feed the newly transformed juveniles only cultured or commercially obtained algae and rear their animals in de-chlorinated city water or well water (McMahon and Bogan 2001). Slow growth and very low survival of juveniles in the laboratory is an indication that they may not be getting the necessary nutrients they need for normal growth.

Little is known about freshwater mussel nutrition and diet preferences (Nichols and Garling 2000). As filter feeders, they ingest whatever is in the water column, and reject as pseudofeces what they do not send to the stomach (McMohan and Bogan 2001). The mussel's gills have been shown to sort out food particles from non-food particles (McMohan and Bogan 2001), but not all particles that are sent to the stomach are necessarily digested and of nutritional value. It is widely accepted among malacologists that mussels eat various types of algae, but studies have shown some mussels may be getting their nutrition from bacteria in their natural environment (Nichols and Garling 2000, Christian *et al.* 2004).

Nichols and Garling (2000) have shown that bacteria are an important food resource for some species of mussels. Juvenile rainbow mussels (*Villosa iris*) have also been shown by Yeager and coworkers (1994) to ingest primarily bacteria. Bacteria that are available to mussels for food are found attached to the particulate organic matter suspended in the water column. Nichols and Garling (2000) suggest that as freshwater mussels filter feed, they strip the bacteria from fine particulate organic matter (FPOM) detritus, as observed in marine bivalves (Prieur *et al.* 1990). Therefore, FPOM detritus may play an important role in freshwater mussel nutrition.

Fine particulate organic matter in streams is created in part by decomposition of leaves, and this process is accelerated by shredding invertebrates. Cummins and coworkers (1973) found that the percent dry weight loss of leaves after 40-60 days was higher with shredding insects present than in controls with no animals (40-64% versus 26%, respectively).

Although there are many detritus shredders in a given aquatic ecosystem, the dominating three shredding insects in most areas of North America are the larvae of some caddisflies, craneflies, and stoneflies (Anderson and Sedell 1979). In North Carolina, common shredding genera among these groups are *Pycnopsyche*, *Tipula* and *Pteronarcys*. *Pycnopsyche* and *Pteronarcys* are typically found in low order mountain streams. *Tipula* are ubiquitous and can be found in most stream orders and throughout North Carolina. All of these insect genera are primarily aquatic; their larvae are found in stream water, but they emerge as flying adults. Hereafter, I will refer only to the shredding larval form of these insects.

Shredding aquatic invertebrates play an important role in river ecosystem function by accelerating the decomposition of leaf litter in streams (Vannote *et al.* 1980). The shredders' detrital feeding process results in FPOM suspended in the water column, which consists of feeding fragments and feces. *Pteronarcys* have been shown to break down leaves at a rate of 29% of their dry body weight per day, and egest about 18% (McDiffett 1970). They have low assimilation efficiency, but are highly productive in terms of amount of particulate material processed. In one study, Cummins and coworkers (1973) found that shredders converted 60% of ingested food to feces. Since shredders can make up 20% of the

macroinvertebrate biomass in streams (Cummins *et al.* 1989), their contribution to the FPOM is significant.

Aquatic insect shredders accelerate the detritus breakdown process by increasing the surface area for microbial colonization, which supports further decomposition of the material and releases nutrients (McDiffett 1970). Shredding insects choose their food not by the leaf species, but by the degradation state of the leaves, preferring leaves with higher levels of microbial colonization (Cummins *et al.* 1989). The same microorganisms are reported to colonize on all species of leaves that fall into the stream (Cummins *et al.* 1973). The detritus particles that shredding insects cause are generally not very nutritious as food for other organisms, but the increase in surface area of the detritus facilitates further colonization by microbes and improves food quality (Anderson and Sedell 1979).

Shredding insects' major contribution to the FPOM in streams may have an impact on the detritus based bacterial food source of freshwater mussels. Anderson and Sedell (1979) reported that bacteria are relatively sparse on detritus until it is passed through invertebrate guts. Bacterial populations in digested food of *Tipula* and *Pycnopsyche* have been shown to be 2-5 orders of magnitude more dense than bacterial populations in ingested food (Cummins and Klug 1979). Cummins and coworkers (1973) suggested that shredder feeding converts detritus into particles that undoubtedly contribute to the food source for collectors. Collectors depend on the microbes attached to the FPOM and their metabolic products for their nutrition (Vannote *et al.* 1980). The inefficient upstream processes provide energy input for downstream processors, and the stream communities are structured to take advantage of this process (Vannote *et al.* 1980). Shredding insects located upstream of

mussels convert large organic material that is unavailable to mussels as food, to FPOM that becomes nutrient- and energy-rich from the attached microbes and accessible to mussels for food. These insects could be playing an important role in the diet of freshwater mussels. Accordingly, the primary hypotheses of these studies were that

(1) Shredding aquatic insects will increase energy-rich particulates in the water column, and

(2) Juvenile mussel growth will increase in the presence of shredding insects in the laboratory.

To address these questions, we first examined the conversion of large leaf matter to particulate material from three common North Carolina shredding insects: *Pteronarcys*, *Pycnopsyche*, and *Tipula*. Jars containing each of the different shredding insects and an insect-free leafpack control were assembled in the laboratory, and water samples were collected 4 times over the course of 3 weeks. Water samples were examined by flow cytometry, and absorbance and fluorescence spectroscopy. Flow cytometry data determined the number of particles <200μm in each treatment. Absorbance data estimated the concentration of DOM and the relative molecular weight of molecules. We used fluorescence spectroscopy to explore chemical composition changes of the samples over time. Sub-adult and juvenile freshwater mussel growth and survival in the presence of shredding insects were measured to assess the potential role these insects may play in the diet of freshwater mussels.

CHAPTER 1: The Unique Contribution of the Aquatic Shredding Insect, *Tipula*, to Organic Matter in Streams.

Introduction

Shredding aquatic insects play an important functional role in river ecosystems (Vannote *et al.* 1980). They break down leaf litter when feeding, which creates fine particulate organic matter (FPOM). The FPOM suspended in the water column cumulatively has a large surface area, making it an important carrier of nutrients in streams (Yoshimura *et al.* 2008). Through fragmentation and feces, the shredding process provides energy and nutrients for consumption by other organisms downstream.

Leafpack is the largest component of course particulate organic matter (CPOM) in temperate streams (Abelho 2001). It leaches the most within the first 24 hours of being submerged in the stream, and has been shown to lose 5-27% of its dry weight in this time (Anderson and Sedell 1979). Leaf leachate consists of polysaccharides (carbohydrates), amino acids (protein) and fatty acids (lipids) (Volk *et al.* 1997), and can compose up to 42% of the total dissolved organic matter (DOM) in a stream reach (McDowell and Fisher 1976). After leaching, microbes colonize the leaf surface, a process which is mostly complete within two weeks (Cummins 1974). The microbial transformation of the leaf material is a key determinant of stream DOM composition (Yoshimura 2010). Pathways for removal of DOM in streams include flocculation, microbial utilization, and photodegradation (Helbling *et al.* 2003).

Decomposition of CPOM is faster with the help of shredding insects. In one study, leaves lost 50% of their weight in 70-90 days when held without shredding insects, but lost

that same weight in 40 days when shredding insects were present (Anderson and Sedell 1979). Cummins and coworkers (1973) found that 20% of initial leaf weight loss went to shredder processing and approximately 10% was lost due to leaching. A large amount of FPOM is generated by the feeding of shredding insects on course particulate organic matter (CPOM) and their defecation (Wallace and Webster 1996). Thus, shredding insects play a large role in leaf decomposition and FPOM production.

The presence of shredding insects also accelerates microbial growth in streams by the release of nutrients from the shredding process (Covich *et al.* 1999). Microbial colonization is similar for all species of leaves that fall into a stream, and shredding insects generally select leaves for feeding based on the degree of degradation (Cummins *et al.* 1973). Anderson and Sedell (1979) found that bacteria are relatively sparse on detritus until it is passed through the invertebrate gastrointestinal tract, and showed that maximum microbial activity on FPOM is reached after 2-3 days of passing through the animal. Crowl and coworkers (2001) completed a study that demonstrated increased microbial production on FPOM in the presence of detrital shrimp. The acceleration of microbial growth by shredding insects on detritus probably increases energy availability to other aquatic organisms (Heard and Richardson 1995).

Different insects contribute to the organic matter in streams in different ways. Covich and coworkers (1999) suggest that the presence or absence of one species can dramatically affect decomposition rates. The three groups of taxa containing the dominate shredding insects in North America are Trichoptera, craneflies, and Plecoptera (Anderson and Sedell 1979). Cummins and coworkers (1973) found that *Tipula* decreased leafpack dry weight

more than all other insect detritivores tested (including *Pycnopsyche* or *Pteronarcys*). In the Cummins and coworkers studies, *Tipula* more than doubled the dry leaf weight loss from leaching (64% to 26%), and it was estimated that *Tipula* and *Pycnopsyche* alone could process at least half of all detritus into fine particulates for their study stream (Cummins *et al.* 1973).

Although the role of aquatic shredding insects in the mechanical and microbial processing of leaves (and FPOM production) is well documented, their affect on chemical composition of DOM over time is less well understood. Our objective for this study was to compare both the quantitative and qualitative processing of CPOM into FPOM and DOM by three sympatric genera of shredding insects: *Tipula*, *Pteronarcys* and *Pycnopsyche*. Flow cytometry and absorbance and fluorescence spectroscopy have been used widely to study quantitative and qualitative water chemistry (Shapiro and Leif 2003, Helms *et al.* 2008, Fellman *et al.* 2010). We used flow cytometry to estimate FPOM creation by these species. Absorbance and fluorescence spectroscopy was used to examine changes in the concentration, relative molecule molecular weight, and chemical composition of DOM over time.

Methods

The study was conducted in August and September of 2009. All insects and leafpacks used in the study were collected from four North Carolina piedmont and mountain streams (located in Caldwell, Chatham and McDowell Counties) within a week before experiments began. Leafpacks were collected in stream locations where shredding insects

were present. We hand-selected leaves that had a similar degree of degradation as packs in which the shredding insects were found.

Experimental Design

Twelve 1-liter glass jars were used to hold four different treatments, each with three replicates. A treatment designated as the control included leafpack but no insects. The remaining three treatments included each of the three insect genera, *Pteronarcys*, *Tipula*, and *Pycnopsyche.* Each insect treatment included three individuals of the same genus per jar and leafpack matter. The jars were held indoors in a temperature- and light-controlled facility. Full spectrum bulbs were set on a 12-hour on, 12-hour off cycle. The air temperature ranged from 20-23ºC during the experiment.

Study jars were thoroughly cleaned and rinsed with an acid wash and tap water and allowed to dry prior to their use. Each treatment jar contained 500mL of unfiltered well water and was aerated with an airstone. Leafpack was homogenized by pulsing with a blender and then filtered through a 200μm sieve, and particles larger than 200μm were used for the experiment. After blending and filtration, 15g wet weight of leafpack were added to each jar. Aluminum foil was placed over the jar top to reduce evaporation. The airstone air flow was adjusted every day to maintain consistency among jars. Insects were acclimated in well water for at least 24 hours before adding to the experiment jars, and were not disturbed for the duration of the experiment.

Water samples

The same day insects were added and 2 days after tanks were initially set up (day zero), 35mL of water was removed from the control treatment for analysis by flow

cytometry, absorbance and fluorescence. This water was replaced with the same volume of fresh well water. The sample was removed by drawing the water off slowly near the surface to avoid disturbing the settled material (including the leafpack) on the bottom of the jar. The samples taken from all 12 jars at day 5, 13, and 21 were retrieved the same way. A 250mL water change was completed on day 8, with collection of the samples on that day.

Part of each water sample was filtered through a 200μm sieve before passing through the flow cytometer to estimate particle count. The remaining water was passed through a clean 0.7μm Whatmann glass microfiber filter (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) to examine the dissolved component through absorbance and fluorescence of the sample. Particles larger than 200μm were considered CPOM, particles between 200μm and 0.7μm as FPOM, and anything smaller than 0.7μm as DOM.

Analytical methods

Flow cytometry was conducted using a FACS Calibur (Becton, Dickinson and Company, Franklin Lakes, NJ), running CellQuestPro Software. Total particle count was received per flow run time, which was held constant throughout the experiment at 60μL per minute for two minutes.

Absorbance of DOM was determined using a Cary 300 UV spectrophotometer (Agilent Technologies, Santa Clara, CA). Each 800-200nm scan was run at 600nm/min. Data was collected at 1nm intervals. A 10cm cylindrical cuvette was used to hold the sample. Clean MilliQ (Millipore, Billerica, MA) water was used as a blank and subtracted from the sample data to get the raw absorbance value $\Delta A(\lambda)$, where λ is wavelength in nm, which was then converted to the absorption coefficient, $a(\lambda)$ as below:

$$\Delta A = A_{sample}(\lambda) - A_{MilliQ}(\lambda),$$

$$a(\lambda) = \frac{2.303 \Delta A}{D},$$

where D is the pathlength in meters (Kirk 1994).

Spectral slope (*S*) value is often used as a characterization tool for light-absorbing DOM (referred to as colored dissolved organic matter, or CDOM) and has been correlated to molecular weight of CDOM (Twardowski *et al.* 2004, Helms *et al.* 2008). The *S* value is a measure of steepness of the absorbance over a range of wavelengths. In general, *S* values are lower for samples with high molecular weight DOM, due to the increased absorption of these molecules at longer wavelengths. There are many different ways of calculating and examining spectral slope values (Twardowski *et al.* 2004). In this study, *S* was calculated as the slope of log transformed absorbance coefficients of three different wavelength ranges: 300-600nm, 275-295nm, and 350-400nm (Osburn *et al.* 2001). The slope values were determined by fitting a regression line to the log-linearized absorption spectra in Microsoft Excel (Microsoft Corporation, Seattle, Washington).

A Cary Eclipse spectrofluorometer (Agilent Technologies, Santa Clara, CA) was used to obtain the fluorescence contouring data to create excitation-emission matrices (EEMs). Lamp intensity was 900 volts, and emission spectra from 300-600nm was measured at excitation wavelengths incrementally from 240 to 450nm at 5nm intervals. Emission wavelengths were sampled every 2nm. A 1cm pathlength cuvette was used to hold the sample. A sample with MilliQ water was conducted before every set of samples to normalize the data for the Raman water scatter peak. Results are reported in Raman Units (RU).

Fluorescence contouring data can be used to identify different types of fluorescent compounds in a water sample (Fellman *et al.* 2010). The EEMs produced show peaks for different compounds (fluorophores) and their position identifies generally what type of compounds they are. We focused on two basic types of DOM fluorescent signals in this study; humic-like (peaks C and A) and protein-like (peaks T and B). Humics exhibit emissions at longer wavelengths and have broad emission maxima due to their complex molecular nature (Fellman *et al.* 2010). Peaks T and B (tryptophan-like and tyrosine-like, respectively) are a mixture of amino acids with similar fluorescence characteristics (Fellman *et al.* 2010). There is some ambiguity in determining these peaks (Coble 1996). For the humic-like peaks, fluorophore C was identified at 350nm excitation and 420-480nm emission, and fluorophore A was identified at 260nm excitation and 380-460nm emission (Coble 1996). For the protein-like peaks, fluorophore T was identified at 275nm excitation and 340nm emission, and fluorophore B was identified at 275 nm excitation and 310 nm emission (Coble 1996). We used Matlab (Mathworks, Natick, MA) to create the 3-D EEMs for our samples and obtained the peak fluorescence values for T, B, C and A at their respective regions of the graph. We measured changes in chemical composition of DOM by examining fluorescence data at designated wavelengths over time. Composition changes are demonstrated by shifts in the position of the excitation or emission maxima over time (Del Castillo *et al.* 1999).

All absorbance and fluorescence data were exported as CSV files and post processed with Microsoft Excel or Matlab. Standard deviations for flow cytometry and absorbance data were calculated and included for each reported treatment average.

Results

Flow cytometry

Particle count was highest at every time point for the *Tipula* treatment (Figure 1). The presence of *Tipula* increased <200µm FPOM more than other shredding insects or leaves decomposing without insects. There were no significant differences between the control treatment and either *Pycnopsyche* or *Pteronarcys*. All particle counts declined after 8 days because of dilution from the water changes in the tanks on that day.

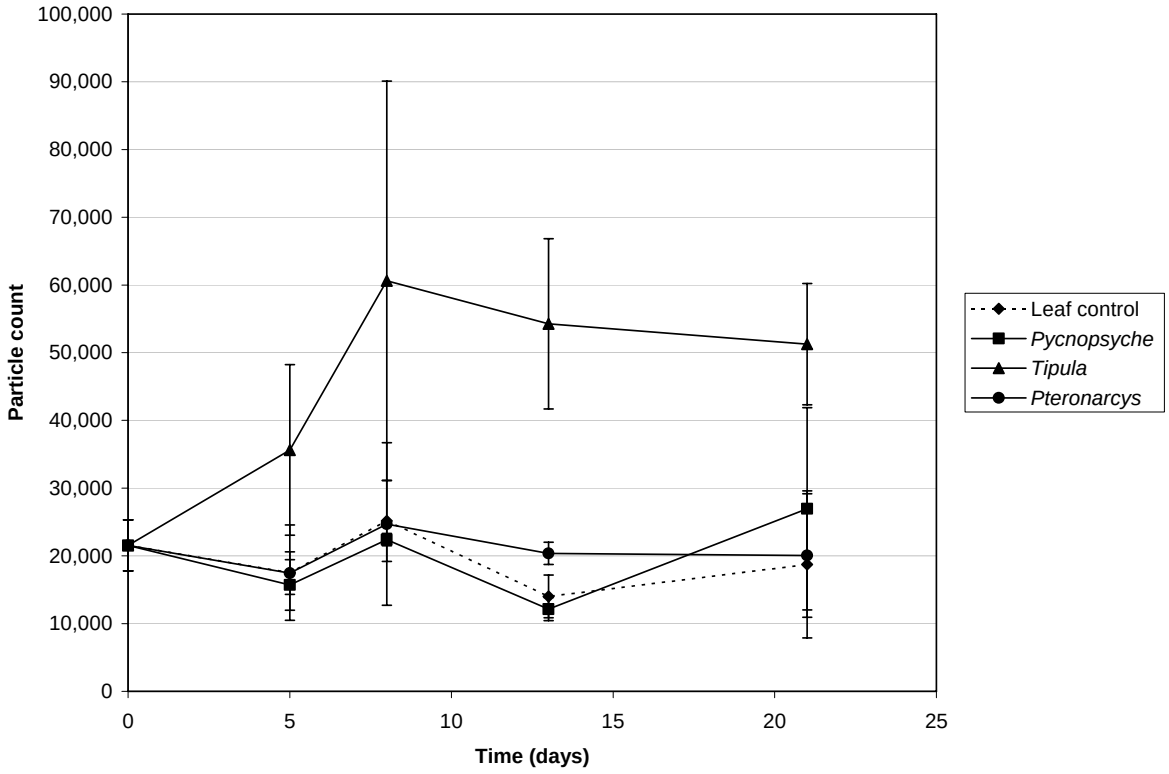

Figure 1. Changes in mean (+/- standard deviation) number of particles <200µm over time in water samples among three replicates of each of three shredding insect genera with leafpack matter and a control with only leafpack matter.

Absorbance of DOM

Water from the *Tipula* treatment demonstrated considerable increased absorption across the range of wavelengths over the other insect treatments and the control (Figure 2). This was consistent across all replicates and time, but most obvious in the day 21 samples taken at the end of the study.

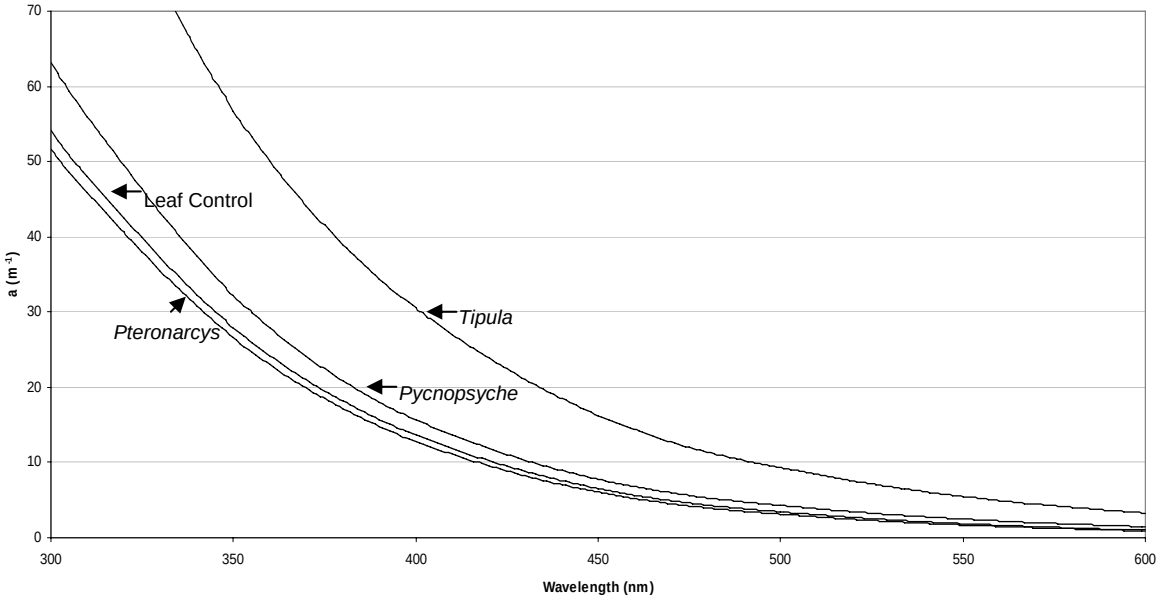

Figure 2. Representative absorption spectra for each treatment, taken from a single replicate from the day 21 samples.

Absorption coefficients at 350nm were examined as a reference point for all absorption data and to indicate the quantity of CDOM in each treatment over time (Figure 3). The *Tipula* treatment was the only treatment that had absorption coefficients significantly above the control values. Because absorbance is directly related to CDOM concentration, we were able to relatively quantify the different contributions by the different insects. The

Tipula water samples had increased levels of CDOM over all the treatments tested in our laboratory.

Tipula were fast and efficient processors of leafpack under laboratory conditions. The *Tipula* water samples were visibly darker in color than any of the other samples by day 13 and 21. When the experiment was disassembled one month after the last data were obtained, *Tipula* had shredded all of their leafpack into a fine silt; no large pieces remained. The leafpacks in the other tanks were still mostly intact with large pieces still apparent.

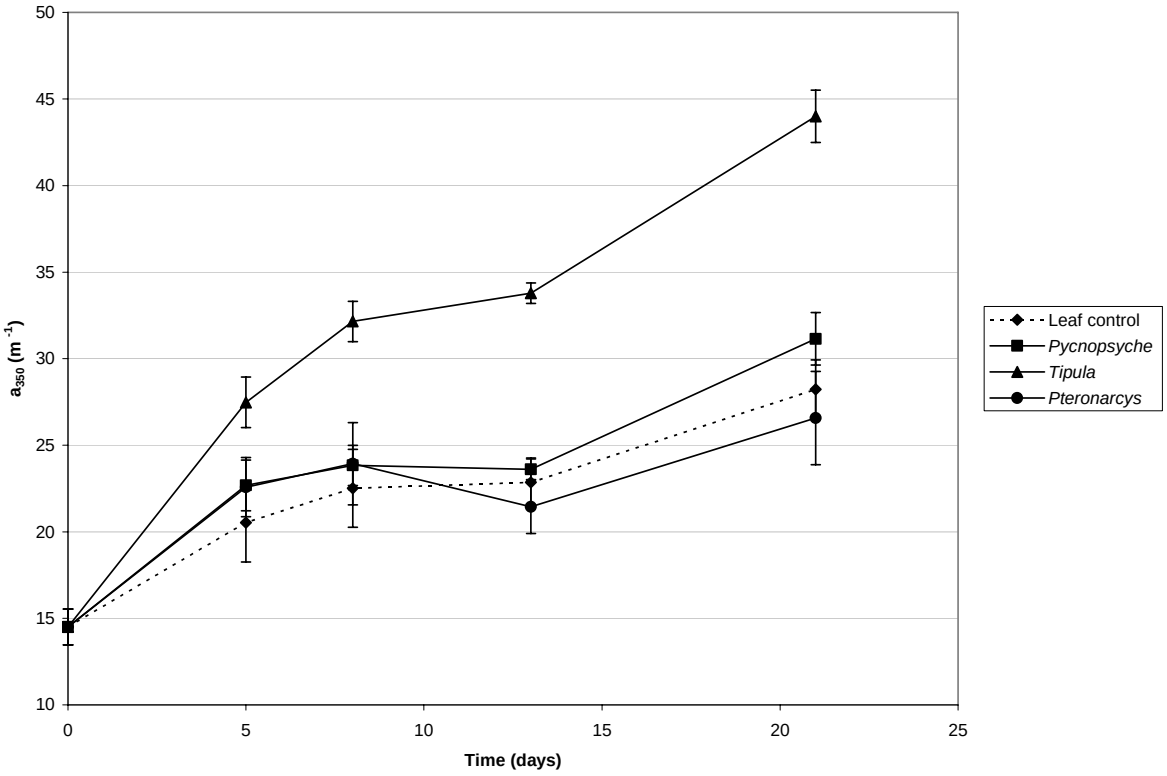

Figure 3. Mean (+/- standard deviation) absorption coefficients at 350nm of water samples taken over time of three shredding insect genera with leafpack matter and a control with only leafpack matter.

Spectral slope value in natural fresh water ranges from 0.012 nm^{-1} to 0.020 nm^{-1} (Kirk 1994). In this study, the values were on the low end of this range (Figure 4). The *S* values

increased over time in all of the treatments (Figure 4, A-C). However, the *S* values for the *Tipula* treatment were lower (less steep) than those from the other insects and the control at every time point. The standard deviation bars on the graphs show that the other shredding insects tested did not demonstrate *S* values very different from the control treatment.

A ratio of the slopes from 275-295nm to 350-400nm, slope ratio or S_R, was used to examine changes in DOM quality by inherent sensitivity to changes in long wave absorption (Helms *et al*. 2008). The *Tipula* treatment had higher slope ratio values at every time point (Figure 4, D).

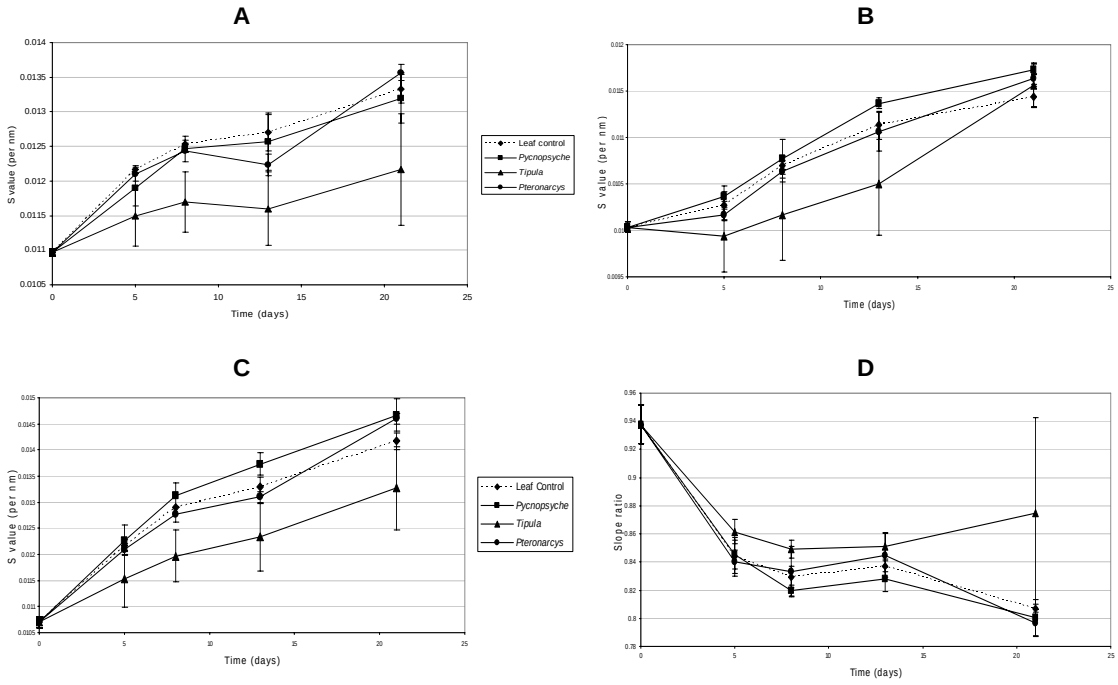

Figure 4. Mean (+/- standard deviation) *S* values for the different treatments over time for three different ranges: 300-600nm (A), 275-295nm (B), and 350-400 (C). Slope ratio is the ratio of $S_{275-295}$ to $S_{350-400}$ (D).

DOM Fluorescence

The fluorescence values for the specific regions of the EEM (T, B, C and A) increased in all treatments over time (Table 1.1). However, the values increased in the *Tipula* treatment more rapidly (by day 8) and were larger than any of the other insect treatments or control values, especially by the end of the study (Table 1.1, bold and underlined data).

Table 1.1. Mean peak fluorescence values and standard deviations (SD) of the different treatments over time for four fluorophores (in RU). Fluorophores labeled T and B are protein-like and fluorophores labeled C and A are humic-like molecules. Bolded numbers in the T and B columns are two times the starting values and bolded numbers in the C and A columns are three times the starting values. Bolded and underlined data are the highest values overall.

	Time (day)	**T**	T SD	**B**	B SD	**C**	C SD	**A**	A SD
Leaf control	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.32	0.07	0.17	0.04	0.93	0.14	0.90	0.15
	8	0.40	0.08	0.24	0.03	1.18	0.15	1.23	0.19
	13	0.35	0.09	0.20	0.03	1.13	0.08	1.22	0.12
	21	**0.50**	0.10	**0.24**	0.05	**1.71**	0.16	**2.09**	0.24
Pycnopsyche	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.34	0.01	0.19	0.02	1.02	0.02	0.99	0.01
	8	0.38	0.01	0.22	0.01	1.13	0.06	1.25	0.02
	13	0.40	0.04	0.22	0.03	1.14	0.04	1.40	0.04
	21	**0.73**	0.07	**0.33**	0.02	**1.87**	0.05	**2.62**	0.09
Tipula	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.43	0.06	0.22	0.01	1.15	0.12	1.15	0.15
	8	**0.54**	0.05	**0.31**	0.04	1.34	0.14	**1.53**	0.21
	13	**0.58**	0.13	**0.31**	0.08	1.38	0.23	**1.71**	0.36
	21	**0.99**	0.47	**0.43**	0.13	**2.26**	0.54	**3.92**	1.77
Pteronarcys	0	0.23	0.02	0.13	0.02	0.54	0.05	0.45	0.03
	5	0.33	0.01	0.19	0.01	1.04	0.06	0.98	0.05
	8	0.40	0.07	0.22	0.02	1.14	0.09	1.24	0.11
	13	0.38	0.05	0.20	0.03	1.06	0.05	1.19	0.12
	21	**0.53**	0.05	**0.29**	0.03	**1.66**	0.14	**2.13**	0.25

A shift in excitation maxima for some of the *Tipula* and *Pycnopsyche* replicates was observed from 325nm to 240nm by the end of the experiment (Table 1.2, bold and underlined

data). This suggests a change in the chemical composition of those samples from C-type humics to A-type humics. This change happened faster for *Tipula*, with day 13 showing the first account of the shift. Not all replicates showed the shift. The emission maxima however, showed no difference over time for any treatment, staying around 420-430nm.

Table 1.2. Excitation maxima (nm) obtained for each of the three replicates (jars) for each treatment over time. Bolded and underlined data represent a shift in the location of excitation maxima, indicating a change in chemical composition of the CDOM.

	day 0	day 5	day 8	day 13	day 21
Leaf control	335	330	325	315	325
	325	335	330	315	315
	335	330	320	325	315
Pycnopsyche	335	330	325	325	**240**
	325	320	325	315	310
	335	330	325	310	320
Tipula	335	325	325	325	320
	325	330	325	**240**	**240**
	335	335	315	330	**240**
Pteronarcys	335	325	325	325	330
	325	330	325	315	320
	335	325	310	325	310

Discussion

The *Tipula* treatment water had the highest concentration of <200µm FPOM and CDOM of the three insects tested and the leaf control. *Tipula* were the most efficient processors of CPOM in the laboratory of the three insects we tested. Due to the accelerated decomposition of CPOM, water containing leaf matter shredded by *Tipula* may have the greatest potential nutritional value to other aquatic fauna.

The spectral slope data showed that all treatments were likely undergoing intense microbial degradation and producing smaller molecular weight molecules over time, as indicated by the increase in *S* value over time (Figure 4) (Helms *et al.* 2008). The lower

spectral slope values for the *Tipula* treatment may have resulted from increased longwave absorption due to the presence of larger molecules in the *Tipula* treatment. Spectral slope values increased over time for all treatments, suggesting continued microbial degradation of DOM. The *S* value remained consistently lower for the *Tipula* treatment over time compared to the other treatments. This indicated that high molecular weight DOM continued to be created or persisted for the *Tipula* treatment. The general decreasing trend of S_R values for all treatments except *Tipula* was consistent with Helms and coworkers (2008) who demonstrated decreasing S_R values with DOM undergoing increasing microbial degradation (Figure 4). The S_R graph demonstrates a difference in DOM quality for the *Tipula* treatment when compared to the other treatments.

The high molecular weight CDOM indicated by the absorption results in the *Tipula* treatment likely is a result of the unique gut structure of *Tipula.* *Tipula* have a distinct bacteria-loaded intestinal anatomy that is different from the other shredding insects (Sinsabaugh *et al.* 1985). The symbiotic bacteria in their gut probably play a role in the increased molecular weight of the DOM. More research is needed to determine what role the different intestinal bacteria have in the creation of larger molecular weight DOM molecules.

The fluorescence data further supported the conclusion that *Tipula* change DOM quality. A change in the chemical composition of *Tipula* samples from C-type humics to A-type humics indicated a shift from labile to more aromatic humic molecules (Fellman *et al.* 2010). Additionally, the *Tipula* treatment had higher levels of both humics and protein than the other shredding insects or the leafpack control over time. In fact, the *Tipula* treatment had nearly a two-fold increase in the amount of both humic and proteinaceous material by the

end of three weeks time over the other treatments. The protein-like fluorescence peaks have been used to label DOM as derived from microbial sources or linked to bacterial production or respiration (Fellman *et al.* 2010). Humic-like fluorescence is usually controlled by hydrology, whereas protein-like components of DOM are controlled by biological processes (Fellman *et al.* 2010). Humics are also the end product of microbial mediated degradation of terrestrial plant material (Aitkenhead-Peterson *et al.* 2003). From this information, we can deduce that the *Tipula* treatment demonstrated elevated levels of microbial decomposition in conjunction with the presence of higher molecular weight DOM. The increase in protein-like material in the *Tipula* treatment was likely excretory, since the T region fluorophores indicate compounds of recent biological origin (Parlanti *et al.* 2000). Protein in adequate quantities and of appropriate form and digestibility is essential for bacterial growth. Simon and Azam (1989) state that protein concentrations can be used to estimate the amount of bacteria present. An increase in protein fluorescence also can indicate an increase in bacteria; Tanoue and coworkers (1995) showed that bacterial membranes may be a major source of dissolved protein in sea water. Regardless of what the protein compounds are, the significant addition of protein to natural waters is ecologically important to bacterial populations and potentially the health of aquatic macroinvertebrates and other fauna. *Tipula's* increased contribution to protein production should be quantified and further studied to determine the importance of *Tipula,* specifically, in protein cycles in streams.

The other shredding insects tested did not demonstrate changes in the molecular weight of DOM different than normal leaf decomposition. Nor did they produce the increased levels of decomposition or protein production like *Tipula*. It is possible that these

species were not as actively feeding in our laboratory due to sub-optimal environmental conditions. *Pycnopsyche* were the smallest of the shredding insects used in this study. Their lower biomass may have lessened their effect compared with other shredding insects. Also, some of the *Pycnopsyche* did not fare well during the experiment and up to 30% of animals may not have been shredding by day 21. Smaller biomass and lower survival may be reasons that *Pycnopsyche* did not have as strong of an effect as *Tipula* on processing of FPOM and DOM in this study. Future research should equilibrate *Tipula* and *Pycnopsyche* biomass and determine the effects on DOM production and quality. *Pteronarcys*, which are generally found in faster and cooler water temperatures, may not have shredded as actively during this experiment due to the higher temperatures in the laboratory. It is also possible that this animal does something different to the DOM that is not able to be determined by the detection methods used in this study. Cummins and coworkers (1973) found that *Tipula* had maximized their leaf conversion at elevated temperatures, similar to our laboratory conditions. *Pteronarcys* may need cooler temperatures and/or higher dissolved oxygen to maximize their processing of detrital material, and may actually have been using some nutrients in the water of this experiment for general body maintenance.

The studies were conducted under controlled laboratory conditions. The natural environment in which these animals live is much more variable and inconsistent. Seasonal variability in food availability and quality for shredding insects may alter the structure of shredding insect populations and their local abundance. Temperature, physical environmental factors, as well as changes in the presence and structure of microbial communities and other invertebrates may dramatically affect the ability and efficiency with

which shredding insects process leafpack. The subsequent contribution to the ecosystem of these shredding insects will also vary with the variable environmental conditions. The implications for *Tipula* in stream ecosystems raised by this study should be thoroughly tested under field conditions.

Covich and coworkers (1999) proposed that some species can have a disproportionately large impact on food-web dynamics and other ecosystem dynamics. The presence or absence of a single species can dramatically alter ecological processes such as decomposition or food-web dynamics (Covich *et al.* 1999). *Tipula* are fairly ubiquitous and tolerant, but their presence may provide a more important ecological contribution than previously thought. The "rivet hypothesis" (Ehrlich and Ehrlich 1981) states that each species has the potential to perform an essential role in the persistence of the community and the ecosystem, and that some species may remain as the sole representatives of a particular functional group (Ehrlich and Walker 1998). *Tipula's* unique processing of CPOM into FPOM and DOM possibly provides some of the nutrients, energy, and trophic pathways important for the overall health of other aquatic species in river ecosystems. More experimentation is needed to determine the composition of DOM generated by *Tipula's* shredding of leafpack and their role in supporting the health of aquatic ecosystems that they inhabit. Additional research is required to more thoroughly elucidate *Tipula's* unique contributions to the FPOM and DOM of river ecosystems. The byproducts of *Tipula* leaf shredding may be more important for the overall health and functionality of river ecosystems than previously recognized.

CHAPTER 2: Shredding Insects' Potential Contribution to Freshwater Mussel Nutrition.

Introduction

Freshwater mussels are likely the most endangered group of taxa in North America (Williams *et al.* 1993). Captive propagation of freshwater mussels has been adapted as a conservation tool to help mitigate the systemic decline that has been observed in freshwater mussel species (Neves 2004). Captive reared juvenile mussels are conventionally fed commercially available or cultured algae (McMahon and Bogan 2001) because algae have been found to be an important component to freshwater mussel diets and are routinely found in their stomachs (Nichols and Garling 2000). But growth and survival of many species of juvenile mussels in the laboratory is poor. Our limited understanding of unionid diets (Nichols and Garling 2000) is likely contributing to the poor success of some species in propagation facilities.

Mussels ingest and filter algae, detritus, and other particulate and dissolved matter from the water column when they feed (McMahon and Bogan 2001). Cilia on the mussel gills sort potential food particles by size as the inhalant material is flushed over the gills, and send the right size particles to the stomach (McMohan and Bogan 2001). With other aquatic species, the examination of stomach contents is routinely used to identify what the animals are consuming. However, the presence of a food item in the stomach of a freshwater bivalve does not confirm that the food item is digested, assimilated, and playing a role in the animals nutritional health. Even if algae are selected for ingestion, Nichols and Garling (2000) found that algal carbons may not be assimilated in body stores. Many particles pass through the gut

untouched (Miura and Yamashiro 1990), and it is difficult to determine beneficial food items of mussels by dissection and examination of stomach contents.

Studies using stable isotope analysis have demonstrated that some mussels use bacteria in the fine particulate organic matter (FPOM) rather than algae as a main food resource (Nichols and Garling 2000, Christian *et al.* 2004). Algae may be important for immediate energy needs or provide a good substrate for beneficial bacteria that are contributing to the nutritional health of mussels (Nichols and Garling 2000). But bacteria may be providing the nutritious and energy-storing component of freshwater mussel diets. Only bacteria derive vitamin B12 and specific fatty acids that are key dietary components for mussels (Nichols and Garling 2000, Vaughn *et al.* 2008).

Food items in stream FPOM are highly varied and come from many sources, but shredding aquatic insects are significant contributors to the particulates in the water column, and can be found in the same habitats or upstream of freshwater mussels. In one study, shredding insects only used 40% of the course particulate organic matter (CPOM) they process (Cummins 1974); the rest is egested as feces. Shredder feeding is also known to create small pieces of leaf detritus that are not ingested. These processes contribute greatly to the amount of FPOM in the stream (up to 2-7mg in feces alone per large shredder per day, McDiffett 1970). Fragmented, well-conditioned CPOM and invertebrate feces probably represent the highest quality components of native stream detritus (Cummins and Klug 1979). Since shredding insects can make up 20% of the macroinvertebrate biomass in streams (Cummins *et al.* 1989), their contribution to FPOM is significant.

Aquatic insect shredders accelerate the detritus breakdown process by increasing surface area for microbial colonization, which further decomposes the material and releases nutrients (McDiffett 1970). Shredding insects have been shown to accelerate microbial growth (Covich *et al.* 1999), which includes bacteria, a potential food source for mussels. It has been suggested that bacteria are relatively sparse on detritus until it is passed through invertebrate guts (Anderson and Sedell 1979). After 2-3 days of passing through the gastrointestinal tract, it has been proposed that maximum microbial activity on FPOM is reached (Anderson and Sedell 1979). These microbes enhance detrital food quality (Tenore *et al.* 1982). It has been determined that bacterial populations in digested food of *Tipula* and *Pycnopsyche* are 2-5 orders of magnitude denser than bacterial populations in ingested food (Cummins and Klug 1979). These bacteria, feces, and other microbes brought about by shredding insect feeding may provide a source of nutrition for freshwater mussels.

There is an intimate relationship between shredding insects and collecting insects in that shredding insects increase growth and survival of collectors through their particle production (Heard and Richardson 1995). A substantial body of work has previously focused on the role of shredding aquatic insects in the life history and diet of filter feeding insects (Heard and Richardson 1995). For instance, Short and Maslin (1977) found that the presence of shredding insects increased food availability to collecting insects. They state that shredding insects are of great importance in leaf processing with respect to nutrient availability to collectors. Cummins and coworkers (1973) found that filter feeding mayflies (*Stenonema*) grew in the presence of shredding insect feces due to coprophagy. Wallace and coworkers (1977) described detritivore feces they detected in the gut of a filter feeder.

Cummins and coworkers (1973) stated that the FPOM created by shredders "undoubtedly" constitutes a high quality food source for collectors. The role of shredding insects in the life history and diets of freshwater mussels, however, is essentially undescribed.

Shredding insects, in their largest numbers, are generally found in headwater streams, and the FPOM they create is carried downstream (Vannote *et al.* 1980). Mussels are found in higher order streams. Covich and coworkers (1999) proposed that filter feeders are typically located downstream of the shredding insects and that their loss from the system would alter food availability for suspension feeders. As water flows down river, local terrestrial input is less important to the health of the ecosystem than upstream input (Vannote *et al.* 1980). Streams generally shift from heterotrophy to autotrophy at about 3rd or 4th order, then revert back to mostly upstream inputs at higher stream orders. Mussels are most common in higher order streams and rivers and likely depend on upstream inputs for their food supply.

The FPOM and possibly DOM generated by shredding insects could play an important role in the diet, growth, and survival of freshwater mussels. In previous studies, we compared and contrasted the FPOM and DOM created by *Pteronarcys*, *Tipula*, and *Pycnopsyche.* The FPOM and DOM generated by *Tipula* processing exceeded that of the other tested species (Greiner *et al.* in manuscript). In addition, *Tipula* generated higher molecular weight DOM molecules and greater levels of microbial-derived protein than the other insect species. *Tipula* possibly provide some of the nutrients, energy, and trophic pathways important to support the health of other aquatic species in river ecosystems. Accordingly, in these subsequent studies we focused on the contribution of the products of *Tipula* leaf shredding to the growth and survival of juvenile freshwater mussels. *Tipula* were

held in containers that concurrently housed freshwater mussels, and survival and growth of the mussels was measured over time.

Methods

Preliminary study

A preliminary study was conducted to measure the potential benefit of a combination of three different shredding insects, *Pteronarcys*, *Tipula* and *Pycnopsyche*, to the growth and survival of freshwater sub-adult mussels. This study was completed at the Table Rock Fish Hatchery in Morganton, NC. Insects and leafpack for the study were collected from various local sites. Leafpack was hand-selected based on the degree of degradation, using leafpack where shredding insects were found as an example.

Four plastic troughs were set up from June-Sept 2009. Three different species of mussels approximately 2 years of age were used in this study: *Lampsilis fasciola* (from the Pigeon River, NC), *Lagumia nasuta* and *Lampsilis radiata* (both from the Broad River, SC). Troughs labeled 1 and 3 held the three species of mussels and leaf packs and insects; troughs 2 and 4 served as controls with no leaf packs or insects (Figures 1 and 2). The insects used were common North Carolina shredders from the genus *Pteronarcys*, *Tipula*, and *Pycnopsyche*. The troughs had one inch of sand in the bottom and were full of water to about one inch below the rim of the tank (approximately 4 inches of water). All tanks had large (¼ inch) size mesh barriers in the middle to hold the leafpack and insects upstream of the mussels in test tanks (screens were present but not jammed by leafpack in control tanks). Troughs were set up so that water came in at one end and out at the other to create a somewhat linear flow (Figure 1). The tanks held approximately 8 gallons of water each. The

water input was set to approximately 2 gallons per minute, from the fish hatchery (unfertilized) water holding pond. Water temperature varied with the natural environment, and stayed between 16 and 24ºC for the duration of the experiment. Troughs had a screen top to let in natural light, but to prevent animals from getting in or out. After one month, the flow in each tank was reduced to about 1 gallon per minute, and a shade screen was added to cover all tanks after filamentous algae became a problem. The shade screen prevented filamentous algae problems for the remainder of the experiment.

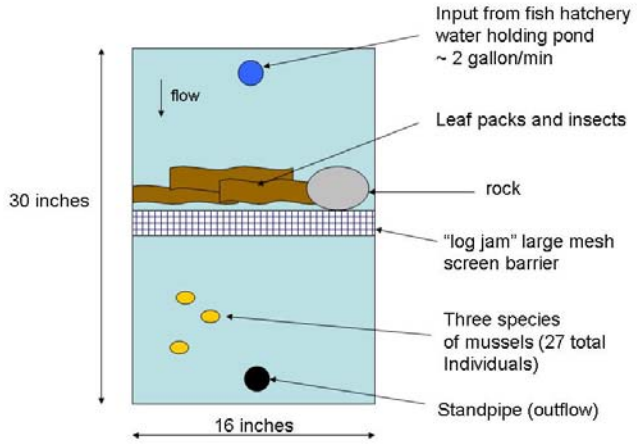

Figure 1. Tank design of preliminary study test tanks. Water flowed in a linear path past shredding insects and leaves before flowing over the filter feeding mussels.

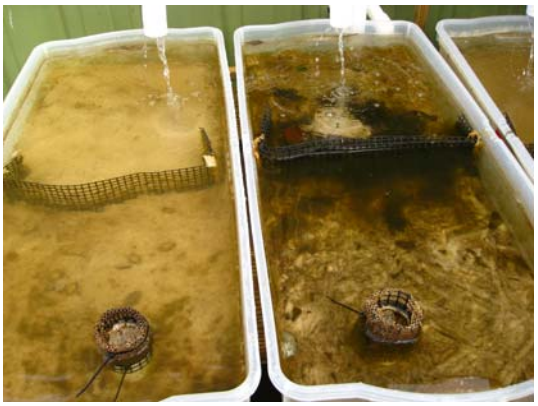

Figure 2. Preliminary study control tank (left) and test tank (right). Shredding insects and leaves (in test tanks only) were kept upstream of the filter feeding mussels, which were at the downstream end of all tanks.

The tanks were examined biweekly. Each time, all leafpack and insects from the experimental tanks were removed and counted. Insect densities were maintained at approximately 50 *Pycnopsyche*, 30 *Tipula*, and 15 *Pteronarcys* per test tank, adding fresh insects and leafpack as necessary. Tanks were cleaned thoroughly once a month by stirring the sand and cleaning the wire mesh barriers. During the other biweekly maintenance visits, tanks were lightly cleaned by waiving away the organic material buildup over the mussel section of the tank, but the mussels were otherwise not disturbed.

The mussels were all measured at the beginning of the experiment. Measurements were taken once a month for all months during the study to monitor growth (this was done during the same trips where the tanks were thoroughly cleaned). At the end of the experiment, tanks were disassembled and the shell length of each mussel was measured a final time.

Laboratory Studies

To examine growth advantages of shredding *Tipula* for the captive rearing of juvenile mussels, a laboratory study was designed to determine if freshwater mussels grow faster in the presence of *Tipula*.

Freshwater mussels are particularly sensitive to ammonia, and ammonia toxicity is lethal to juvenile mussels (Augspurger *et al.* 2003). To ensure that ammonia produced by *Tipula* would not affect mussel health, a preliminary study of *Tipula* ammonia production was conducted. Five *Tipula* held in a 2.5 gallon tub of water produce an untraceable amount of ammonia (mg/L) over a period of 9 days. Tank water was not replaced during the trial. The ammonia concentrations were measured in three separate tanks of craneflies at 0, 3, 6, and 9 days after craneflies were added to the tanks. Water samples were collected and absorbance was measured using the Salicylate method on a Hach spectrophotometer (model DR2800, Hach Company, Loveland, CO). The pH of these tanks was measured on day 7 (model UB-10, Denver Instruments, Bohemia, NY). Although the water was increasingly brown throughout the study, the pH of the water was not different than control water.

Two and a half gallon plastic tanks were used for the laboratory studies (Figure 3). Light was automatically controlled by a 12 hour on, 12 hour off cycle. The air temperature of the laboratory varied daily in a cyclic manner, with cooler temperatures at night. Although the facility has central heating and cooling, facility temperatures did fluctuate at times with outdoor ambient temperatures. Tanks were filled with city water treated with sodium thiosulfate and Ammo Lock (Aquarium Pharmaceuticals, Chalfont, Pennsylvania) to remove chlorine and chloramines. A small fish net with 1mm size mesh was suspended over

the side of the bucket so that the net was submerged, but the rim was out of the water, keeping anything larger than 1mm in size from escaping into the rest of the tank (Figure 3). Autoclaved play sand >300µm in size and 150g of weight covered the bottom of each tank. Two clean airstones were placed in each tank; one in the suspended net and one on the sediment at the bottom of the tank. Mussels were added after the tanks were set up, with attempts to vary the different sizes evenly across all treatments and tanks. Three *Tipula* were added to the submerged net in each tank and 35g of wet processed leafpack was added to each net (except for control tanks).

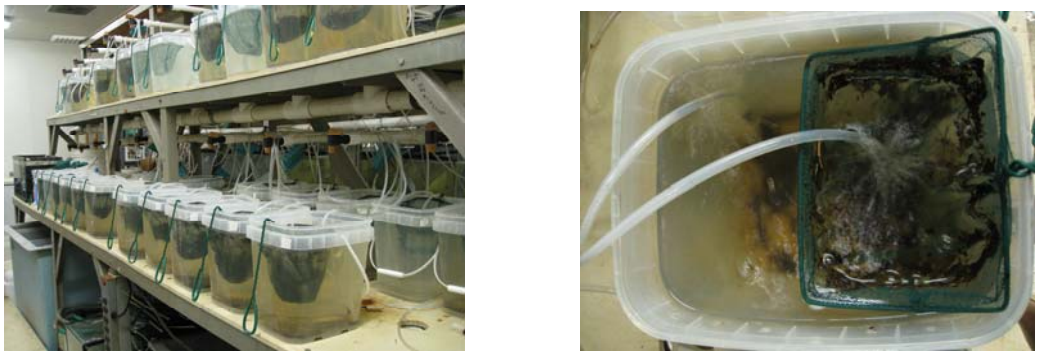

Figure 3: Study tanks holding shredding *Tipula* and juvenile mussels (left), and a close-up of the test tank (right) during studies conducted to assess the growth of juvenile mussels when insect generated FPOM is available.

Fresh leafpack collected from the field was periodically added to replenish the food supply for the *Tipula*. This leafpack was collected from piedmont North Carolina field sites where mussels were also present. All tanks with leafpack were treated the same, including controls with no insects. Before adding fresh, recently collected leafpack to the tanks, it was thoroughly searched and all *Tipula* were removed. The leaves were added to a blender and pulsed a couple times to break up the leaves into smaller pieces and distribute the different species and components of the leafpack. The resulting leafpack was then rinsed through one

of the unused small fishnets (1mm size mesh) to remove all particles smaller than 1mm before adding to the tanks; so that only leafpack pieces larger than 1mm were used.

Tipula assessments were completed once a week to maintain the same number of *Tipula* in each tank. At all times, 2-4 *Tipula* were in the test tanks, making sure that all treatments and replicates had the same number of *Tipula* at any one time. *Tipula* survival was fairly high over the course of the experiments; on average only one new fly larva was added to an individual tank once every few weeks. Different *Tipula* sizes were distributed evenly across all tanks and treatments. *Tipula* were collected at field sites where mussels were also present in the north piedmont region of North Carolina.

The commercial algae used were a mixture of Nanno 3600 (*Nannochloropsis spp.*) and Shellfish diet (*Isochrysis spp., Pavlova spp., Tetraselmis spp.,* and *Thalassiosira weissflogii*), both from Reed Mariculture Inc. (San Jose, CA). One drop of 1:3 Nanno to water and 1 drop Shellfish diet was fed once a day on weekdays to each tank. A 75-80% water change on all tanks was performed once a week, slightly agitating the water to stir up and remove the settled organic matter but not to disturb the sediment or mussels.

Experiment 1

Experiment 1 commenced the first week of May 2010. Four treatments were set up in 5 replicates for each of the two species of mussels:

(1) Commercial algae only, no *Tipula* or leafpack

(2) *Tipula* and leafpack and commercial algae

(3) *Tipula* and leafpack with no commercial algae

(4) Leafpack without *Tipula* and no commercial algae

Lampsilis fasciola juveniles were obtained from the Aquatic Wildlife Conservation Center mussel propagation facility in Marion, Virginia. Juveniles were propagated from adults from the Clinch River in Russell County, Virginia. The mussels were approximately 8 months to a year old when removed from the facility and used in this experiment. They varied in size from 3.3mm to 7.6mm (measured 50 of the 200 animals used). Second generation *Villosa delumbis* were propagated at North Carolina State University (Raleigh, NC) and used in the study. The original wild-caught *V. delumbis* were from the Deep River, NC. The mussels were four months of age and ranged in size from 1.0mm to 1.4mm (measured 50 of the 200 animals used). Ten mussels were added to each tank, and tanks contained only one mussel species. This made 40 tanks total for this experiment.

In the last week of May, *Tipula* were found in the leafpack only (no *Tipula*) treatment. The flies were immediately removed from this treatment. After this event, all fresh leafpack brought into the laboratory was quarantined for two weeks before going through and picking out *Tipula* for addition to tanks, to prevent future contamination of controls.

Mussel length was assessed in 2 of the 5 replicates for *V. delumbis* (8 tanks) and 1 of the 5 replicates for *L. fasciola* (4 tanks) the first week of June (at the 30 day time point). These tanks were harvested and the animals were removed from the experiment, leaving 28 tanks in the experiment after this assessment.

Starting July 15, leafpacks were stirred up with the daily feeding to release particles generated by *Tipula* that were less than 1mm from the nets and into the tank. Also at this time, the replicates were pooled so that only 8 tanks remained, one for each treatment for

each species. All remaining mussels were kept in the experiment at this time. Mussels were assessed for length (70 day time point), and then placed back into the tank.

Because control tanks in experiment 1 were contaminated with larval *Tipula* that were too small to see when the tanks were set up (noted above), a second experiment was conducted. As noted above, leafpacks used in the second experiment were held for two weeks prior to use in the experiment to ensure the larval *Tipula* were of sufficient size to see and remove them from the leafpacks before addition to tanks.

Experiment 2

Experiment 2 commenced July 30, 2010. Three treatments were included for this study, again set up in 5 replicates for each of the two species of mussels:

(1) Commercial algae only, no *Tipula* or leafpack

(2) Leafpack and commercial algae with *Tipula*

(3) Leafpack and commercial algae without *Tipula*

Animals for experiment 2 were propagated at North Carolina State University (Raleigh, NC). *Elliptio lanceolata* were obtained from propagated adults collected from Swift Creek (Tar basin), NC. Juvenile mussels were 1 month old before placing in the experiment. Lengths ranged from 0.2mm to 0.7mm (50 of 150 animals used were measured). Ten mussels were placed in each tank. The *Lampsilis fasciola* used were from propagated adults from the Pigeon River, NC. These juveniles were 2 months old at the start of the study. Individuals ranged from 0.6mm to 1.1mm in length (50 of 450 animals were measured). Thirty *L. fasciola* were placed in each tank. One mussel species was placed in each tank, yielding 30 tanks total for this experiment.

Both experiment 1 and experiment 2 were harvested on Dec 7 (at 210 days for experiment 1 and 130 days for experiment 2). The investigator was not informed of the identity of the individual treatments. The identifying treatment tags were covered up by a third party and not revealed until after all length data were acquired. All mussels in all tanks were measured. An analysis of variance (ANOVA) was conducted to test the potential difference in length of the test treatment and control animals. A Tukey Honest Significant Difference test determined which treatments were different from one another.

Dissolved Organic Carbon samples

At 50 days, we collected 30mL water samples from one random replicate for each treatment from experiment 1. A control of treated laboratory water was also collected. Samples were placed in the refrigerator for two days before filtering and acidifying (with H_3PO_4) the samples for DOC concentration analysis. At 210 days for experiment 1 and 130 days for experiment 2, we collected 8 samples from the experiment 1 tanks, 2 laboratory water samples, 2 samples of laboratory water treated with Ammo Lock, and one from each of two replicates for each of the experiment 2 treatments (12 samples from experiment 2). Samples were refrigerated until tested. Ten days later, samples were filtered and acidified for DOC content analysis.

Results

Preliminary study

Mussel lengths for the preliminary study were recorded and averaged per species and treatment (Tables 2.1 and 2.2). No significant differences were observed between tanks or treatments (Figures 1 and 2 and Tables 2.1 and 2.2). Mussel survival was 100%.

Table 2.1. Mean initial length (mm) and standard deviations (SD) of mussels used in preliminary studies with shredding insects.

L. fasciola		*L. nasuta*		*L. radiata*	
average	SD	average	SD	average	SD
26.93	1.40	37.97	1.58	21.78	1.83

Table 2.2. Mean length (mm) and standard deviations (SD) of mussels at the termination of preliminary studies.

	L. fasciola		*L. nasuta*		*L. radiata*	
	average	SD	average	SD	average	SD
Tank1 test	30.3	1.1	42.5	1.5	26.7	2.4
Tank2 control	30.5	1.7	44	1.2	26.7	1.8
Tank3 test	29.6	1.3	43.1	1.6	26.4	1.7
Tank4 control	30.1	1.4	43	1.3	27.8	1.9
ANOVA p-value	0.5526		0.1449		0.5489	

The preliminary studies helped guide the design and implementation of the laboratory studies. Frequently during the two week maintenance checks of the hatchery study, *Tipula* were found buried in the sand or otherwise missing (and therefore not shredding). Consequently, *Tipula* were held in nets suspended in the tanks during subsequent laboratory experiments. Since this specific genus was found to make the most FPOM and DOM in the laboratory (Greiner *et al.*, in prep), their effect on mussel growth may have been weakened for the hatchery study.

Water temperature at the hatchery may have remained too low for significant mussel growth. Also, frequent handling has been shown to temporarily arrest mussel growth and the delay in growth can last for several weeks (McMohan and Bogan 2001). We delayed measurements during the second laboratory experiment to minimize the effect of handling on mussel growth.

Flow in the hatchery tanks may have been too fast for shredder generated particles to gain bacterial attachment before going down the drain. Although the shredding insects may have been generating food particles for mussels, these particles may need time to become nutritionally beneficial for mussels. Static tanks were set up in the subsequent laboratory studies to allow time for microbial attachment.

Laboratory studies

Experiment 1

Experiment 1 had an assessment of mussel length at 30 days (those animals were removed from the experiment), another assessment of length at 70 days when replicates were combined, and the final assessment at 210 days.

Villosa delumbis held in the presence of *Tipula* and fed commercial algae grew faster than *V. delumbis* receiving just algae, just *Tipula*, or leafpack without algae (Table 2.3 and Figure 4). The lengths of mussels in treatments that were not fed commercial algae did not differ from the algae only control. The addition of *Tipula* and leafpack to tanks that were fed commercial algae increased growth of the *V. delumbis* mussels more than just feeding them commercial algae alone.

Lampsilis fasciola showed no difference between treatments (Table 2.3). The range of lengths of *L. fasciola* at the beginning of the experiment convoluted any discrepancies that may have arisen from the different treatments.

Table 2.3. Mean length (in mm x 10^{-1}) and standard deviations (SD) of mussels held in tanks with or without *Tipula* and with or without the commercial algal diet for experiment 1. 'Algaeonly' is the treatment fed commercial algae, but no *Tipula* or leafpack were in the tanks; 'cranefliesA' is the treatment with *Tipula* and leafpack, and fed algae; 'cranefliesNA' is *Tipula* and leafpack but not fed commercial algae; 'leavesNA' is leafpack without *Tipula* and not fed commercial algae.

	V. delumbis			***L. fasciola***		
30 day harvest						
	average	SD	TukeyHSD	average	SD	TukeyHSD
algaeonly	17	1.6	A	54	13.5	A
cranefliesA	19	2.2	B	44.8	12.3	A
cranefliesNA	15.5	1.6	AC	56.5	12.4	A
leavesNA	15	1.3	C	55.1	13.3	A
ANOVA p-value	1.12E-06			0.46		
70 day						
	average	SD	TukeyHSD	average	SD	TukeyHSD
algaeonly	18.3	2.1	A	60.7	12.1	A
cranefliesA	32.4	5.6	B	61	12.6	A
cranefliesNA	20.1	3.3	A	54.8	11	A
leavesNA	17.6	3.2	A	52.9	10.9	A
ANOVA p-value	2.20E-16			0.07		
210 day						
	average	SD	TukeyHSD	average	SD	TukeyHSD
algaeonly	39	6.9	A	66.4	12	A
cranefliesA	68	11	B	70.1	14.3	A
cranefliesNA	32.3	6.9	A	66.7	9.5	A
leavesNA	-	-	-	54.5	14.8	A
ANOVA p-value	1.28E-11			0.45		

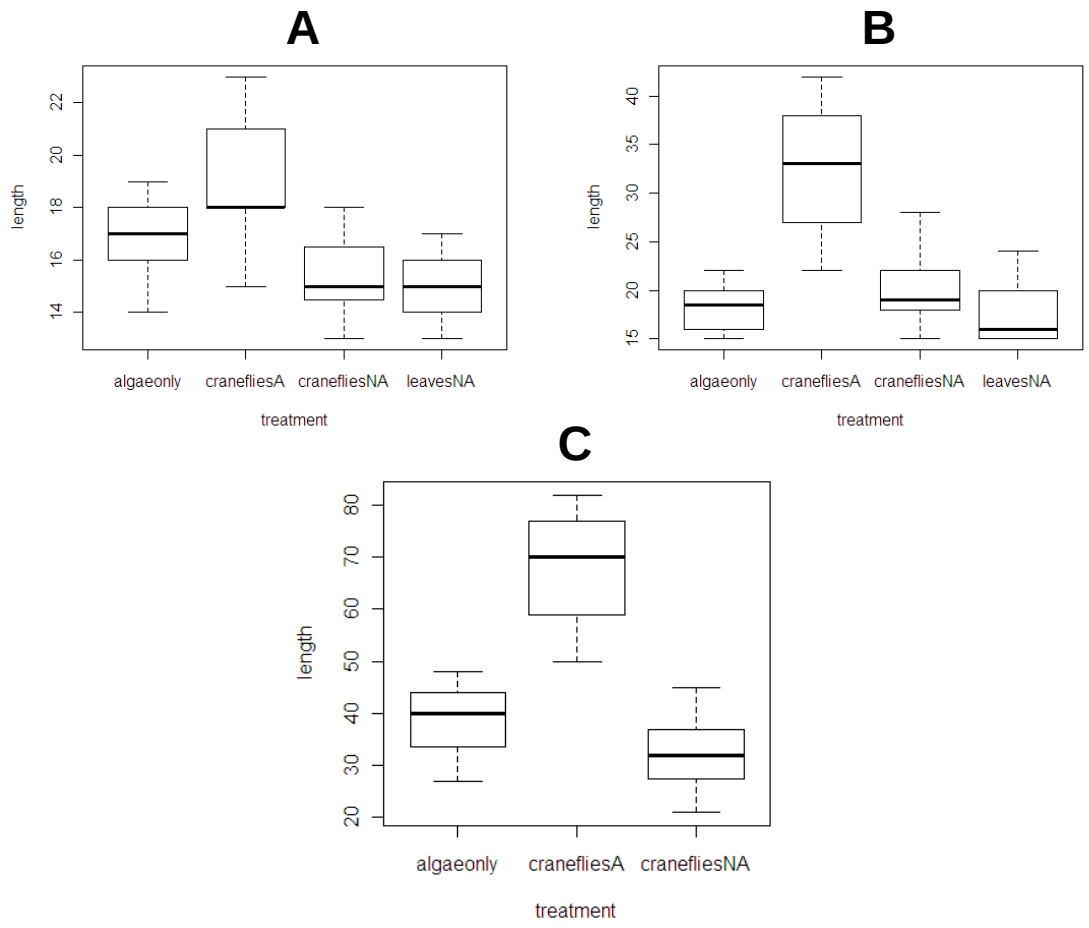

Figure 4. Box plots of *Villosa delumbis* length (in mm x 10^{-1}) held with or without *Tipula* at the 30 day harvest time point (A), 70 day time point (B), and 210 day endpoint (C) in experiment 1. 'Algaeonly' is the treatment fed commercial algae, but no *Tipula* or leafpack were in the tanks; 'cranefliesA' is the treatment with *Tipula* and leafpack, and fed algae; 'cranefliesNA' is *Tipula* and leafpack but not fed commercial algae; 'leavesNA' is leafpack without *Tipula* and not fed commercial algae. There was no survival (and therefore no length data) in the leavesNA treatment at 210 days.

Survival was very low for the leavesNA treatment for both species of mussels (0% for *V. delumbis* and 5% for *L. fasciola*). For *V. delumbis*, survival in the cranefliesNA treatment was not different than treatments fed commercial algae (37% versus 40% and 43% for algae treatments). *Lampsilis fasciola* had decreased survival in both treatments that were not fed commercial algae (10% and 5% versus 38% for the algae treatments).

Experiment 2

Mussels in experiment 2 had their lengths assessed only at the end of the experiment to eliminate the potential effect of handling on mussel growth. *Elliptio lanceolata* showed no difference between the *Tipula* treatment and the algae only control, however the algae only control showed better growth than the leaves control (Table 2.4, Figure 5).

Lampsilis fasciola demonstrated higher growth rates in the presence of *Tipula* than mussels fed only the commercial diet (Table 2.4, Figure 5). The control treatments were not different from one another; and the leaves control was not different from the *Tipula* treatment (Table 2.4). As shown with experiment 1, the addition of *Tipula* and leafpack to tanks that are already fed commercial algae increased growth of the mussels more than just feeding them commercial algae alone.

Table 2.4. Mean length (in mm x 10^{-1}) and standard deviations (SD) of mussels held with or without *Tipula* at 130 days for experiment 2. 'Algae only' is the treatment fed commercial algae, but no *Tipula* or leafpack were in tanks; 'craneflies' was the treatment with *Tipula* and leafpack and fed commercial algae; 'leaves' was the treatment with leafpack and fed algae, but without *Tipula*.

	E. lanceolata			*L. fasciola*		
	average	SD	TukeyHSD	average	SD	TukeyHSD
Algae only	24.4	4.9	A	18.3	4.1	A
craneflies	22.6	3.7	AB	22.4	3.2	B
Leaves	17.8	1.7	B	19.5	3.7	AB
ANOVA p-value	0.01			5.49E-03		

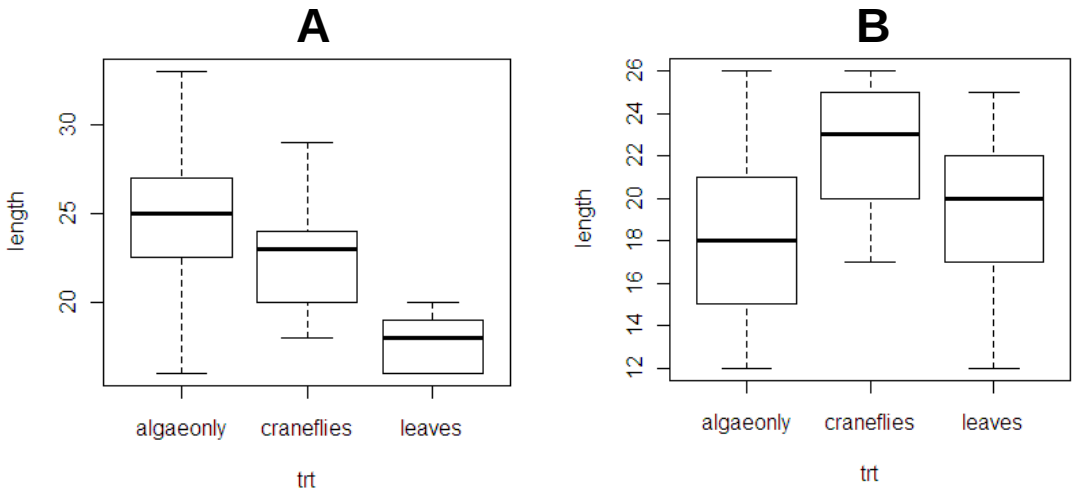

Figure 5. Box plots of mussel length (in mm x 10^{-1}) of *Elliptio lanceolata* (A) and *Lampsilis fasciola* (B) held with or without *Tipula* at 130 days in experiment 2. 'Algae only' is the treatment fed commercial algae, but no *Tipula* or leafpack were in tanks; 'craneflies' was the treatment with *Tipula* and leafpack and fed commercial algae; 'leaves' was the treatment with leafpack and fed algae, but without *Tipula*.

Survival was best in the algae only treatment for both *L. fasciola* (up to 57% versus up to 13% for the *Tipula* treatment and up to 37% for the leaves treatment), and *E. lanceolata* (10-50% versus up to 40% in the *Tipula* treatment and up to 30% in the leaves treatment).

Dissolved organic carbon

In both experiments, tanks with *Tipula* exhibited higher DOC concentrations than tanks without *Tipula* (Tables 2.5 and 2.6). Dissolved organic carbon values were higher in tanks with leafpack than control tanks (Table 2.7).

Table 2.5. Dissolved organic carbon concentrations in experiment 1 comparing the growth of mussels held with or without *Tipula* (mg/L) at 50 and 210 days. 'Algaeonly' is the treatment fed commercial algae, but no *Tipula* or leafpack were in the tanks; 'cranefliesA' is the treatment with *Tipula* and leafpack, and fed algae; 'cranefliesNA' is *Tipula* and leafpack but not fed commercial algae; 'leavesNA' is leafpack without *Tipula* and not fed commercial algae.

	L. fasciola		***V. delumbis***	
	50 days	210 days	50 days	210 days
craneflies and algae	6.24	4.53	6.73	3.87
craneflies no algae	7.38	3.85	7.74	3.88
leaves no algae	6.49	3.24	5.98	3.10
algae only	3.71	2.69	3.65	2.24

Table 2.6. Dissolved organic carbon concentrations in experiment 2 comparing the growth of mussels held with or without *Tipula* (mg/L) at 130 days. 'Algae only' is the treatment fed commercial algae, but no *Tipula* or leafpack were in tanks; 'craneflies' was the treatment with *Tipula* and leafpack and fed commercial algae; 'leaves' was the treatment with leafpack and fed algae, but without *Tipula*.

	L. fasciola		***E. lanceolata***	
	replicate 1	replicate 2	replicate 1	replicate 2
algae only	2.22	1.65	2.71	2.55
leaves	3.81	3.57	3.43	3.23
craneflies	4.37	3.68	4.18	5.02

Table 2.7. Dissolved organic carbon concentrations (mg/L) of city water, laboratory water, and laboratory water treated with Ammo Lock (a compound used to eliminate ammonia from treatment tanks).

Controls

	replicate 1	replicate 2
city water (experiment 1, 50 days)	7.17	not done
lab water	2.40	2.38
lab water with ammolock	5.50	6.06

Discussion

Lampsiline juvenile mussels reared in the presence of shredding *Tipula* demonstrated greater growth than mussels fed commercial algae diets alone. The greater growth demonstrated by *V. delumbis* and *L. fasciola* in the presence of *Tipula* indicates that *Tipula* somehow contribute to an increase in food quality or abundance for some species of mussels. In experiment 1, we saw that *V. delumbis* survival in the cranefliesNA treatment was not

different than treatments fed commercial algae. This suggests that although those mussels grew slowly without the addition of commercial algae food, there is some nutrition provided by the *Tipula* that supports survival and sustenance for this species. Experiment 1 also demonstrated that there was not a density dependence effect on mussel growth since the treatment with the best growth was also the treatment with the best survival (Figure 4). Since the best growth was seen in treatments that were also fed commercial algae, as previously recognized, algae do appear to play an important role in the diet of freshwater mussels (McMahon and Bogan 2001). The commercial algae diet may provide an immediate energy source or serve as a vehicle for the bacteria or other small particles generated by the insects to be gathered by the gills as food for the mussel. Juvenile mussel diets in freshwater mussel propagation facilities may benefit from including both shredding *Tipula* and algae in the diet. In this manner, both the nutritional value and physical presence of algal particles will be supplemented with FPOM, dissolved protein, and other nutrients generated by *Tipula* during feeding (Greiner *et al.* in prep). Filtered water from separate *Tipula* tanks could be added to young juvenile mussel tanks as food on a daily basis to decrease susceptibility to predators, as seen by the decreased survival in treatments with the fresh leafpack in experiment 2. Studies building on this work have been initiated to examine how various filtrates of the products of shredding *Tipula* can be used to supplement juvenile mussel diets. Also, leafpack and shredding *Tipula* could be added to separate chambers of mussel rearing containers to provide a continual dietary supplement. Until we determine the specific elements of the material *Tipula* generate that contribute to mussel growth, some species of

juvenile mussels may benefit from adding shredding *Tipula* to the propagation grow-out systems.

Elliptio lanceolata showed no difference in growth between the *Tipula* treatment and the algae only control. This pleurobemine species may use algae as a bigger component of their diet for growth than the other lampsiline species tested. Nichols and Garling (2000) found that some species of mussels are more herbivorous than others, and retain a higher fraction of algal carbons in their tissue stores. They found that *Lampsilis* species specifically had a lower percentage of algal carbons in their body stores and attribute this difference possibly to this animals' different digestive capabilities.

Tipula generate more FPOM and DOM than other shredding insects tested (Greiner *et al.* in prep). Additionally, we found evidence that *Tipula* treatments created larger molecular weight DOM molecules and increased levels of microbial-derived protein than was observed with the other species tested. *Tipula* have a highly efficient digestion system (Martin *et al.* 1980). Although it is accepted that most gastrointestinal tracts of shredding insects house bacteria that aid in digestion and energy extraction of detritus (Klug and Kotarski 1980), Sinsabaugh and coworkers (1985) found that only *Tipula* (of *Tipula*, *Pycnopsyche* and *Pteronarcys*) have endosymbiotic bacteria in their gut. These bacteria have been found to be cellulose-degrading bacteria (Sinsabaugh *et al.* 1985). Other shredding insects obtain the necessary bacteria needed for digestion from their diet. Also, *Tipula* have unique hindgut morphology with an extra fermentation chamber (Sinsabaugh *et al.* 1985) that houses a diverse community of epimural and loose bacteria (Klug and Kotarski 1980). The region just anterior of the rectum is the densest with bacteria (Klug and Kotarski 1980). It follows that

Tipula probably excrete these bacteria in feces to some degree. With the abundance and ubiquity of *Tipula* in streams, it is possible that *Tipula* mediated bacteria production contributes to the diet of freshwater mussels (and probably other aquatic animals) in nature.

Tipula appeared to increase DOC concentrations in the tanks. When the breakdown of leaves was unaided by shredding insects, DOC concentration also increased in the tanks (as demonstrated by the "leaves only" control), but not as much as with shredding *Tipula* present. Adding Ammo Lock raised DOC values to levels present in *Tipula* treatments. Ammo Lock was added with the weekly water changes, but after the DOC samples were taken from the experiment tanks. It is likely that either the DOC from the Ammo Lock broke down within a week or it was getting used by the mussels and then not replenished until the weekly water change. In saltwater oysters, soluble organic nutrients are directly absorbed and used for growth and metabolism (Kennedy *et al.* 1996). Roditi and coworkers (2000) found that zebra mussels get up to 50% of their carbon needs from DOC. A study designed to determine DOC uptake from unionids would be useful.

Limitations of the study

Experiment 2 was shorter than experiment 1 by 1.5 months, and the following other differences may have contributed to a weakened response from the different treatments in experiment 2:

(1) Generally speaking, older mussels use suspension feeding whereas younger mussels also use pedal feeding of the sediment (McMahon and Bogan 2001). The younger mussels in experiment 2 may have shown less of an effect from *Tipula* due to their limited access to the nutritious *Tipula*-generated matter that was only in suspension.

(2) Most mussels grow the most during the spring in the wild (Negus 1966). Experiment 2 was not assembled until late July.

(3) The earlier life stages of *Tipula* may have a greater impact on leaf litter breakdown due to higher densities and faster feeding (Cummins *et al.* 1973). These earlier life stages were seen in experiment 1, but not experiment 2. This could potentially decrease the effect of *Tipula* in experiment 2.

(4) *Tipula* (along with many other shredding insects) have a summer "shut-down" period (Cummins 1974). This may have coincided with a larger time block of experiment 2 than experiment 1, decreasing the effect of *Tipula* treatment in experiment 2.

(5) In experiment 2 (and the second half of experiment 1), the leafpack used was quarantined for 2 weeks before addition to mussel tanks. This process may have changed the quality of the microbial community, making the *Tipula* less effective eaters, possibly generating less nutritious particles for mussels.

Further studies

This study documented that the presence of other macroinvertebrates may contribute to the growth of freshwater mussels. Additional studies are needed to understand the manner in which *Tipula* contribute to the diet of juvenile and adult mussels. Stable isotope analysis of mussel tissue and *Tipula* generated particulates would determine if the mussels assimilate the *Tipula*-mediated material in their body stores. A nutrient analysis on the *Tipula* generated particulates (such as percent carbohydrates, protein, lipids, cholesterol, etc), in addition to research on the dietary needs of freshwater mussels, would help us further

understand the potential need for *Tipula* in freshwater mussel habitats and the role their co-culture can play in mussel propagation facilities.

CONCLUSIONS

Tipula generated the most FPOM and DOM of all shredding insects tested. The DOM generated by *Tipula* was larger in molecular weight and included higher levels of microbial degradation and microbial-derived protein than the other shredding insects or controls. This distinctiveness may be attributed to *Tipula's* unique gut structure and associated bacteria. Since shredding insects contribute greatly to the FPOM in streams (Cummins *et al.* 1989), *Tipula's* special shredding process may impact the overall health and function of stream ecosystems.

Lampsiline mussels demonstrated greater growth in the presence of *Tipula* than mussels provided with just a commercial algal diet. Algae also seem to play a role in freshwater mussel nutrition, since mussel growth was best when diets of shredding *Tipula* products were supplemented with algae. *Tipula's* unique contributions to freshwater DOM and FPOM apparently have a positive impact on food quality or abundance, and overall juvenile freshwater mussel nutrition.

Future research

Additional work is necessary to identify and describe what components of the *Tipula* shredding process benefit mussels and how *Tipula* contribute to juvenile freshwater mussel diets and health. Analysis of juvenile mussel feeding would help clarify the specific food items generated by *Tipula* that are used by mussels. Knowing the size of selected food and exactly how the juveniles obtain it from their surroundings would help specialists improve the diets fed to freshwater mussels in captivity. For example, zebra mussels directly absorb DOM and it may play a significant role in their diet and metabolism, especially when FPOM

levels are low (Baines and Fisher 2005). The relationship could be the same for unionids, and suggests that a liquid diet of dissolved nutrients might be a viable adjunct to diet preparation when they are held in aquaria. Stable isotope analysis of *Tipula* products and mussel tissues would help determine a food web connection between these two organisms (DeNiro and Epstein 1978, Fry 2006).

Quantifying the functional assemblage of the microbial community in the *Tipula* products using a test like phospholipid fatty acid analysis (Findlay 1996) would help determine what function the microbes in the *Tipula* products perform. By comparing the *Tipula* mediated microbes with known microbial structures, we could identify different functional groups (like cellulose-degrading bacteria) or look at the microbial community structure. *Tipula's* assimilation efficiency of cellulose was the highest of three shredding insects tested in one study, its digestion aided by its microbial symbionts (Sinsabaugh ***et al.*** 1985). Mussels may be able to use bacteria expelled by *Tipula* in the same way, ingesting the bacteria for aid in digestion of other particulates. Or perhaps the *Tipula* products provide a food source for the bacteria, which are then ingested by the mussels as a direct food source.

In these studies, we did not consider other FPOM contributors like crustaceans or snails (*Gammarus* or crayfish, for example), which may also affect nutrient availability to mussels. *Gammarus* can be significant contributors to FPOM in streams when their numbers are high (Anderson and Sedell 1979), and it has been found that *Gammarus* in aquaria generated FPOM chemically similar to FPOM collected from streams (Yoshimura ***et al.*** 2008). A study examining *Gammarus* production and their potential effect on juvenile freshwater mussel growth would provide an informative comparison to this study.

Application of research

Although we do not know exactly how and why *Tipula* benefit mussels, we have demonstrated a positive effect. Propagation facilities working with rare mussel species will want to consider the use of *Tipula* in their rearing systems for juvenile mussels. Although only the lampsiline species showed positive results in our study, it is possible that other mussel species would benefit as well. Since *Tipula* are relatively easy to find and collect in the field and no detrimental effects of *Tipula* on mussels were found in our study, studies should be conducted to determine how *Tipula* can be integrated into captive rearing protocols for unionids. To avoid predation problems associated with the addition of stream-collected leafpack to early stage juvenile mussel tanks, filtered water taken from tanks with shredding *Tipula* could be added to the juvenile mussel tanks as a food supplement.

Population augmentation, streamside infestations and translocation efforts may benefit by the examination of proposed sites for shredding insects. Increased growth of some species of juvenile mussels in the presence of *Tipula* in our laboratory indicate the possibility of the same effects in the field. Until this is tested, it seems justified to choose sites with communities of *Tipula* in close proximity. Some relocation efforts of freshwater mussel populations have been unsuccessful (Nichols and Garling 2000). Dunn and Sietman (1997) state that the poorly understood ecology of mussels limits the success of translocation efforts. We are uncertain of all the necessary requirements for their habitat, and have no accepted set of guidelines for site selection for mussel population enhancement (Layzer and Scott 2006). The identification of natural food resources is critical for successful choosing of suitable sites for translocated or augmented populations (Vaughn *et al.* 2008). If *Tipula* are indeed

contributors to freshwater mussel diets, surveys should show the presence of *Tipula* at potential sites before they are selected for mussel population augmentation.

Tipula may provide a more important ecological function in stream ecosystems than previously recognized. Their release of unique nutrients into the overall ecosystem likely impacts other aquatic species. Freshwater mussels have been shown to benefit from shredding *Tipula*, but there are probably other unknown effects contributing to the intricate food web relationships between this common shredding insect and filter feeders. The relationship between shredding insects and filter feeding insects has been examined in several different studies (Heard and Richardson 1995), but we need to broaden our scope and look across different types of stream macroinvertebrates to see new, undiscovered relationships. These studies have begun the investigation of this required work, and found even more questions that need answers.

Not only have we expanded our understanding of *Tipula* DOM generation and the complexity of freshwater mussel diets, but we have supported the importance of biodiversity by identifying new food web links across different types of animals. Certainly there are more connections like this one that have yet to be discovered. Each species has the potential to affect the health and survival of other species. Until those links are found, we need to aggressively argue for conservation of the biodiversity in aquatic ecosystems. These systems continue to fascinate and surprise even the scientists that work and think about these environments every day.

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