RESEARCH & DEVELOPMENT

Investigation of Factors Contributing to the Decline in the Nutritional Health of *Alasmidonta raveneliana* in the Little Tennessee River, Franklin, North Carolina

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16. Abstract

The Appalachian elktoe (*Alasmidonta raveneliana*) is found at geographically fragmented locations in Western North Carolina and Tennessee. The species is imperiled and was federally listed as endangered in 1994. Populations in the Little Tennessee River appear to have suffered approximately a 90 percent reduction and additional impacts to this population could result in its extirpation. Field observations had suggested that a decline in food resource availability could be contributing to the continued decline in the Little Tennessee River *A. raveneliana* population. Numerous factors including changing sediment loads, the presence of an invasive species (e.g. *Corbicula fluminea*), geochemical changes or the chronic release of pollutants could be associated with food resource depletion. Studies were conducted to examine the likelihood that the nutritional health of Appalachian elktoe populations has been impaired. Sentinel *A. raveneliana* and *Lampsilis fasciola* were held at three sites in the Little Tennessee River and three sites in the Tuckasegee River. The survival and growth of both species was measured and their nutritional health status assessed relative to available seston and other food resources supporting their diets in the two rivers systems. Concurrent assessment of water column and sediment microbial populations was conducted to develop profiles of microbial communities and examine how relative changes over time may be contributing to the decline in *A. raveneliana* populations in the Little Tennessee and Tuckasegee Rivers. Metabolic profiles of gill tissue were developed to examine the health status of the sentinel animals. Scanning electron microscopy of gill tissue and the complementary histopathologic assessment of *A. raveneliana* tissues was used to identify pathologic changes that could potentially be associated with the decline. Little growth was observed in the *A. raveneliana*. In contrast, the *L. fasciola*, which were markedly smaller in size at the onset of the project, grew rapidly during the study period. Mortality was observed in *A. raveneliana* at five of the six sites and in both rivers. No mortality was observed in *L. fasciola.* Microbiome studies of the water column and sediment documented marked seasonal and site variability in prokaryotic and eukaryotic stream microfauna and microflora. Similar variability was noted in stream metabolic profiles and the presence of dissolved and fine particulate organic matter, but was not specifically correlated with mussel mortality. Metabolic profiles of the *A. raveneliana* indicated the animals were in a state of tissue catabolism. Scanning electron microscopic studies revealed the presence of a "biofilm" on the gills of *A. raveneliana* that could be impeding respiration and food particle collection. Specific recommendations for continued study and species conservation were provided. A website, the Little Tennessee Water Quality Initiative was created to make study data publically available.

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Executive Summary

A rapid decline in *Alasmidonta raveneliana* populations in the Little Tennessee River prompted theses studies. High densities of the Asian Clam, *Corbicula fluminea* in the river suggested that the decline could be related to the presence of this invasive species and competition for food resources. These studies were conducted to examine the hypothesis that impaired nutritional health has contributed to the decline of *A. raveneliana* populations. Accordingly, we compared and contrasted the nutritional health of *A. raveneliana* populations in the Little Tennessee and Tuckasegee Rivers. Sentinel *A. raveneliana* were held in cages at three selected sites in both rivers. Sentinel *Lampsilis fasciola* were held at the same sites as a control. The survival and growth of both species was measured and their nutritional health status assessed relative to available seston and other food resources supporting their diets in the two rivers systems. Ambient water quality parameters, pH, dissolved oxygen, turbidity and temperature were obtained approximately biweekly at each study site. In addition, water temperature was continuously monitored at each site using digital temperature data loggers. Water samples were also obtained for characterizing the presence of fine particulate organic matter, conducting stable isotope analysis, water column and sediment biochemistry and to develop water and sediment profiles of the prokaryotic and eukaryotic communities in both rivers at the sentinel study sites. The health of the sentinel animals was evaluated by performing gross necropsies and histopathologic evaluation of the harvested tissues. Scanning electron microscopy was used to examine the gills of selected species. In addition, metabolomic profiles were generated to assess the overall metabolic health of the sentinel mussels.

Alasmidonta raveneliana mortality was observed at five of the six study sites. All *A. raveneliana* exhibited little growth throughout the study. In contrast, no mortality was observed in the *L. fasciola*. The *L. fasiciola* animals were captive reared and smaller at the onset of the study and they displayed substantial growth throughout the study period. There was no correlation between *Corbicula* density at the study sites and mussel mortality. On average, water temperature was higher in the Little Tennessee River than the Tuckasegee River throughout the study period. Marked seasonal variability was observed in both prokaryotic and eukaryotic organisms in the water column and the sediment. Metabolic profiles documented the poor nutritional health of *A. raveneliana* and their where biochemical differences observed in glycogen, amino acid metabolism and other metabolic parameters in the animals held at Tuckasegee site one when contrasted with that of the other study sites. Gross examination of the *A. raveneliana* suggested marked tissue catabolism and histopathologic assessment identified a paucity of immunologically important hemocytes. Scanning electron microscopy indicated the presence of a "biofilm" coating the gills of the sentinel *A. raveneliana*, which could be impairing respiration and particle filtering. The data garnered during this study has enhanced our understanding of the nutritional health of *A. raveneliana* populations in the Little Tennessee and Tuckasegee Rivers, as well as the role food resource availability may be playing in population declines. However the mortality observed did not appear correlated with the density of *Corbicula* in the rivers. Although the mortality observed did not appear correlated with the density of *Corbicula* in the Rivers, we have concluded that the nutritional health of the *A. raveneliana* has

been compromised. Specific recommendations included: 1) conducting additional studies to identify the potential origin of the "biofilm" coating observed on *A. raveneliana* gills; 2) refining the use of ecosystem health assessment techniques used during these studies; 3) developing a specific protocol for identifying sites for potential population augmentation; and 4) working with state and federal agencies to establish a Rapid Response Team to investigate episodes of precipitous decline in freshwater mussels and other species.

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INTRODUCTION

Background and Literature Review

The Appalachian Elktoe (*Alasmidonta raveneliana*) is found at geographically fragmented locations in Western North Carolina and Tennessee (Fridell 2003). In Tennessee, it is only found in a short reach of the Nolichucky River. In North Carolina, relatively small, scattered populations still remain in the Nolichucky, Upper French Broad, and Little Tennessee River systems (USFWS 2003). The species is imperiled and was federally listed as endangered in 1994.

The relatively thin-shelled Elktoe predominately resides in lotic rocky streams with a stable substrate (Fridell 2003). No single specific factor has been associated with its extirpation from rivers systems in Tennessee and NC. However, water impoundments that limit stream flow, and construction related activities that disrupt and alter stream sediments have been suggested as reasons for their decline (Fridell 2003).

The Little Tennessee River is essential to the recovery of the Appalachian Elktoe in NC. The River, in Western North Carolina has its origins in the Blue Ridge Mountains in Georgia, from where it flows North into NC and then northwest into Tennessee (NCDWQ and LTBA 2008). However, the Little Tennessee River Elktoe population has experienced a precipitous decline since 2004. Prior to 2005, the Little Tennessee River population of the Appalachian Elktoe had been considered the healthiest of remaining populations. The quality of habitat in the river and the overall number and year classes of *A. raveneliana* suggested that the population was stable. However, in 2005, biologists with the NC Wildlife Resources Commission documented a substantial decline in the numbers of Appalachian Elktoe at several sites scattered throughout the occupied reach of the river (Steve Fraley, NCWRC, pers. comm. 2005). The cause(s) of this decline was unknown, but the decline appeared to be continuing (Steve Fraley, USFWS, pers. comm. 2010) prior to the initiation of these studies and continues today (Jay Mays, USFWS, 2015). To date, this population appears to have suffered approximately a 90 percent reduction in numbers. Any additional impacts to this population could result in its extirpation.

Figure 1: General map showing the location of the Little Tennessee River Basin.

Critical habitat is defined in the Endangered Species Act as habitat that is essential to the conservation of the species. The US Fish and Wildlife Service (USFWS) designated the reach of the Little Tennessee River, from the dam at Lake Emory downstream to the backwaters of Fontana Reservoir as critical habitat for the Appalachian Elktoe (NC DWQ/LTWA-2008).

The primary hypothesis that prompted these studies was that an invasive species, the Asian clam (*Corbicula fluminea*), may contribute to the decline due to direct competition with *A. raveneliana* for food resources. *Corbicula* feeding is relatively nonselective, and their active pedal feeding may provide them with an ecologic advantage when food resources are scarce (Boltovskoy et al. 1995). Atkinson and coworkers (2011) had documented that freshwater bivalve diets may be broader than previously considered, but noted that the diet of *C. fluminea* may place it in direct competition with native unionids for seston food resources. Belanger and coworkers (1990), and later Phelps (1994) noted that when *Corbicula* were found at high densities unionid growth rates declined. *Corbicula* densities in the Little Tennessee have increased and could significantly be impacting the availability of food resources for native unionids in the River. Others factors identified that appear temporally coincident with the decline of *A. raveneliana* in the Little Tennessee include heightened storm flows that occurred during hurricane Francis and Ivan, which inundated the region in September 2004 (Fraley and Simmons 2006), and its antithesis, drought conditions present in NC since 2006 that have possibly reduced stream base flow.

Prior Studies

Since *A. raveneliana* is an endangered species and initial studies focused on the examination of moribund animals for evidence of pathogen infection. The body condition of the collected specimens was poor, with less than anticipated body mass for the size of the shell (Fraley, pers. comm.). Histopathologic examination of tissues from a small number of animals detected no pathogens or lesions compatible with chronic toxic changes. In another study the nutritional health of *A. ravenenliana, E. dilitata* and *Lampsilis fasciola* from the Little Tennessee was compared with *A. varicosa* obtained from two other systems. Protein values were similar, and reflected values previously documented in other freshwater mussel species. But trace mineral analysis of body tissues and shell from *A. raveneliana* were comparable with *A. varicosa* and the other species with one exception. Manganese levels in *A. raveneliana* were 2-4 times that observed in the other species. Additional laboratory studies confirmed the nonselective feeding habitats of *C. Corbicula* and suggested that the invasive clam could be competing for the same food resources as *A. raveneliana.*

Definition of Need

The health of an aquatic ecosystem and its ability to support native fauna is defined by the complex interactions of its biotic and abiotic components. Individual species have different thresholds of tolerance and may decline in health and abundance when the mix of variables that define a system change and alter water quality, food-

resource availability, or stream substrates. The introduction of non-native species may alter the availability of food resources, and possibly out compete native species for spawning sites or alter water quality (Atkinson et al. 2011). The Little Tennessee River population of the Appalachian Elktoe is critical to the conservation of the species. When an endangered or threatened species is present at a site proposed for a crossing structure, additional environmental considerations must be met to minimize potential additional impact to resident imperiled species. In addition, the review process is often delayed and construction costs elevated. Factors contributing to the decline of the Appalachian Elktoe need be identified and if possible, the decline reversed to prevent its extirpation from the Little Tennessee River. In addition, documenting the cause(s) of the decline will assist resource agencies in preventing similar declines in other streams supporting *A. raveneliana* populations.

Purpose and Scope

Alasmidonta raveneliana populations in the Little Tennessee River have declined markedly since 2004. Land-use changes and other anthropogenic factors are often suggested as reasons for declines such as those being observed in the Little Tennessee *A. raveneliana* populations. However, the presence of *C. fluminea* in the river suggested that the decline could be related to high densities of this invasive species rather than directly associated with anthropogenic factors. Prior studies have documented that *C. fluminea* may compete with native mussels for food resources, and these studies were conducted to examine the hypothesis that impaired nutritional health is contributing to their decline. In these studies we compared and contrasted the nutritional health of *A. raveneliana* populations in the Little Tennessee and Tuckasegee Rivers. Sentinel *A. raveneliana* were held at three selected sites in both rivers. Their survival and growth were measured and their nutritional health status assessed relative to available seston and other food resources supporting their diets in the two rivers systems. Concurrent assessment of water column and sediment prokaryotic and eukaryotic communities in both rivers facilitated examination of their potential contribution to the diet of *A. raveneliana* at the study sites. The data garnered during this two-year effort has enhanced our understanding of the nutritional health of *A. raveneliana* populations in the Little Tennessee and Tuckasegee Rivers, as well as provided critical information about the diets of mussels and the role food resource availability may be playing in population declines

RESEARCH APPROACH/ORGANIZATION OF REPORT

Field surveys conducted by NC Wildlife Resources Commission personnel had suggested a potential relationship between the introduction and recent increase in *C. fluminea* populations in the Little Tennessee River and the decline of *A. raveneliana* populations. Initial laboratory studies in our laboratory documented that *C. fluminea* ingests particles of a similar size, and field studies but Atkinson and coworkers (2011) suggested that *C. fluminea* compete for food resources in streams where they are sympatric. In initial field studies (noted above) that compared the growth of a small number of *A raveneliana, E. dilitata* and *Lampsilis fasciola* held at sites with different

densities of *C. fluminea,* only a small difference in mussel growth was observed, and only at one study site. *A. raveneliana* obtained from the Tuckasegee at the time the study was completed were in better body condition than those obtained from the Little Tennessee River. The basic hypothesis driving these studies was that the decline of *A. raveneliana* in the Little Tennessee River was associated with an increase in density of *C. fluminea*. Consequently, we hypothesized that the population decline of the Appalachian Elktoe was food-resource related. Since populations of *C. fluminea* were believed greater in the Little Tennessee River and *A. raveneliana* populations in the Tuckasegee River were thought to be thriving we conducted a multi-seasonal in-situ sentinel mussel study in the Little Tennessee River and the Tuckasegee Rivers. Appalachian Elktoe were harvested from the Tuckasegee River and placed in cages in both rivers. If our overall hypotheses that the *C. fluminea* in the Little Tennessee River were out-competing *A. raveneliana* for food resources was correct we anticipated that caged *A. raveneliana* in the Little Tennessee River would experience a decline in nutritional health and display greater mortality than those held in cages in the Tuckasegee River. **In this report we describe these studies that were conducted with the following objectives:**

Objective 1: Compile and review existing environmental resource data for the Little Tennessee watershed;

Objective 2: Compare and contrast the survival, growth and nutritional health of Alasmidonta raveneliana held in cages in the Little Tennessee and Tuckasegee Rivers;

Objective 3: Measure the availability of food-web resources in the Little Tennessee and Tuckasegee Rivers at selected study sites;

Objective 4: Develop a microbial profile of the Little Tennessee and Tuckasegee River sediment at the selected study sites;

Objective 5: Compare and contrast the use of fine particulate organic matter, bacteria, and algae by juvenile A. raveneliana and Corbicula fluminea;

Objective 6: Develop recommendations for the conservation of Alasmidonta raveneliana in the Little Tennessee Watershed.

The body of the report is organized into chapters based on these stated study objectives.

CHAPTER 1

Compilation and Review of Existing Environmental Resource Data for the Little Tennessee Watershed and Site Characterization

The Little Tennessee River is located in western NC where NC abuts Tennessee and Georgia. The 1,797 sq. mile basin includes more than 2,500 miles of streams and rivers and serves as a water resource for municipalities and a population of more than 94,000 (2010 census). The Little Tennessee River flows North from Georgia into North Carolina and had previously supported a thriving *A. raveneliana* population. However a major die-off of the species was documented in 2004. A continuing decline in the *A. raveneliana* population was noted in 2005 (Fraley and Simmons 2006). Efforts to investigate an event or events contributing to a population health problem begin after the initial cause occurs. Consequently retrospective assessment of previously collected data is needed to potentially identify factors that may have contributed to the problem. Accordingly, we reviewed and cataloged a compilation of existing historical data resources from local municipal (e.g. Macon County SWCD), state (e.g. NC DWQ, NCWRS) and federal agencies (e.g. USGS, USFWS). The data was reviewed to identify potential factors that may have contributed to their decline. A website was established to serve as a repository of the information compiled. It serves as a portal to additional sites with related information. The site, The Little Tennessee Watershed Initiative was created using a user-friendly web site information system, Word Press, and posted to a unique project specific URL (http://littletnwi.org/). As envisioned, the site serves as a research resource for individuals working in, or studying the Little Tennessee Watershed. The site is intended as a fluid resource that will evolve overtime as additional data is added and new links to other studies are created. The following data resources have been posted on the site for open public access (Table 1) and the following links to other water quality and research initiatives in the basin were created.

Site Characterization

Six sites, 3 in the Little Tennessee River and 3 in the Tuckasegee River were selected for housing cages for these studies (Figures 2 & 3, Table 2). These six sites had been used previously for preliminary our studies.

Table 1. Resources linked on the Little Tennessee Watershed Initiative (littletnwi.org) website.

Resources	Link Address
Watershed Resources	
General Information	
LITTLE TENNESSEE RIVER BASIN	http://littletnwi.org/wp-content/uploads/2013/07/final_web_littletennessee.pdf
BASIN 7 LITTLE TENNESSEE	http://littletnwi.org/wp-content/uploads/2013/07/B07_LittleTenn.pdf
15A NCAC 02B .0303 LITTLE TENN RIVER BASIN AND SAVANNAH RIVER DRAINAGE AREA	http://littletnwi.org/wp-content/uploads/2013/07/15a-ncac-02b-.0303.pdf
BASINWIDE ASSESSMENT REPORT LITTLE TENNEESSEE RIVER BASIN	http://littletnwi.org/wp-content/uploads/2013/07/LTN-2010-Basin-Assessment-Report.pdf
Little Tennessee River Basin Ambient Monitoring System Report	http://littletnwi.org/wp-content/uploads/2013/07/LTN-River-2010-Report.pdf
River Restoration Case Study: Hydropower Reform Little Tennessee River Basin Georgia, North Carolina, Tennessee	http://littletnwi.org/wp-content/uploads/2013/07/little_tenn_hydro_case_study_04.pdf
NCDENR: RIVER BASIN RESTORATION PRIORITY PLANS	http://portal.ncdenr.org/web/eep/rbrps/little-tennessee
Water Report Cards	
Caler Fork	http://littletnwi.org/wp-content/uploads/2013/07/Caler_Fork_mini.pdf
Bradley Creek	http://littletnwi.org/wp-content/uploads/2013/07/Bradley_ck_mini.pdf
Barkers Creek	http://littletnwi.org/wp-content/uploads/2013/07/Barkers_ck_mini.pdf
Tellico Creek	http://littletnwi.org/wp-content/uploads/2013/07/Tellico_ck_mini.pdf
Skeenah Creek	http://littletnwi.org/wp-content/uploads/2013/07/Skeenah_ck_mini.pdf
Rabbit Creek	http://littletnwi.org/wp-content/uploads/2013/07/Rabbit_ck_mini.pdf
Peeks Creek	http://littletnwi.org/wp-content/uploads/2013/07/peeks_ck_mini.pdf
Water Quality	
Stream denitrification across biomes and its response to anthropogenic nitrate loading	http://littletnwi.org/wp-content/uploads/2013/07/nature06686.pdf
Quality of suspended fine particulate matter in the Little Tennessee River	http://littletnwi.org/wp-content/uploads/2013/07/10.1023_B-HYDR.0000026482.10915.pdf
Request Approval of the 2012 Hiwassee, Little Tennessee & Savannah River Basinwide Water Quality Plans	http://littletnwi.org/wp-content/uploads/2013/07/WQC2012Presentation_HIW_LTN_SAV.pdf
NORTH CAROLINA STATE UNIVERSITY Water Quality Programs: LITTLE TENNESSE RIVER BASIN	http://www.water.ncsu.edu/litten.html
Tennessee Department of Environment and Conservation: Little Tennessee River Watershed Water Quality Management Plan	http://www.tn.gov/environment/water/watersheds/little-tennessee-river.shtml
Baseflow Turbidity of Tributaries to the Upper Little Tennessee River, North Carolina and Georgia	
BASEFLOW TURBIDITY OF TRIBUTARIES TO THE UPPER LITTLE TENNESSEE RIVER, NORTH CAROLINA AND GEORGIA	http://littletnwi.org/wp-content/uploads/2013/07/martin_leslie_a_200805_ms.pdf
BASEFLOW TURBIDITY OF TRIBUTARIES TO THE UPPER LITTLE TENNESSEE RIVER, NORTH CAROLINA AND GEORGIA presentation	http://littletnwi.org/wp-content/uploads/2013/07/martin.pdf
Educational Curriculum	
Upper Little Tennessee Watershed Water & Habitat Quality Educational Curriculum	http://littletnwi.org/wp-content/uploads/2013/07/10455.pdf
Ecology	
Ecological Significance of the Little Tennessee Basin	http://littletnwi.org/wp-content/uploads/2013/07/Chapter-12.pdf
FISHING FOR ANSWERS: AN ANALYSIS OF BIOMONITORING TRENDS IN SEVEN DIFFERENT WATERSHEDS WITHIN THE LITTLE TENNESSEE RIVER BASIN	http://littletnwi.org/wp-content/uploads/2013/07/10415.pdf
Longitudinal patterns of metabolism in a southern Appalachian river	http://littletnwi.org/wp-content/uploads/2013/07/1468267.pdf
The Land Around the River	
Land-Use Changes in Southern Appalachian Landscapes: Spatial Analysis and Forecast Evaluation	http://littletnwi.org/wp-content/uploads/2013/07/10.pdf
Temporally and spatially uniform rates of erosion in the southern Appalachian Great Smoky Mountains	http://littletnwi.org/wp-content/uploads/2013/07/martin_leslie_a_200805_ms.pdf
Watershed Fauna	
General Information	
Tennessee River Facts	http://littletnwi.org/wp-content/uploads/2014/04/TN_River-Facts.pdf
Little Tennessee River basin	http://littletnwi.org/wp-content/uploads/2014/04/LTRB-Full.pdf
Stream biodiversity: The ghost of land use past	http://littletnwi.org/wp-content/uploads/2014/04/PNAS-1998-Harding-14843-7.pdf
FISHING FOR ANSWERS: AN ANALYSIS OF BIOMONITORING TRENDS IN SEVEN DIFFERENT WATERSHEDS WITHIN THE LITTLE TENNESSEE RIVER BASIN	http://littletnwi.org/wp-content/uploads/2014/04/10415.pdf
Fish	
Effects of Riparian Forest Removal on Fish Assemblages in Southern Appalachian Streams	http://littletnwi.org/wp-content/uploads/2014/04/j.1523-1739.1999.98172.x.pdf
Reintroduction of Four Imperiled Fishes in Abrams Creek, Tennessee – Imperiled Fish and Mussel Management in the Little Tennessee River of Western North Carolina	http://littletnwi.org/wp-content/uploads/2014/04/1528-7092%282005%29004%5B0093%3Arofiff%5D2.0.co%3B2.pdf
Invertebrates	
Invertebrate food webs along a stream resource gradient	http://littletnwi.org/wp-content/uploads/2014/04/j.1365-2427.2002.00786.x.pdf
Production of benthic macroinvertebrate communities along a southern Appalachian river continuum	http://littletnwi.org/wp-content/uploads/2014/04/j.1365-2427.1997.d01-578.x.pdf
Longitudinal changes of macroinvertebrate communities along an Appalachian stream continuum	http://littletnwi.org/wp-content/uploads/2014/04/f95-247.pdf
Mussel Surveys Associated with the Duke Power – Nantahala Area Relicensing Projects in the Little Tennessee and Hiwassee River Systems	http://littletnwi.org/wp-content/uploads/2013/05/Final_Mussel_Study_Report.pdf

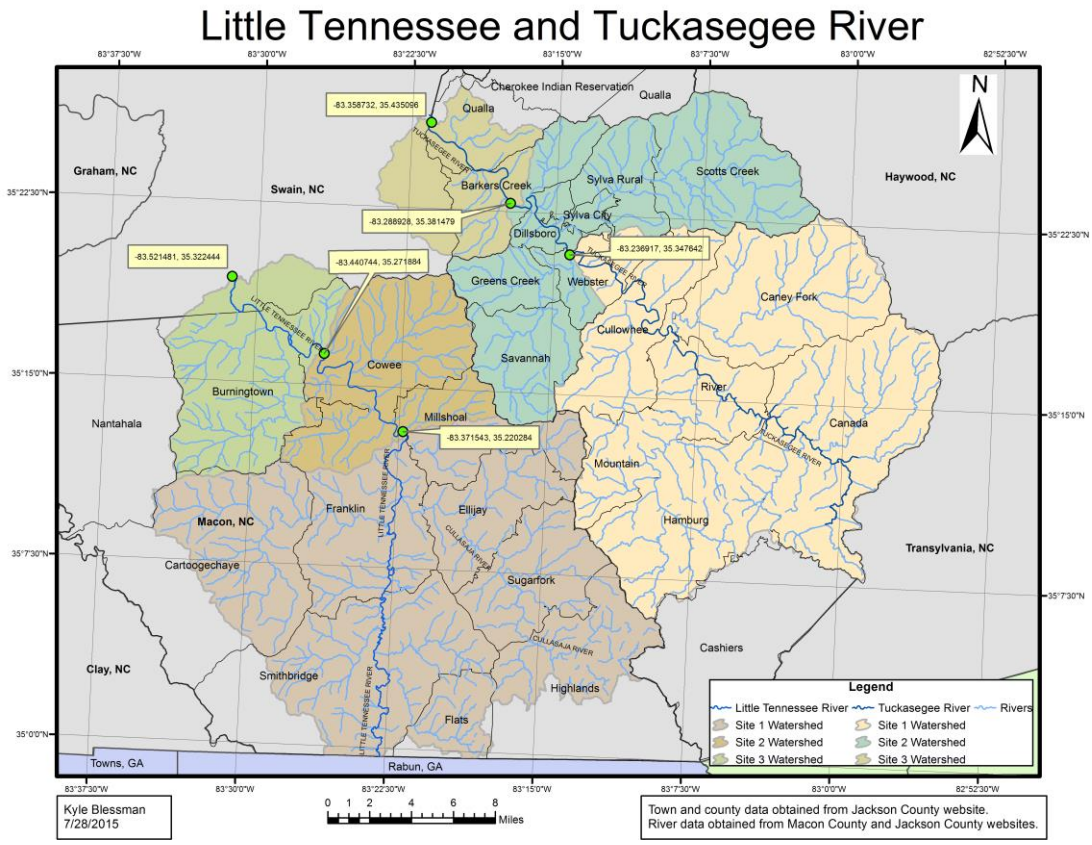


Figure 2: Map of the Little Tennessee River Basin depicting the six study sites.

Table 2: Coordinates of the six study sites at which sentinel animals were held in cages in the Little Tennessee and Tuckasegee Rivers.

Site	Landmarks	Coordinates	
Tuckasegee River Site 1	Dillsboro, NC	35.347642	-83.236917
Tuckasegee River Site 2	Barker's Creek	35.381479	-83.288928
Tuckasegee River Site 3	Whittier Post Office	35.435096	-83.358732
Little Tennessee River Site 1	Franklin Dam	35.220284	-83.371543
Little Tennessee River Site 2	Rosecreek Bridge	35.271884	-83.440744
Little Tennessee River Site 3	Needmore Swinging Bridge	35.322444	-83.521481

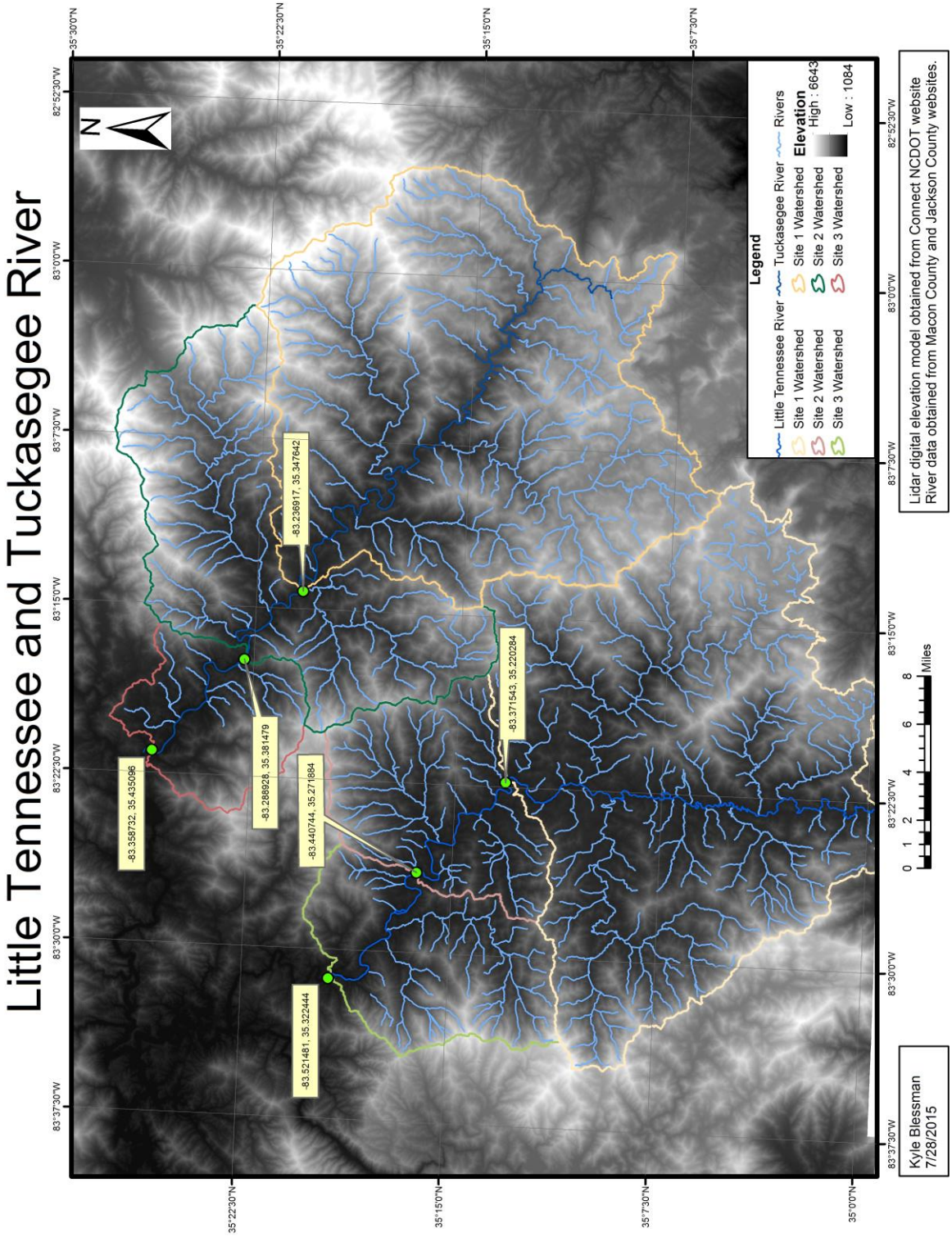


Figure 3: Basin elevation map of the Little Tennessee River Basin and the six study sites.

To further characterize the study sites at which the animals were held we developed a series of geographic information system profiles. These maps were used to document the watershed contributing to each stream reach associated with the study sites (Figures 4, 5) and their proximity to the location of potential point sources of contamination that could impact mussel health. In addition, the soils types within the watersheds were mapped to inform the shell analysis that was conducted (Figures 4, 5) (Appendices 1-7: Soil types maps).

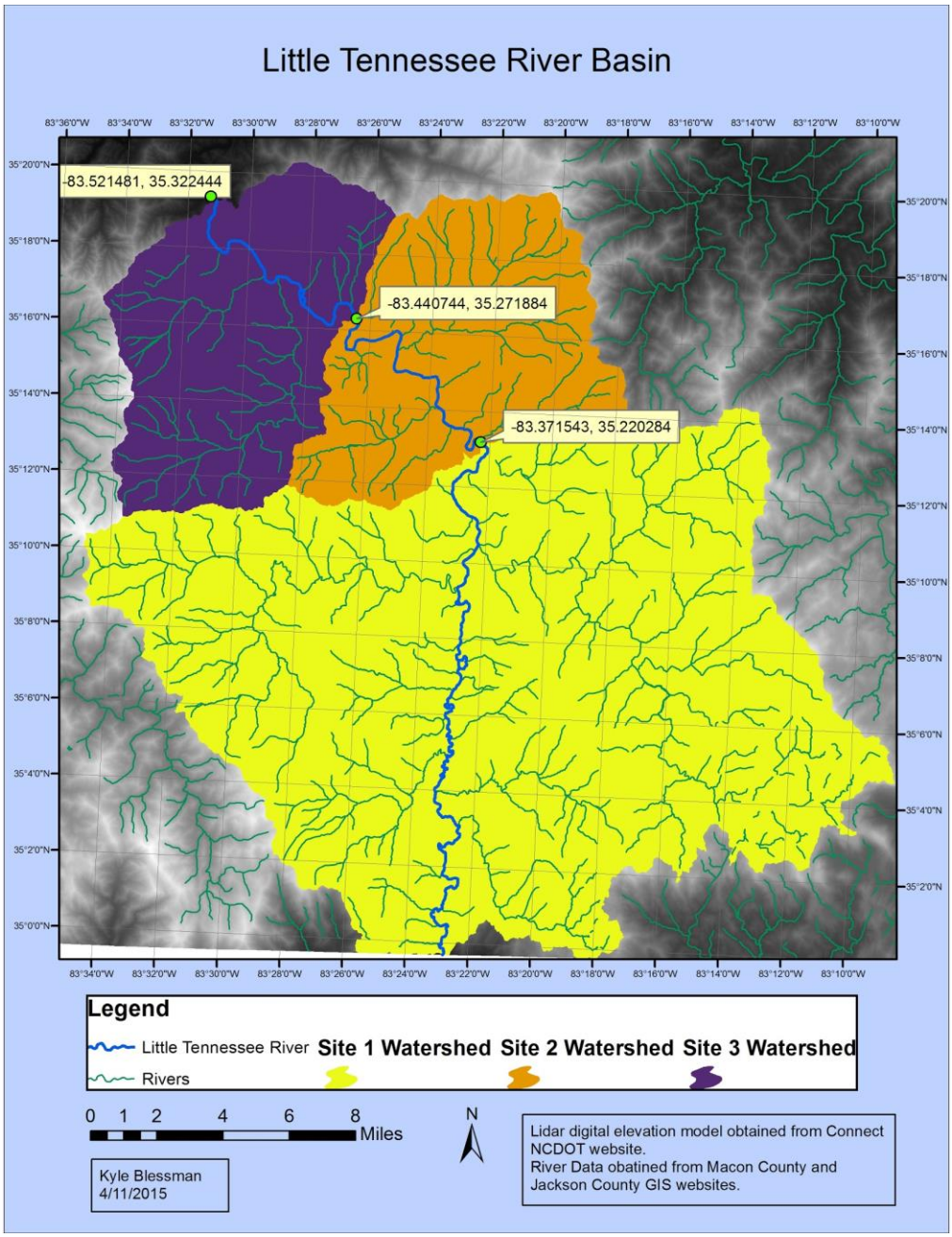


Figure 4: Watershed associated with the three study sites in the Little Tennessee River.

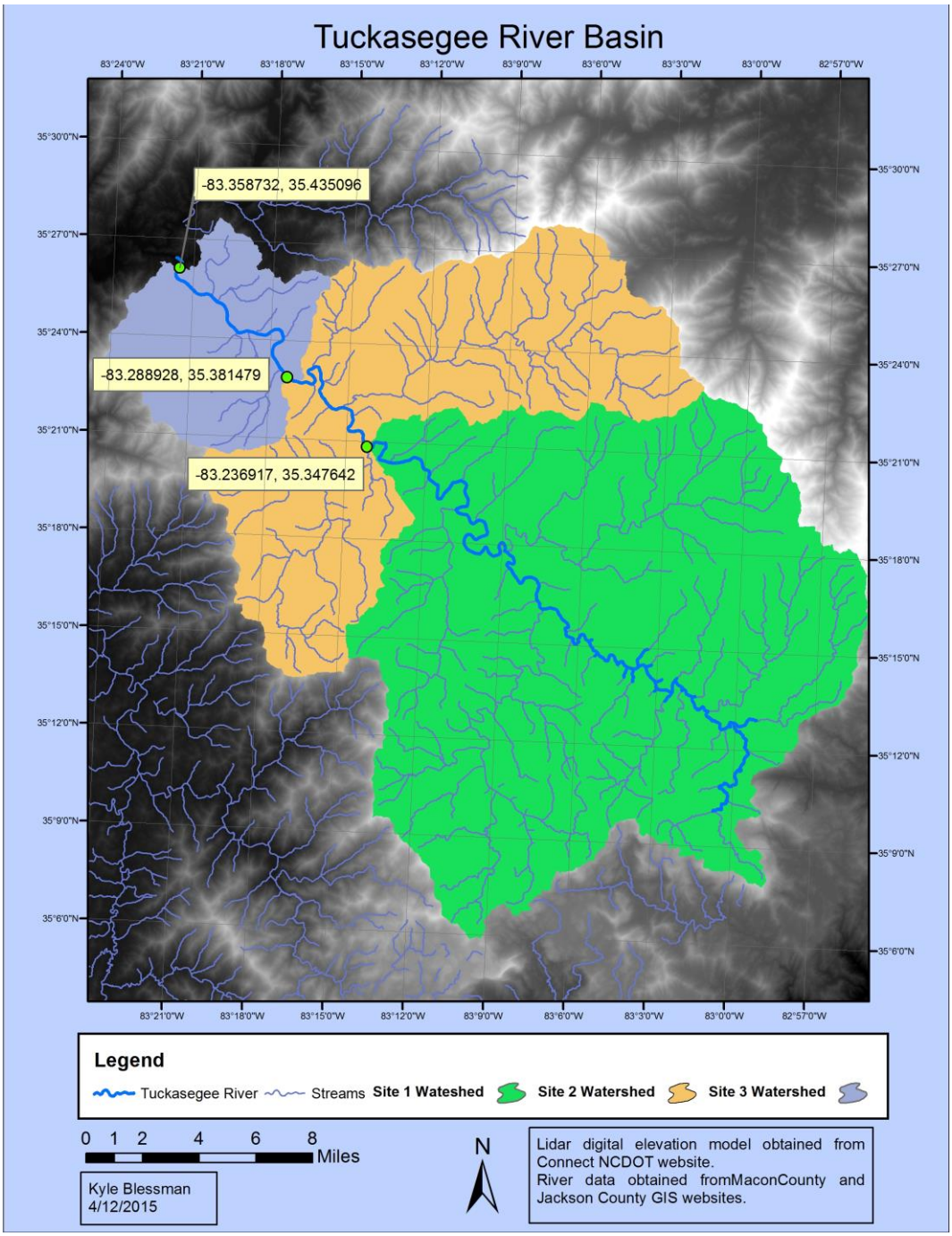


Figure 5: Watershed associated with the three study sites in the Tuckasegee River.

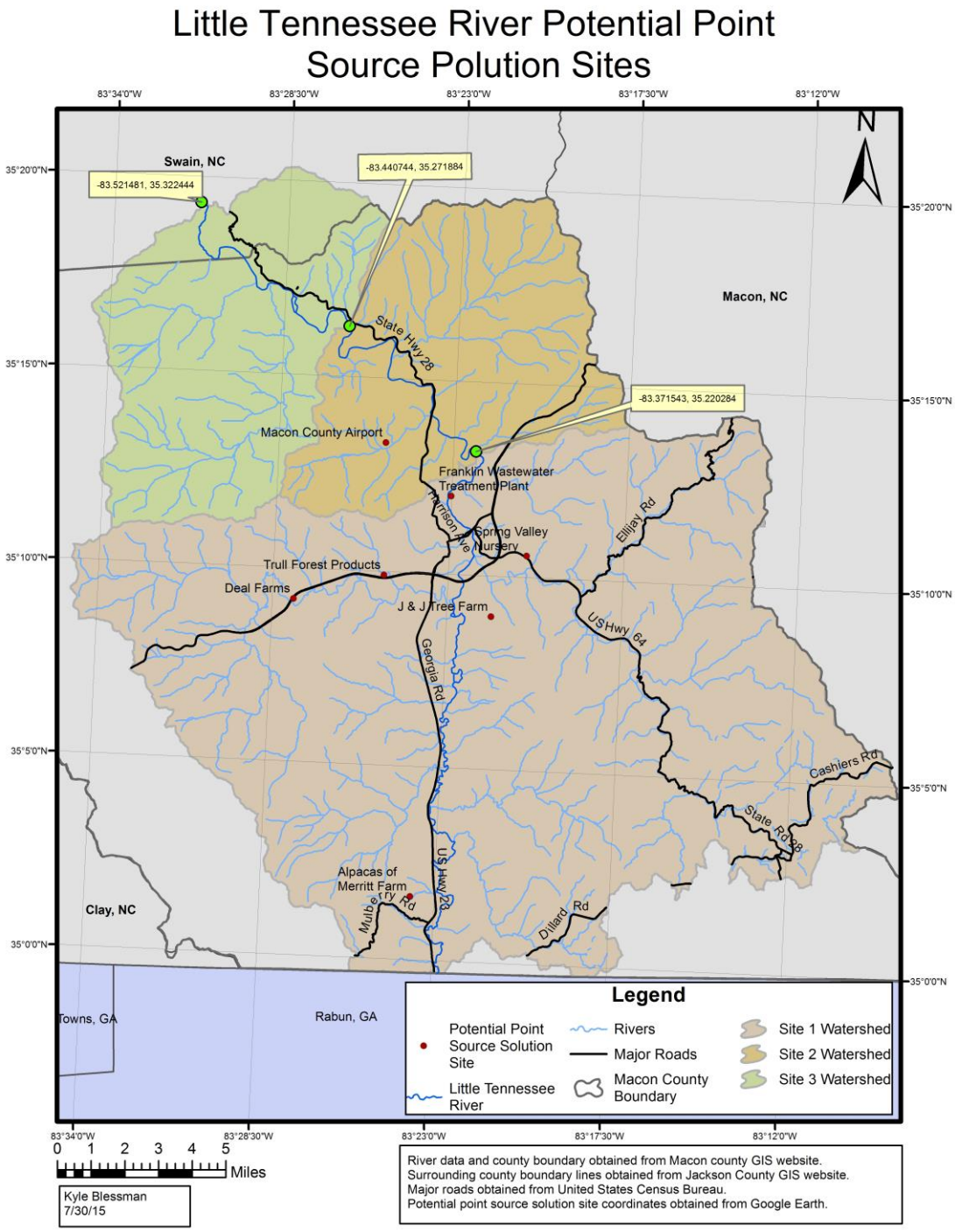


Figure 6: Location of potential point sources of contamination in the Little Tennessee River.

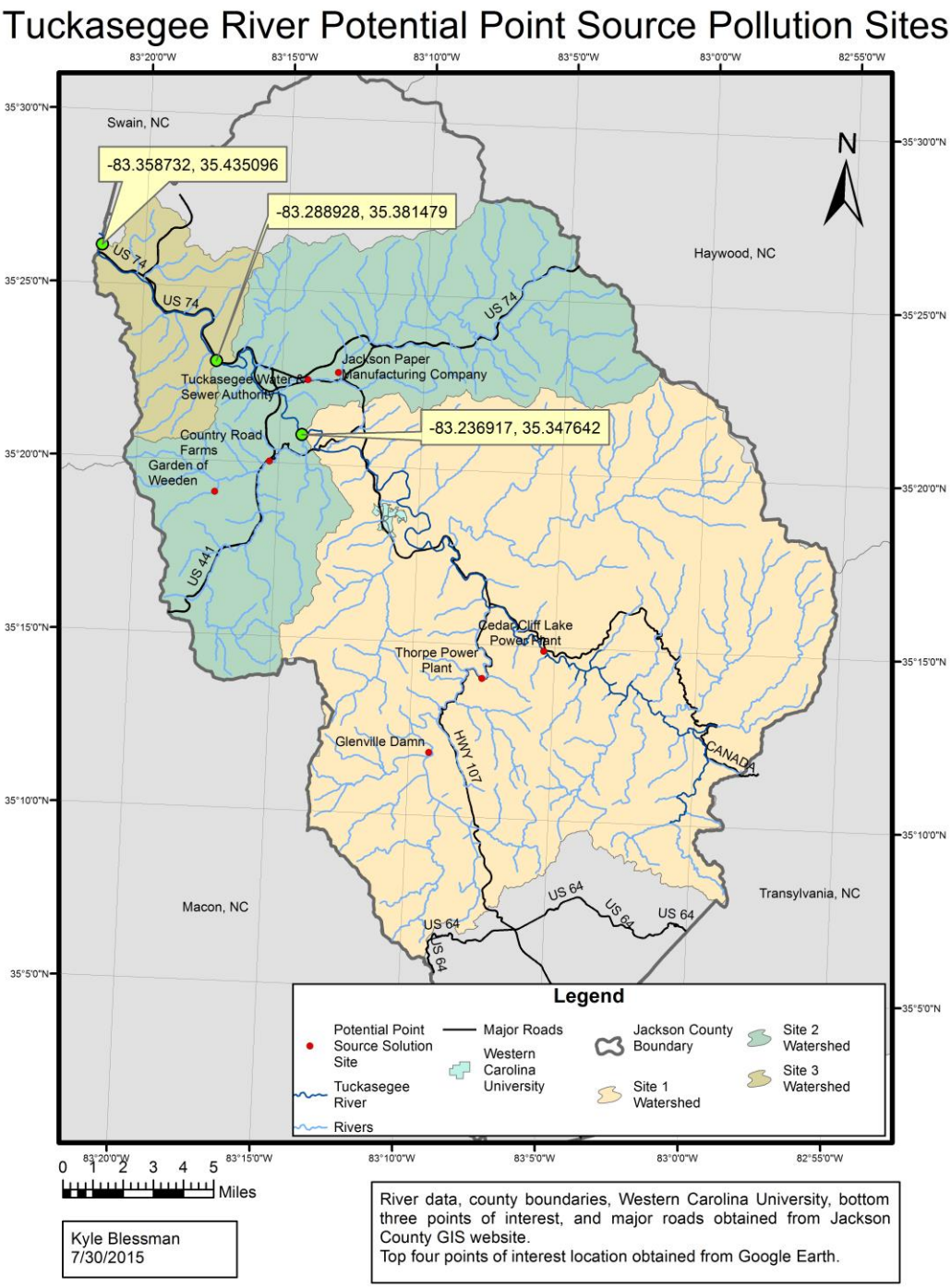


Figure 7: Location of potential point sources of contamination in the Tuckasegee River.

Chapter 2

Survival, Growth and Nutritional Health of Alasmidonta raveneliana Held in Cages in the Little Tennessee and Tuckasegee Rivers

Eighty-two *A. raveneliana* were obtained from the Tuckasegee river near Barkers Creek (35.381479, -83.288928) with a thriving population. Seventy-two animals were randomly assigned to one of six study sites (Figures 2-7), 3 sites in the Little Tennessee (Figure 4) and 3 in the Tuckasegee (Figure 5). Twelve animals were held at each of three sites in both rivers (Tables 2-4). Although relatively similar riverine habitat the relative proportion and distribution of soil types in the watershed are markedly variable (Appendix). *Corbicula fluminea* was present at each of the study sites, but at different densities (Figure 8). The remaining 10 animals were sacrificed and used to develop baseline values for comparative data analysis. In addition to the *A. raveneliana*, we also placed *L. fasciola* separate cages at each site. The *L. fasciola* were captive propagated and reared at the Marion Conservation Aquaculture Center operated by the NC Wildlife Resources Commission. These, captive reared animals were smaller than the field collected *A. raveneliana*.

The mussels were held in plastic mesh cages anchored in the bottom substrate. The *A. ravenliana* were held in cages made of plastic coated mesh (1 x 0.5 inch mesh). The hatchery reared *L. fasciola* were held smaller 0.5 x 0.5 inch mesh. All animals were individually identified with randomly selected numeric identification tags glued to the shells. Shell metrics and weight were obtained before deployment in the cages (Table 2). Each animal was measured (length, weight, height) and a wet weight obtained. A buoyant weight (Alvarez-Molina et al. 2004) was also obtained when subsequent metrics were obtained at the mid-point and end of the study. Thermochron iButton sensors (Maxim Integrated, San Jose, CA) were placed on each cage to monitor water temperature at the sites. Once deployed the cages were examined monthly to remove fouling and assess the general condition of the holding cages and temperature sensors.

Ambient water quality measurements (pH, dissolved oxygen, temperature, turbidity) were obtained biweekly visits to the site. Water samples were also obtained during each visit to each site to measure food-resource availability at the sites (Objective 2). Samples were obtained and placed in coolers containing blue ice for transport and shipment by courier to the laboratory.

The animals were placed in cages at each of the six sites on April 2nd, 2013., At the mid-point of the study the animals were removed from the cage and measured (length, width, height, buoyant weight), and ½ the animals in each cage were randomly selected for necropsy, and nutritional analysis (described below). A post-mortem examination was conducted. General body condition was noted and photographed and gross pathology findings noted. Marked mortality was noted in the *A. raveneliana* in both rivers, and the sentinel studies with *A. raveneliana* were halted after 6 months on October 3, 2013 prevent the loss of all the animals, which would have prevented any tissue analysis and health assessment. Tissues were harvested for the analyses noted below. No losses were observed in the *L. fasciola* and they were left in the cages throughout the duration of the study until they were removed and sacrificed in May 2014 for necropsy and tissue collection for further analysis.

Table 3. Mussel ID, site the mussels were held, and metric data for *A. raveneliana*.

Mussel ID	Site	buoyant weight (g)	length (mm)	width (mm)	height (mm)
C499	Tuckasegee 1	10.3	67.5	35	23.4
C476	Tuckasegee 1	10.7	69.8	32.8	22
C509	Tuckasegee 1	6.7	65.7	30.7	21.7
C522	Tuckasegee 1	2.8	48.8	27.3	16.5
C481	Tuckasegee 1	8	69.2	36.8	22
C475	Tuckasegee 1	9.5	63.7	35.8	21.9
C530	Tuckasegee 1	10.3	71.9	37.1	22.7
C493	Tuckasegee 1	5.9	54.9	30.1	17.6
C467	Tuckasegee 1	6.1	61	32	19.2
C504	Tuckasegee 1	2.2	44.5	22.5	11.3
C484	Tuckasegee 1	12.1	74.1	36.8	24
C501	Tuckasegee 1	13.4	73.5	32.1	26
C519	Tuckasegee 2	12	72.2	37.3	27.3
C507	Tuckasegee 2	13	75.1	38.7	26.6
C496	Tuckasegee 2	9.3	78.8	36.4	27
C472	Tuckasegee 2	9.9	71.5	35	25.1
C500	Tuckasegee 2	8.6	79.1	41.4	29.1
C486	Tuckasegee 2	4.5	53.5	27.2	28.1
C513	Tuckasegee 2	3.9	56	31.1	18
C516	Tuckasegee 2	23	77	39.1	25.5
C505	Tuckasegee 2	8.8	66.7	32.4	23.6
C469	Tuckasegee 2	12.3	76	39.6	24.1
C465	Tuckasegee 2	10.8	73.5	37.5	24.7
C518	Tuckasegee 2	11.7	70.4	36	26.6
C506	Tuckasegee 3	13.3	75.7	37.5	25
C511	Tuckasegee 3	6.9	57.1	29.3	19.5
C489	Tuckasegee 3	3.8	55.5	28.4	19.2
C498	Tuckasegee 3	12.2	73.4	37.8	24.6
C464	Tuckasegee 3	4.1	51.5	27	16.4
C487	Tuckasegee 3	12	71.7	37	24
C471	Tuckasegee 3	15.6	75.1	40.8	30.5
C533	Tuckasegee 3	2.9	51.4	26.2	17.8
C512	Tuckasegee 3	10	71.7	37.7	24
C492	Tuckasegee 3	15.3	77.9	39.5	25.5
C531	Tuckasegee 3	8.7	70.7	33.6	24.7
C478	Tuckasegee 3	14.2	78	36.1	25.3
C463	Little Tennessee 1	12.3	70.9	36.1	24.5
C525	Little Tennessee 1	1.9	43.3	23	13.3
C482	Little Tennessee 1	15.3	78.3	37.4	30.2
C524	Little Tennessee 1	12.4	71	34.4	21.9
C494	Little Tennessee 1	10.1	69	36.5	23.5
C473	Little Tennessee 1	3.7	48	24.5	16.8
C490	Little Tennessee 1	5.6	52.9	29	18.9
C529	Little Tennessee 1	4.1	51.7	27.8	15.9
C466	Little Tennessee 1	9.3	72.3	38	23.4
C514	Little Tennessee 1	13	75.4	34.1	24
C510	Little Tennessee 1	9.6	68.7	24.5	34
C528	Little Tennessee 1	10.7	71.2	24.3	33.7
C495	Little Tennessee 2	7.4	78	35.9	24.6
C488	Little Tennessee 2	8.1	66.8	34.5	23.9
C491	Little Tennessee 2	4.5	51.5	26.3	17.4
C508	Little Tennessee 2	10.9	69.1	36.6	25.5
C517	Little Tennessee 2	10.8	69.2	38	20.8
C502	Little Tennessee 2	3.7	49.9	26	17.3
C497	Little Tennessee 2	11.5	69	36	23
C474	Little Tennessee 2	11.2	70	35.8	24.5
C515	Little Tennessee 2	9.3	65.9	35	24.2
C485	Little Tennessee 2	13.9	77.4	40.4	24.6
C523	Little Tennessee 2	3.2	44.6	23.1	14.9
C479	Little Tennessee 2	13.6	74.8	41.5	26.2
C470	Little Tennessee 3	11.2	79.8	40.6	25.2
C468	Little Tennessee 3	13.2	75.2	39.6	24.1
C520	Little Tennessee 3	2.3	49.3	24.5	16.4
C483	Little Tennessee 3	13.1	69.6	35.9	23.5
C477	Little Tennessee 3	10.6	73	36.2	25
C526	Little Tennessee 3	11	72.2	37.6	25.1
C480	Little Tennessee 3	9.6	72.6	36.5	24.5
C503	Little Tennessee 3	7.2	61.5	34.7	20.9
C534	Little Tennessee 3	2.7	45.3	23.3	14
C532	Little Tennessee 3	2.1	46.6	24.2	15.4
C527	Little Tennessee 3	9.8	68	34.1	26.8
C521	Little Tennessee 3	5.3	55	18.8	19.1

Table 4. Mussel ID, site the mussels were held, and metric data for *L. fasciola.*

Mussel ID	Site	dry weight (g)	length (mm)	width (mm)	height (mm)
P132	Tuckasegee 1	3.7	30.3	17.9	11
P120	Tuckasegee 1	4.4	32	19.6	12.2
C966	Tuckasegee 1	4.3	30.8	18.2	12.5
P097	Tuckasegee 1	3.3	28.9	17.4	10.6
P130	Tuckasegee 1	3.9	30.7	18.6	11.8
P144	Tuckasegee 1	2.6	27.6	16.8	9.2
P139	Tuckasegee 1	2.8	27	16.2	10.7
P149	Tuckasegee 1	1.8	24	14.5	8.7
P147	Tuckasegee 1	2.9	27.5	17.5	10
P108	Tuckasegee 1	6.6	34.4	21.7	14.5
P104	Tuckasegee 1	4.8	32.8	20.8	12.8
P142	Tuckasegee 1	3.1	27.7	17	11
P156	Tuckasegee 2	4.1	31.1	19	11.6
P102	Tuckasegee 2	4.2	30.2	18.5	12.6
P111	Tuckasegee 2	5.1	33.1	20.5	13.3
P140	Tuckasegee 2	2.8	27.1	16.8	10.3
P113	Tuckasegee 2	5.7	33.8	21.1	14.1
P137	Tuckasegee 2	3.2	29.2	17.5	10.3
P100	Tuckasegee 2	3	27.6	17.5	10.6
P103	Tuckasegee 2	4.5	32	18.5	12.5
P122	Tuckasegee 2	4.5	30.7	19.5	12.7
P155	Tuckasegee 2	4.5	32.1	19.4	11.5
P138	Tuckasegee 2	4.4	31.9	19.3	12
P126	Tuckasegee 2	3.7	29.5	18	11.4
P098	Tuckasegee 3	3.9	30.2	18.7	11.2
P112	Tuckasegee 3	5.2	33	20.5	13.3
P133	Tuckasegee 3	2.9	27.3	16.4	10.5
P107	Tuckasegee 3	4.1	31	18.6	12
P135	Tuckasegee 3	5.5	32.7	20.3	13.6
P164	Tuckasegee 3	2.4	27	16.7	9
P129	Tuckasegee 3	3.7	29	17.6	12
P123	Tuckasegee 3	5.7	32.1	20.7	14.2
P096	Tuckasegee 3	3.4	27.9	18	11.6
P152	Tuckasegee 3	4	30	18.8	12
P134	Tuckasegee 3	3.1	28.8	17.7	10.7
P145	Tuckasegee 3	2.9	27.2	17.1	10.7
P118	Little Tennessee 1	3.3	29.2	17.6	10.5
P161	Little Tennessee 1	4.8	29.4	19.7	13.5
P114	Little Tennessee 1	2.4	26.8	16	10
P165	Little Tennessee 1	3.8	29.6	18.3	11.6
P159	Little Tennessee 1	3.6	28.8	18.1	11.8
P143	Little Tennessee 1	2.5	26.2	15.3	10
P115	Little Tennessee 1	4	31	18.5	11.5
P128	Little Tennessee 1	4.4	31	20.2	12
P109	Little Tennessee 1	2.7	27.1	16.5	10.5
P090	Little Tennessee 1	3	27.9	17.3	10.6
P093	Little Tennessee 1	3.8	29.5	18.1	12
P091	Little Tennessee 1	3.9	29	18.1	11.8
P154	Little Tennessee 2	3.4	29	17.9	11
P094	Little Tennessee 2	3.5	28.3	17.7	11.3
P117	Little Tennessee 2	3.3	28	17.2	11.1
P131	Little Tennessee 2	5.2	32.6	20.8	13.1
P168	Little Tennessee 2	1.6	23.3	14	8.6
P148	Little Tennessee 2	1.9	24.7	15	9
P169	Little Tennessee 2	2.1	25.7	16	9.1
P167	Little Tennessee 2	2.5	25.4	15	10
P095	Little Tennessee 2	3.3	31.1	10.9	18.9
P166	Little Tennessee 2	2.9	27.5	16.9	10.7
P105	Little Tennessee 2	4.2	30.7	18.8	12.2
P153	Little Tennessee 2	3	28.2	17	10.3
P127	Little Tennessee 3	4.1	32.3	19.5	11.5
P158	Little Tennessee 3	3.2	27.9	17.4	11.1
P089	Little Tennessee 3	3.1	28.5	18.1	11
P163	Little Tennessee 3	3.4	18.4	18	11
P121	Little Tennessee 3	5	32.6	19.4	13.4
P116	Little Tennessee 3	6.9	34.8	21.9	15.4
P092	Little Tennessee 3	5	32.4	20.2	12.8
P125	Little Tennessee 3	4.4	31	19.8	12.3
P124	Little Tennessee 3	4.3	31.4	19.6	11.8
P110	Little Tennessee 3	7.1	36.7	22.8	14.7
P099	Little Tennessee 3	3	26.8	15.9	11.1
P119	Little Tennessee 3	4.2	30.1	18.6	12

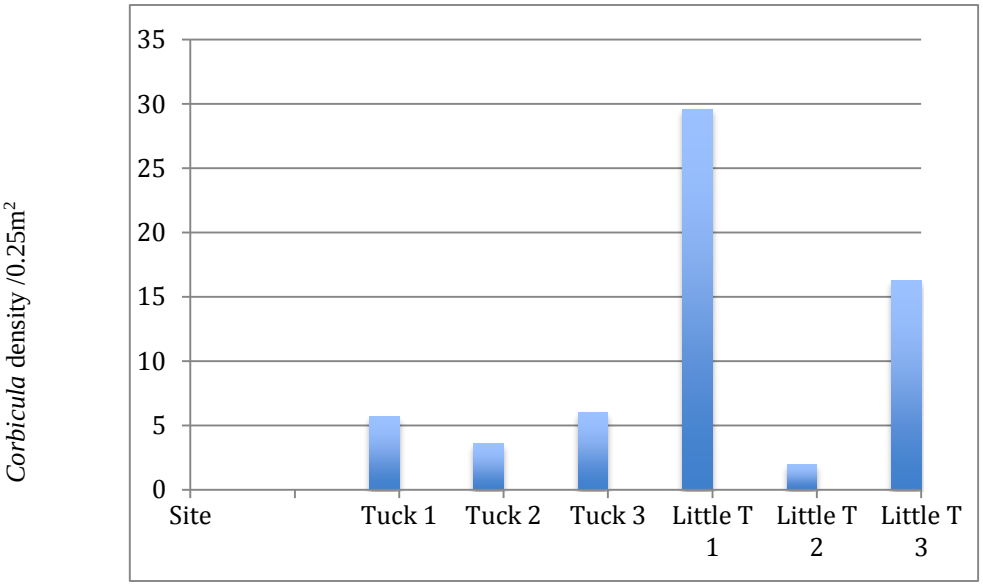


Figure 8: *Corbicula* density at the study sites.

At the time of harvesting from the sentinel sites we obtained a cross section of tissue from each animal for histopathologic analysis and placed the tissue in 10% neutral buffered formalin. The tissues were processed by the CVM Histopathology laboratory into paraffin and sectioned at 5 microns for histopathologic examination. Sections were examined by two board certified veterinary pathologists (Law, Borst) for gender and evidence of disease: 1) hemocyte infiltration and potential pathogens; 2) evidence chronic toxicity (e.g. reproductive tissue integrity). In response to preliminary findings, additional special stains were used to enhance visualization of specific chemical residues in tissue sections taken from two animals. A Von Kossa stain was used to identify the potential presence of tissue mineralization, a Gram stain was used to identify the presence of bacteria, a periodic acid-Schiff stain was used to identify the presence of polysaccharide and Giemsa stain was used to identify the potential presence of protozoans.

A small section of digestive gland and gill tissue was placed in McDowell's and Trumps fixative (4F:1G) and held for scanning electron microscopic assessment of randomly selected tissue specimens from both *A. raveneliana* and *L. fasciola*.

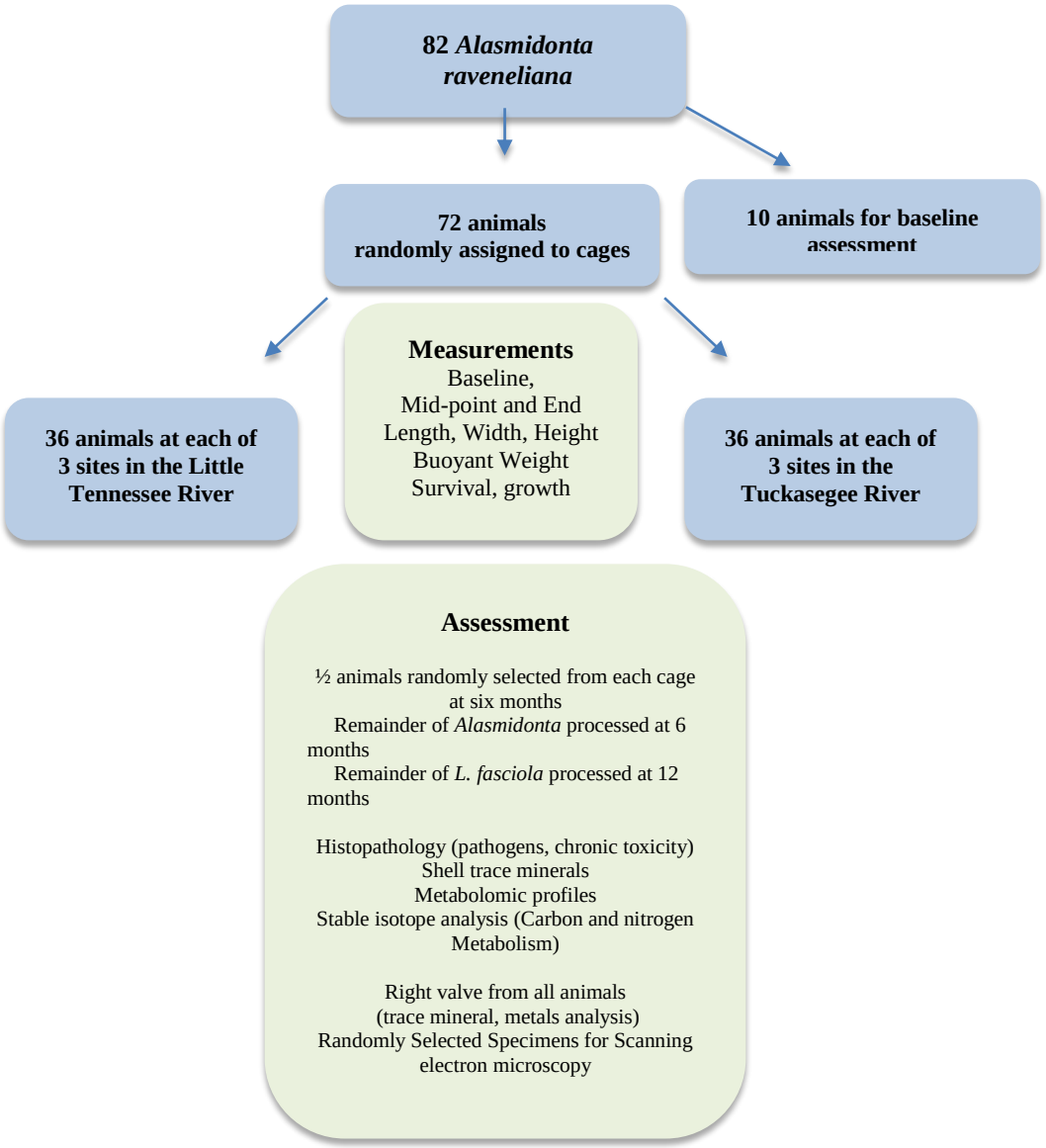
Shells from all animals were collected and identified with the animals's ID number. An approximately ½ inch section of the right valve was removed from each animal, imbedded in plastic and sent to the University of Texas, Austin, Eximer laser ablation laboratory for microchemical analysis. The remainder of the right valve and the left valve were stored for future analysis.

Our effort to understand the metabolic changes that took place in the sentinel animals was fortunate to benefit from the support of the Research Triangle Institute NIH Metabolomics Study Core. Their support markedly expanded the breadth of the analysis of study animals. Although we had proposed to save the *L. fasciola* tissue for future

analysis due to funding limitations, their support accommodated developing metabolomic profiles, of all specimens for which sufficient gill tissue was available (n=100), from both species. A full non-targeted metabolomics profile of each animal was obtained. This facilitated a thorough assessment of the metabolic health of each animal including amino acid and mucopolysaacheride mobilization, During sampling, a small section of gill, adductor, mantle and foot were obtained, flash frozen in liquid nitrogen, and kept on dry ice until being stored at -80º C to await analysis.

Additional tissue was obtained for carbon and nitrogen stable isotope analysis to serve as an indicator of the source of carbon and nitrogen the animals were using for protein metabolism. Additional tissues were obtained, frozen and retained for potential toxin analysis as warranted and supported by additional funding.

A schematic of the various tissues obtained and processed is provided below.



Results

Figure 9: Field design and study sampling

Ambient Water Quality Monitoring

Hourly temperature recordings were also obtained with Thermochron iButton sensors housed in PVC pipe attached to both cages at each site. Ambient water quality samples were taken at each of the sites every two weeks throughout the study period, with several notable exceptions when stream conditions made sampling unsafe (9 July, 15 October, and 26 November 2013 and 7 January, 28 January, 4 February, and 8 March 2014). Stream water temperature (^{o}C), pH (hydrogen ion concentration), turbidity (NTU), dissolved oxygen (mg/dl) was recorded.

The Little Tennessee River showed greater variability and lower average dissolved oxygen levels when compared to the Tuckasegee River. The pH in the Little Tennessee River varied more by site relative to the Tuckasegee. The temperature patterns were similar in the two rivers, but average temperatures in the Little Tennessee River were approximately 1 ^{o}C higher than temperatures in the Tuckasegee River. In particular, water temperature in the summer in the Little Tennessee River averaged 1-2 degrees celcius warmer than water temperatures in the Tuckasegee River. Typical turbidity levels appeared comparable in the two rivers. The spikes in turbidity reflect storm events near sampling times.

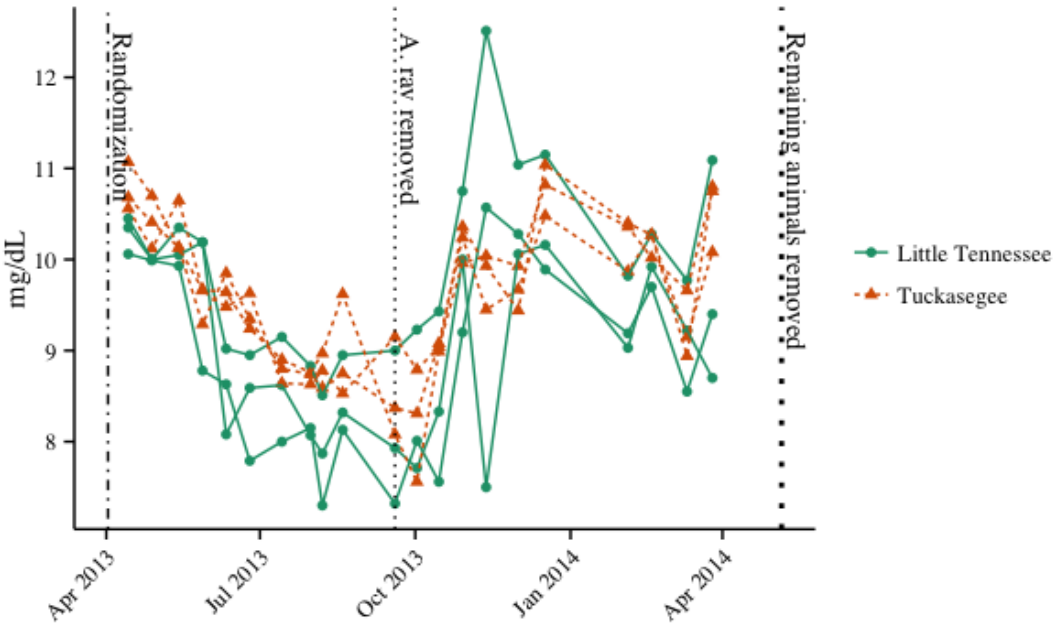


Figure 10. Dissolved Oxygen in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014. Each line represents a study site.

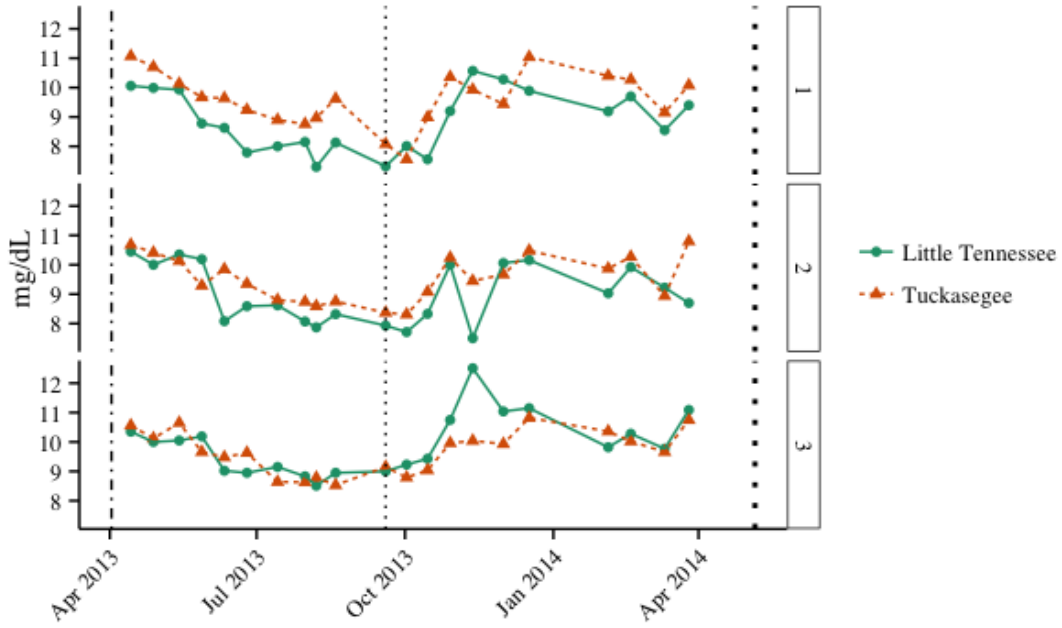


Figure 11. Dissolved Oxygen in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014 by site.

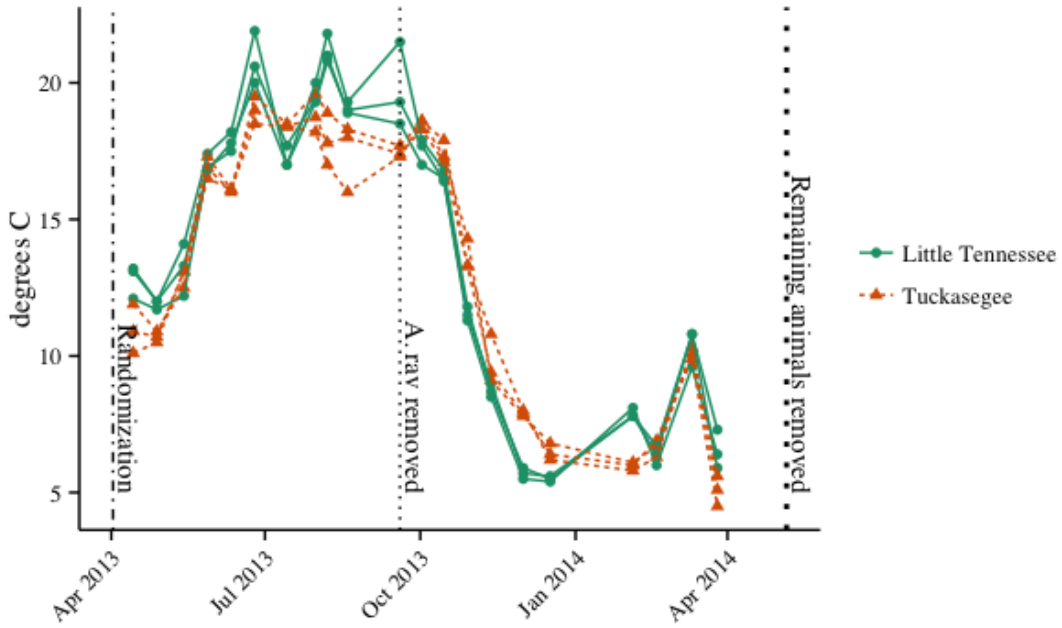


Figure 12. Temperature in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014. Each line represents a study site.

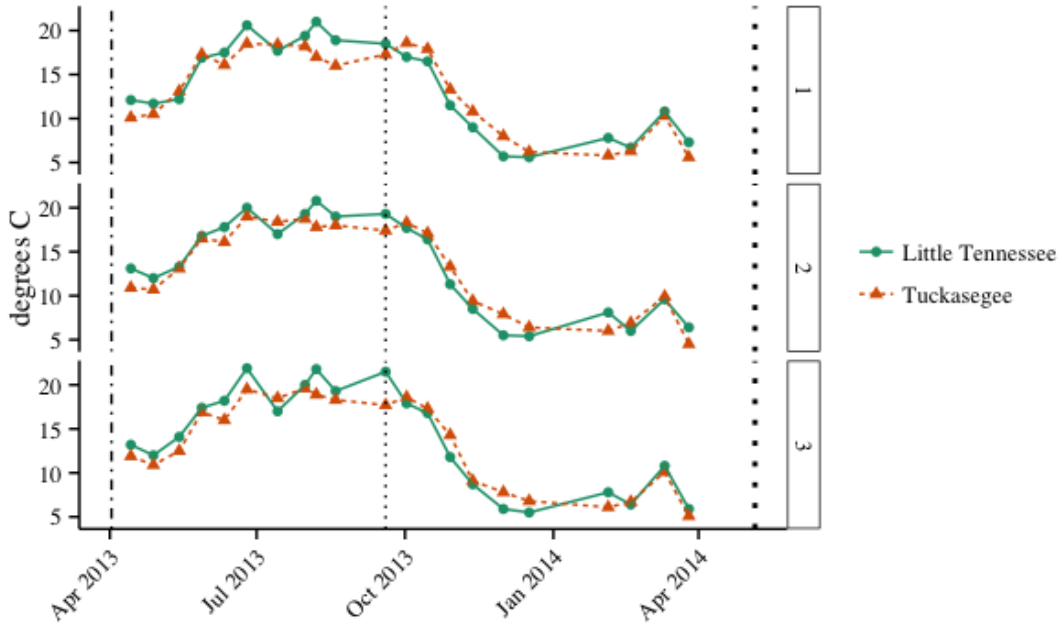


Figure 13. Temperature in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014 by site.

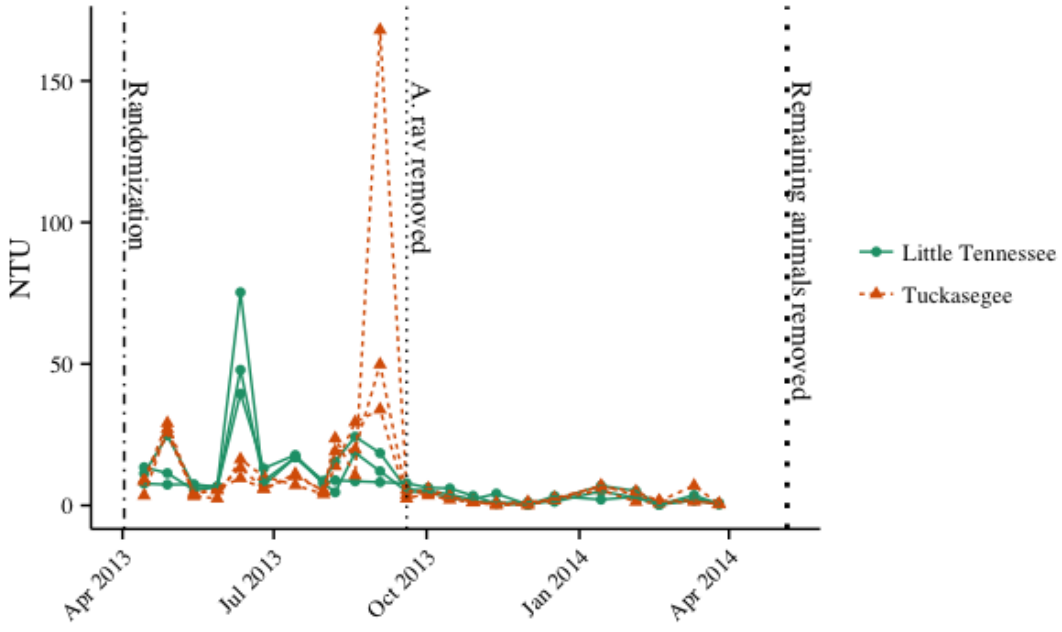


Figure 14. Turbidity in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014. Each line represents a study site.

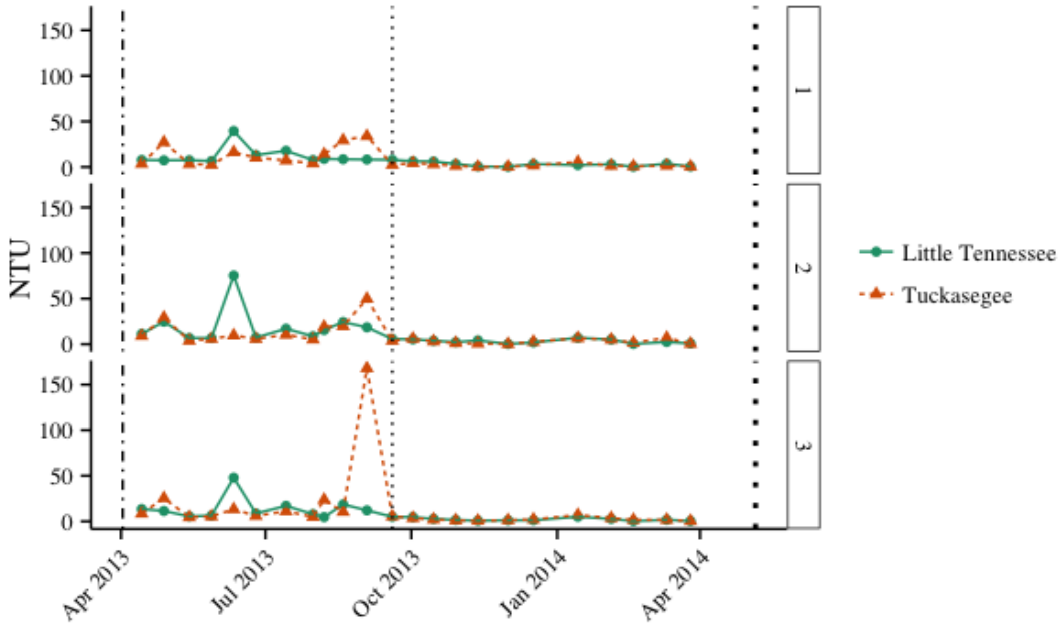


Figure 15. Turbidity in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014 by site.

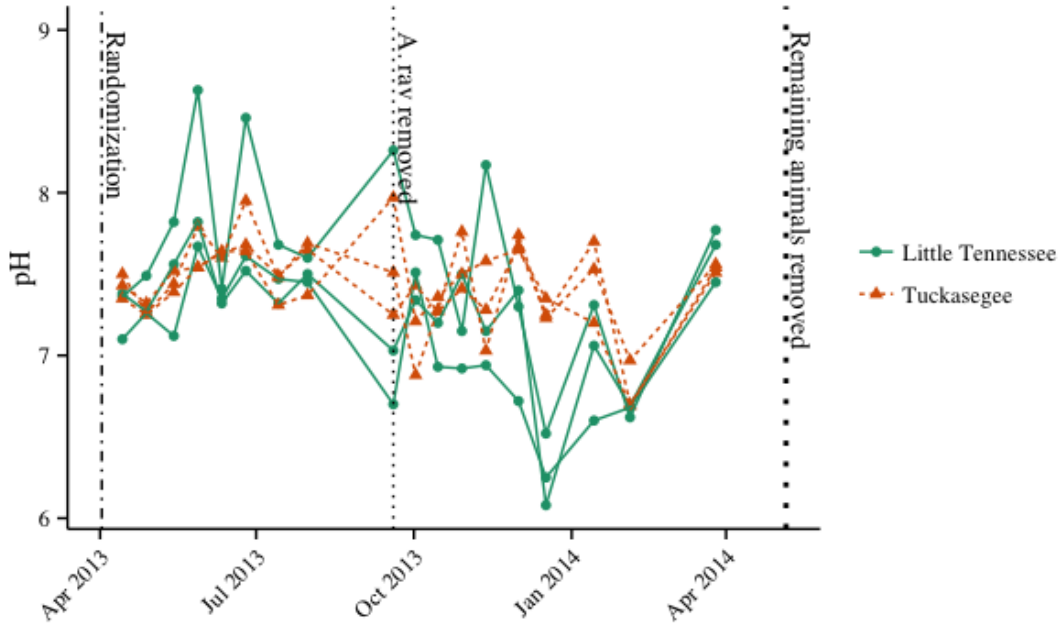


Figure 16. pH in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014. Each line represents a study site.

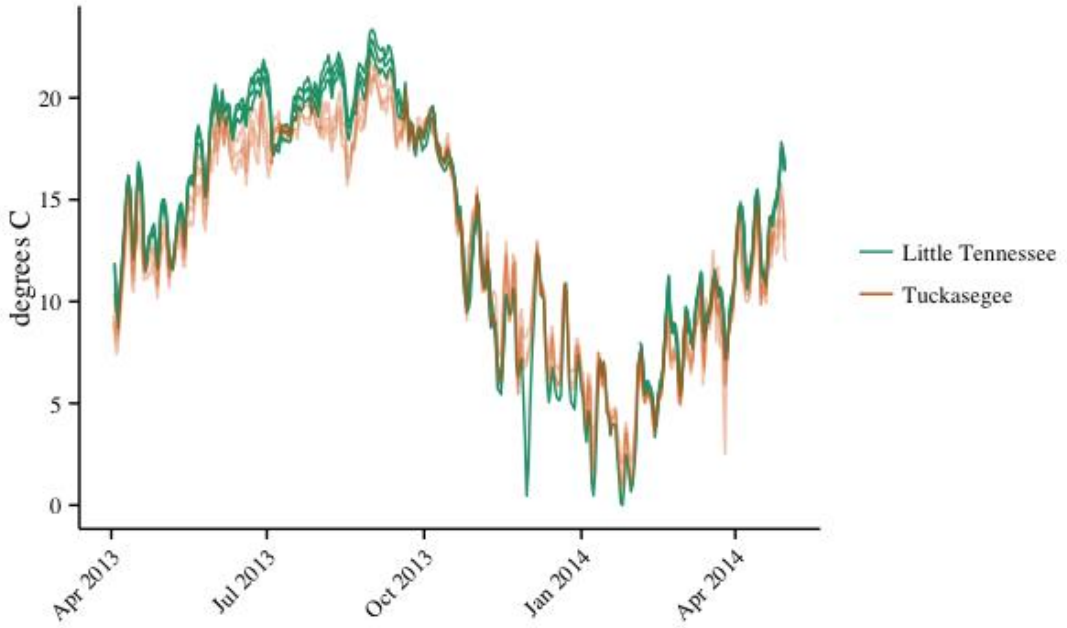


Figure 18. Average daily temperature in the Little Tennessee and Tuckasegee Rivers from April 2013 to April 2014 measured by button logger.

Caged Mussel Survival and Growth

Mussel survival in the cages was assessed monthly unless weather conditions (note above) precluded safe access to the cages. In addition, mussel length (mm), height (mm) and width (mm) as well as wet weight were obtained prior to the mussels being placed in the cages, at 6 months and when they were removed from the cages for necropsy. Buoyant weight was also obtained at 6 months and at the time of necropsy. Mortality was observed at 5 sites, Little Tennessee River sites 1-3 and Tuckasegee River Sites 2&3. No mortality was observed at Tuck site 1. No *L. fasciola* died during the course of these studies. One *A. raveneliana* and 2 *L. fasciola* were lost to followup (Tables 3&4, above).

Gross and Histopathologic assessment

The gills of freshwater mussels play multiple roles. Gills along with mantle tissue play a key role in respiration and gas exchange. In addition, gill cilia play a prominent role in nutrition by sorting and moving captured particles to the digestive glands. Freshwater mussel gills also serve as brood chambers for developing glochidia. In preliminary sentinel studies we noted differences in the morphology of *A. raveneliana* and *L. fasciola*. We hypothesized that the decline in health observed in *A. raveneliana* harvested from the Little Tennessee could be related to gill damage. Animals with impaired gill function could potentially have difficulty making use of dissolved oxygen, collecting and processing food particles and maintaining larval brood chambers.

Healthy gills should display a uniform lawn of independent cilia at their apex supported by singular lamellae and the presence of hemocytes at their base (Fig 18).

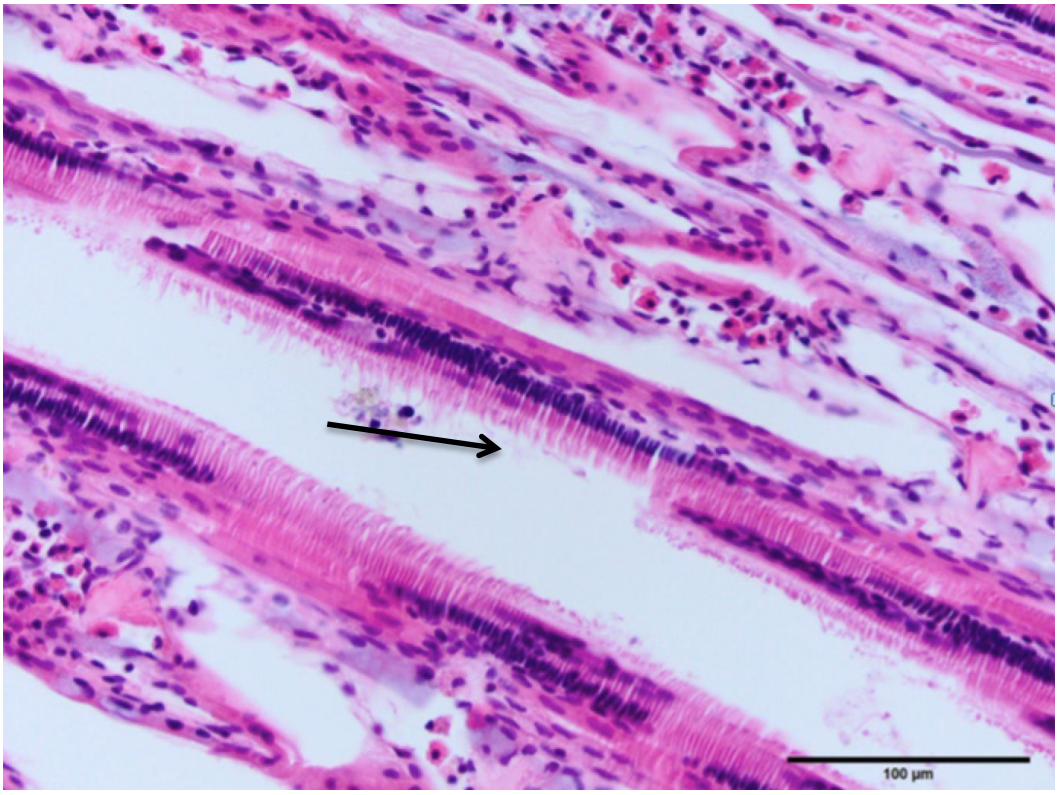


Figure 18: 100um light microscopic image of *Lampsilis fasciola* P125. Cilia are abundant, uniform and although hemocytes (arrow) are present.

A few animals displayed focal injury to the gills, with mild to moderate infiltration of hemocytes and focal fusion of gill lamellae (Fig. 19). Gill fusion can occur in response to chronic irritation of the gill lamellae. However, this was not a widespread or consistent change amongst the specimens examined.

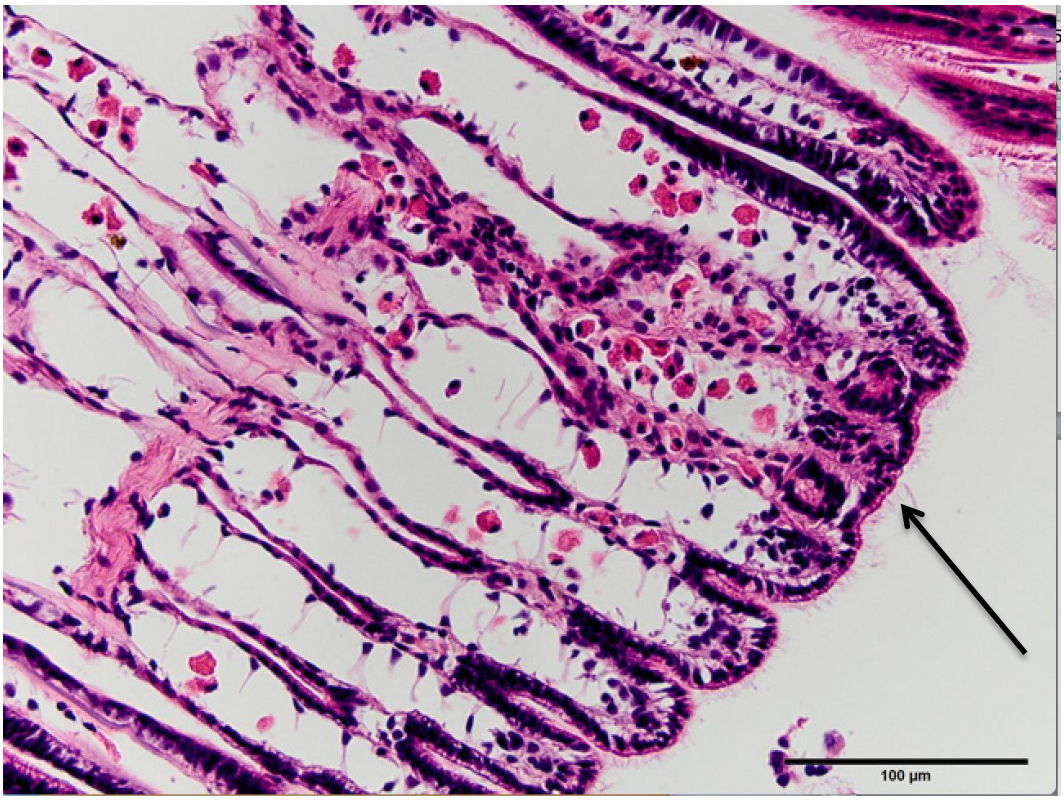


Figure 19: 100 um section of gill from *Alasmidonta raveneliana* C-493 demonstrating fusion of the gill lamellae (arrow) and infiltrated hemocytes.

By far, the most consistent and troublesome change noted is the apparent paucity of hemocytes in multiple tissues of multiple animals, such as at the base of the gills, where "pools" of reserve hemocytes are expected to be found (Figure 20). A paucity of hemocytes could occur in response to: 1) environmental or other disease/stressor that is sapping the immune system (i.e., increased demand) or immunocompromising the mussels; 2) a viral disease that is destroying hemocytes; or 3) chemical pollutants such as estrogenic compounds that may suppress production of cells (hypothetical in bivalves).

A few of the specimens contained various parasites, but the specific types are not of a consistent etiology to suggest that these were the cause for morbidity or mortality (Figures 21-23). In most specimens, parasite numbers were few. However, one *Alasmidonta* had a heavy nematode burden (Fig. 24), with numerous cross-sections of relatively large, adult nematodes, apparently of a single species.

Histopathology sections of mussel tissue can also be used to determine the sex of individual mussels. In Figure 24, glochidia are apparent indicating the animal was female and in Figure 25 spermatids were observed indicating the animal was male.

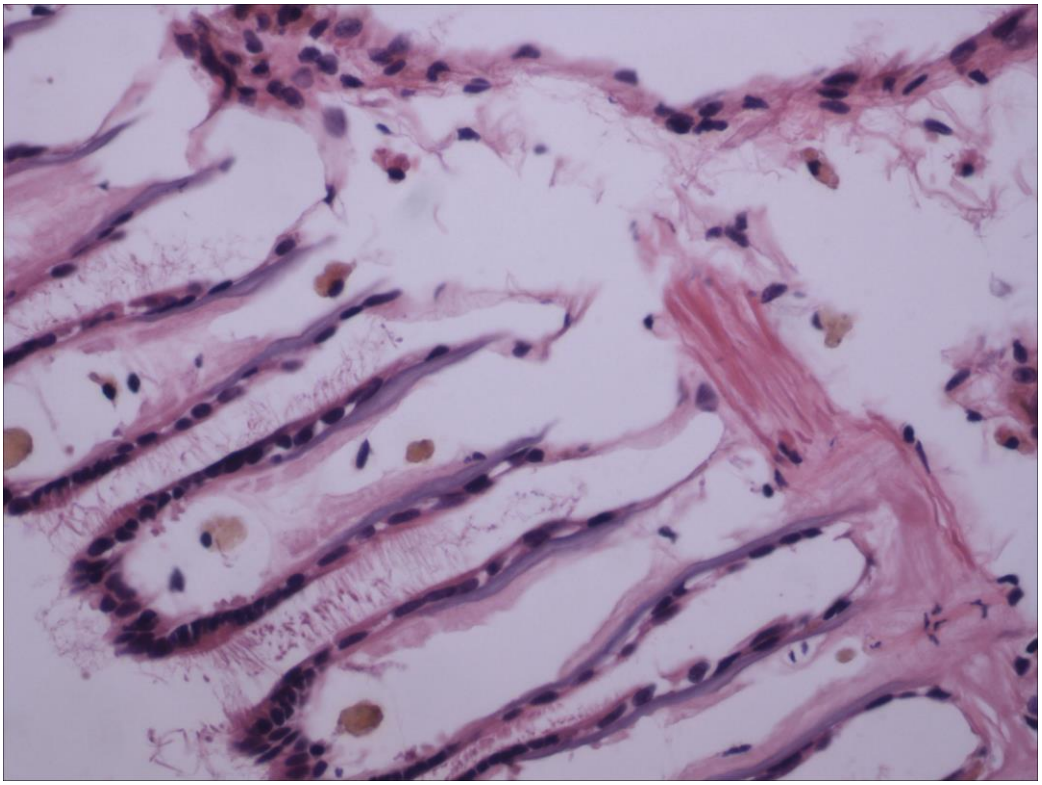


Figure 20: 100 um image of the gill tissue of C-503 displaying a paucity of hemocytes

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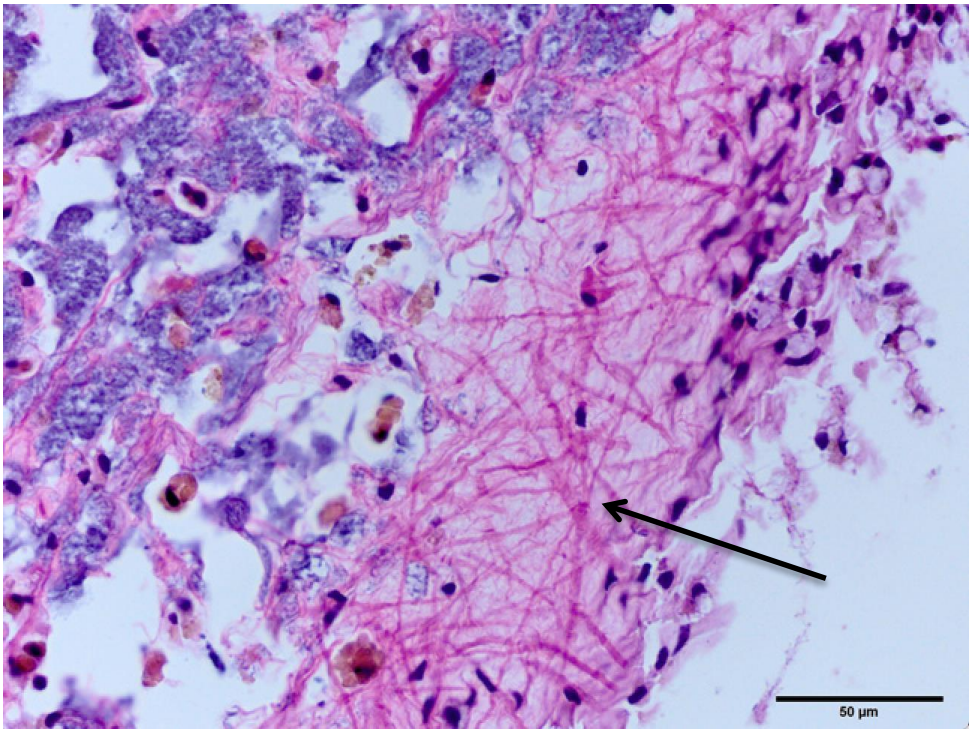

Figure 21: Potential fungal hyphae (arrow) are apparent in the gills of *A. raveneliana* C-515.

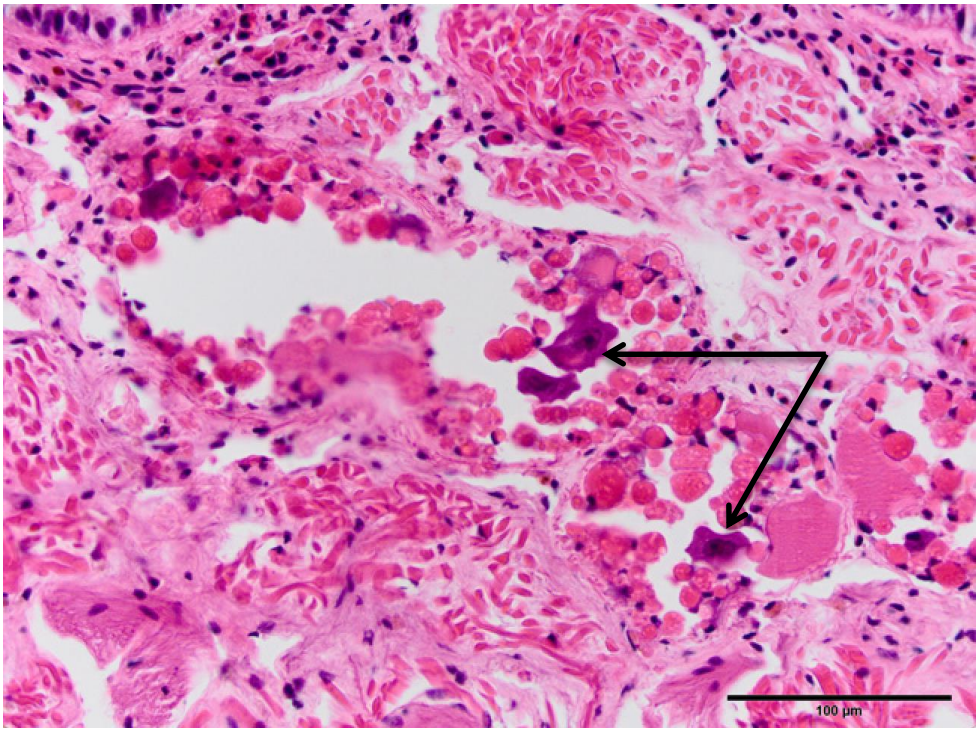

Figure 22: 100 um tissue section showing degenerating digestive gland of *A.raveneliana* C-520 and the presence of an amoeba-like protozoan (arrows).

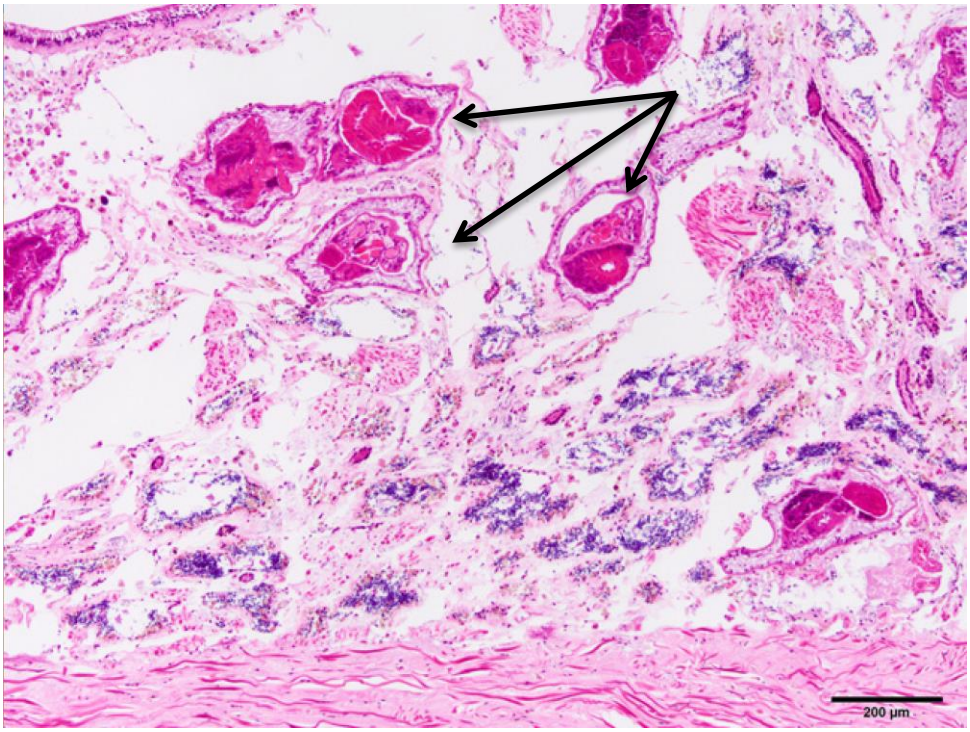

Figure 23: 200 um section from *A. raveneliana* C-485 showing the presence of a heavy nematode burden (arrows).

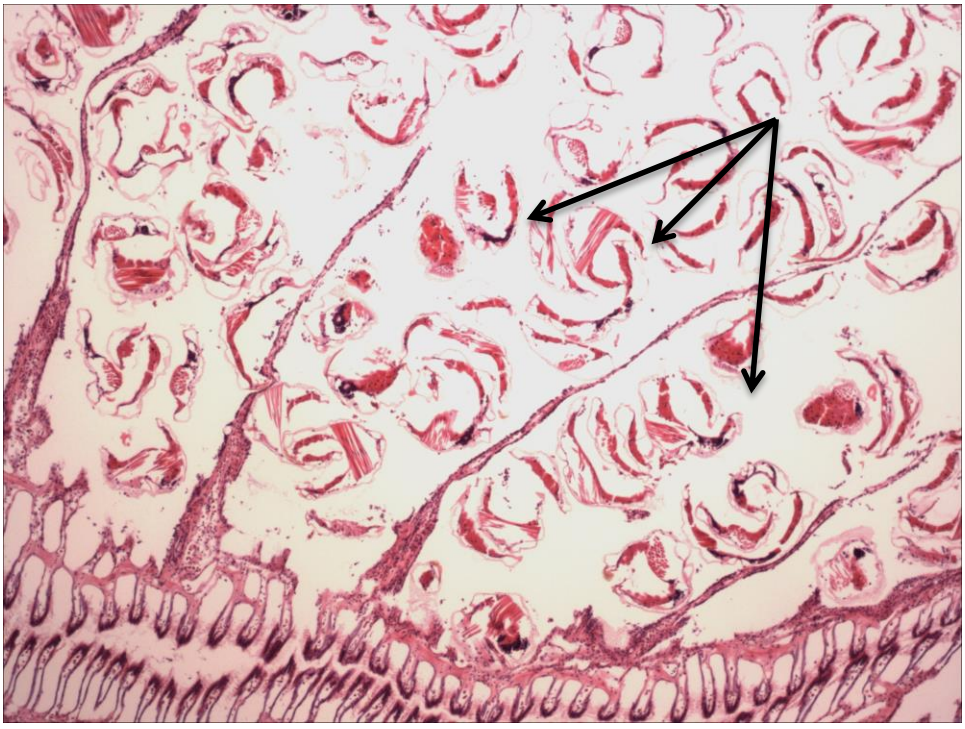

Figure 24: Microscopic section of glochidia (arrows) of a gravid *A. raveneliana*, Ar-10, one of the 10 baseline animals.

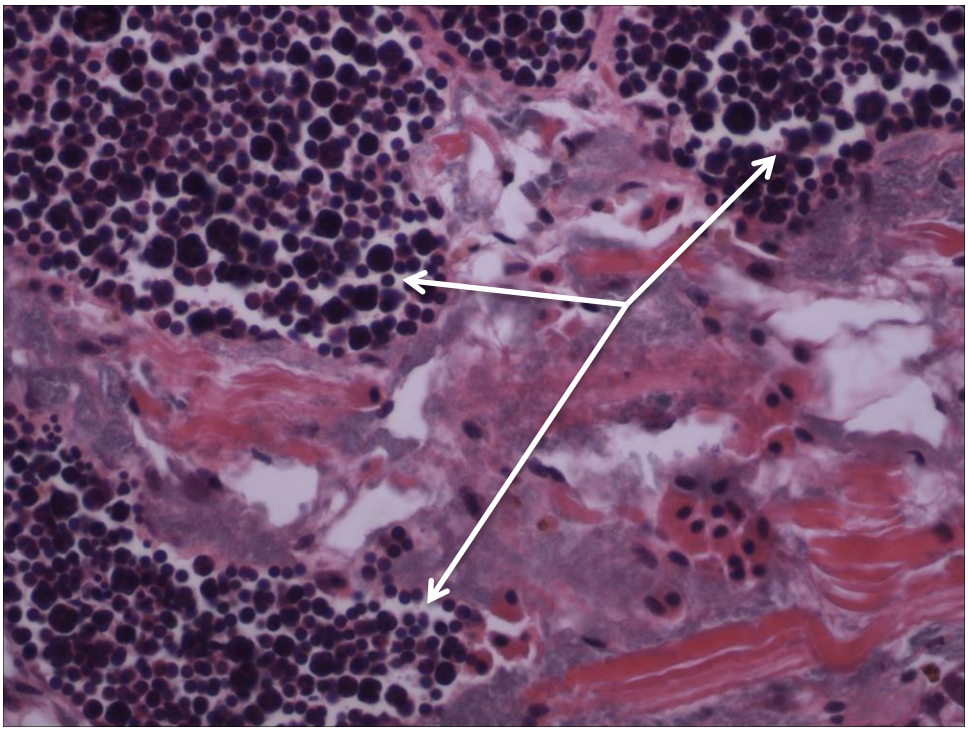

Figure 25: Microscopic section of reproductive tissue showing spermatids (arrows) of a male *A. raveneliana.* C-467.

Scanning electron microscopy

Gill tissues from selected *A. raveneliana* and *L. fasciola* were excised and placed in fixative and held in a refrigerator until processed for scanning electron microscopy.

Sentinel *A. raveneliana* exhibited heavy coatings of an organic film on the gills of animals at each of the sites (Figure 26). The coating was not observed on the baseline animals harvested at the beginning of the study. Although present on several *L. fasciola* specimens, it was not as prominent (Figure 27). Some of the samples had organic films containing masses of fibrin-like filaments that were not seen in any of the *A. raveneliana* samples. Fused gill lamellae were apparent on the gills of many of the animals.

Crystalline lamellar concretions were observed on the gills of many of the specimens (Figure 28). These crystals have previously been described as calcium phosphate crystals (Silverman et al. 1983).

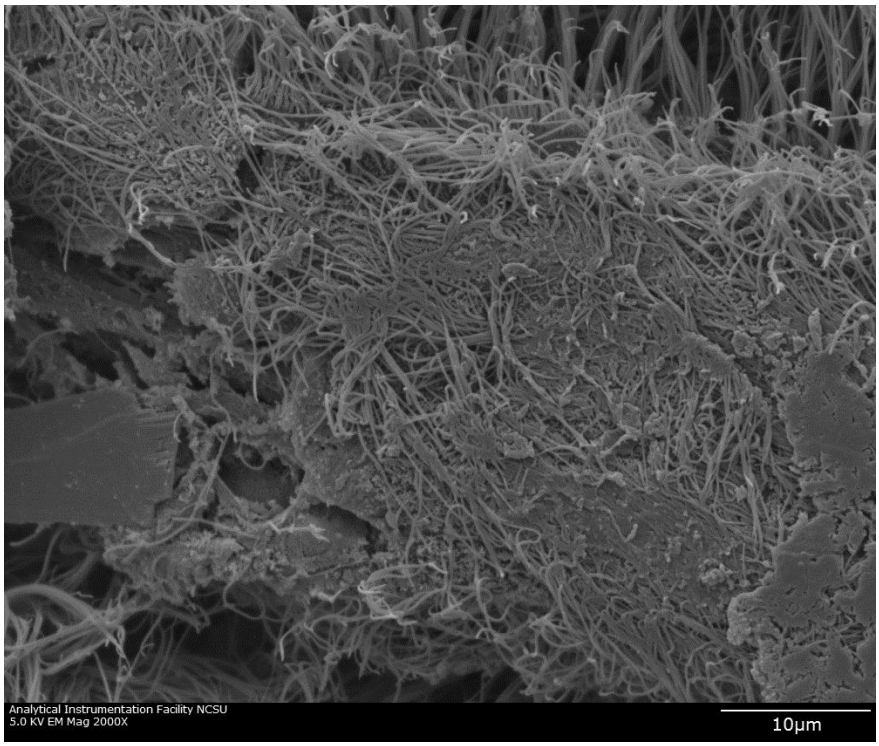

Figure 26: 10 um image of the gills of a sentinel *A. raveneliana* displaying The glue-like coating that was observed to coalesce the cilia of sentinel *A. raveneliana*.

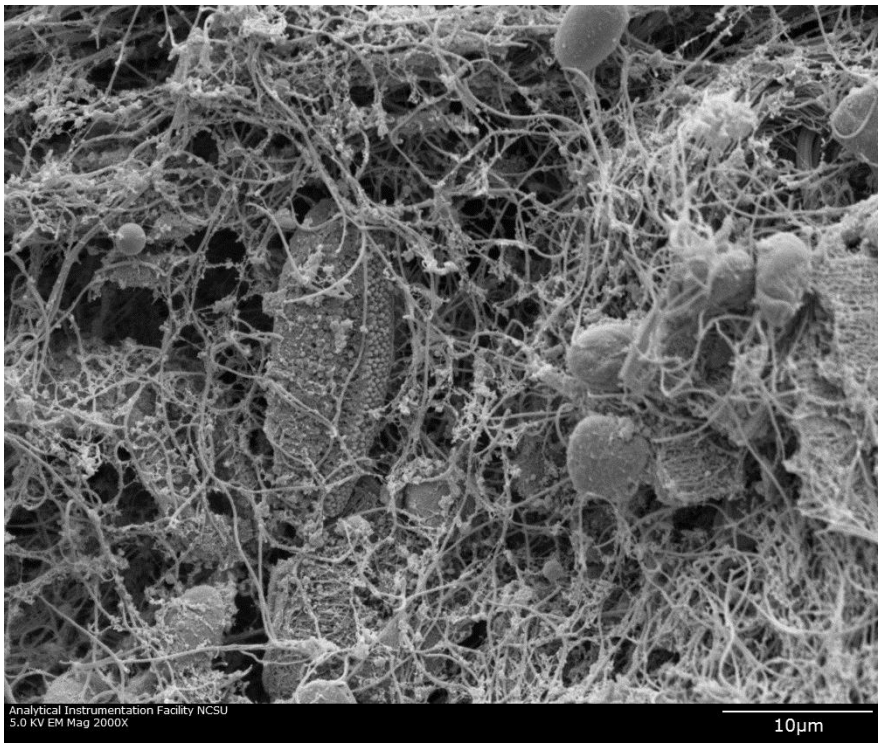

Figure 27: 10 um image of the gills of a sentinel *L. fasciola* displaying relatively circular food particles and a branching fibrin-like lattice covering the gills.

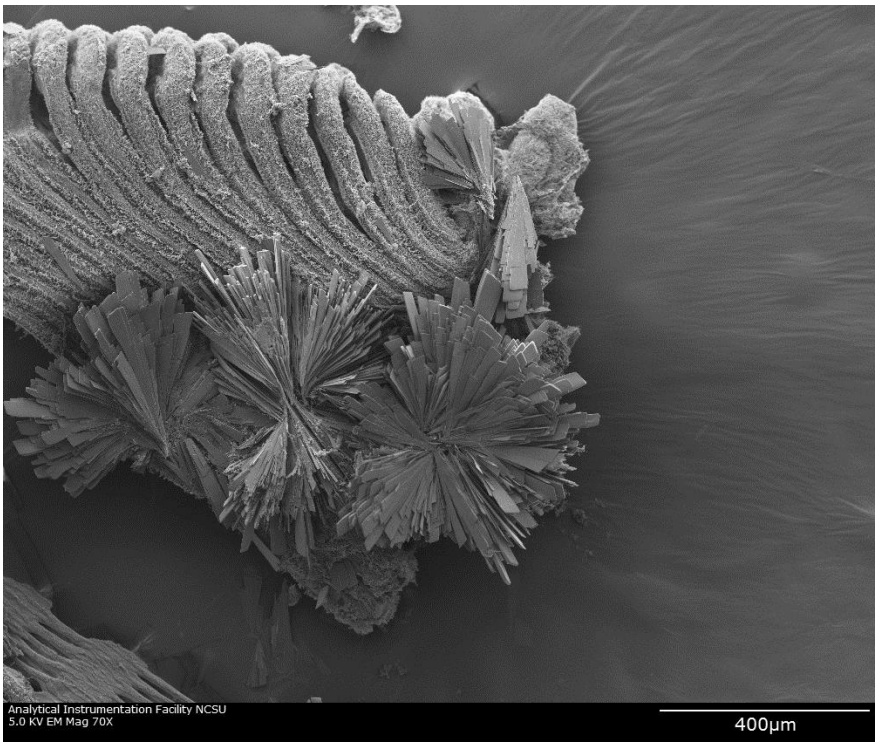

Figure 28: 400 µm scanning electron microscopy image of gill tissue showing crystal concretions on the gills of a *Lampsilis fasciola.*

Shell Analysis

Continuous laser ablation transects were made along the principal growth axes of the *A. raveneliana* and *L. fasciola* shells to discern patterns of metal enrichment during shell growth, particularly the last six months (*Alasmidonta*) or last year (*Lampsillis*). Axial (umbo-to-outer edge) cross-sections of single valves from 161 individuals in thin section were examined. Two or three valves were included in each thin section. Metal concentrations varied laterally across the valve. The majority of industrial metals surveyed (e.g., Co, Ni, As, Se, Mo, Cd, Sn, Hg) occurred near background levels. The results did not support significant uptake during shell growth. Calcium, Mg, Mn, Sr, and Ba were the elements in highest concentrations, and Mn, Sr and Ba covaried within individual growth layers. Some enrichment of Pb in the outer shell layer was seen in both baseline and sentinel animals.

Metabolomic profiles of mussels held at the study sites

The use of metabolomics is a new technique used in environmental assessment. Sometimes termed environmental metabolomics or ecometabolomics (Jones et al. 2013, Lankadurai et al. 2013, Macel et al. 2010, Sardans et al. 2011), little information is available on the metabolic profiles of many wild species. Our goal of this study was to establish a baseline metabolic "fingerprint" as it relates to *A. raveneliana* and *L. fasciola* in the Little Tennessee and Tuckasegee Rivers. We also intended to compare metabolomic

profiles between sample sites and rivers to investigate whether there were nutritional deficiencies or impairments of metabolic pathways that could help to explain the decline in *A. raveneliana* populations in these rivers.

Fifty to 150 mg of gill tissue was excised from 10 baseline *A. raveneliana* and 10 baseline *L. fasciola* at the beginning of the study. Fifty to 150 mg of tissues was also taken from each animal as they were removed from the cages for sampling, either at 6 months or 12 months after the initiation of the study. In total, gill tissue from 48 *A. raveneliana* and 52 *L. fasciola* was examined. Flash frozen samples were analyzed at the RTI International Metabolomics Core. The samples were homogenized in acetonitrile. Analysis was done in reversed phase UPLC-MS using both positive and negative ion methods on a Waters SYNAPT G2 Mass spec platform. Compound identification and quantification were performed using Progenesis QI (Nonlinear Dynamics, Durham, NC) data analysis software. Further analysis was done using MetaboAnalyst 3.0 (Xia et al. 2015).

A total of 450 metabolites were identified in our study. A general metabolic "fingerprint" of the 25 most significant metabolites imparting a difference between species is presented in Figure 25. The compounds 24-Oxo-1alpha,23,25-trihydroxyvitamin D3, 23S,25,26-Trihydroxyvitamin D3 and tetrahydrocorticosterone were downregulated in many *A. raveneliana* baseline and all *L. fasciola* samples but upregulated in all other *A. raveneliana* samples except sample C498 from Tuckasegee River site 3. 24-Oxo-1alpha,23,25-trihydroxyvitamin D3 is a metabolite of vitamin D3. Also known as cholecalciferol, vitamin D3 is synthesized by the body and serves to regulate calcium uptake (Zhang and Naughton 2010). Tetrahydrocorticosterone is a glucocorticoid with anti-inflammatory and catabolic properties. Many phosopholipids (PE, PC, LysoPE, and LysoPC) were downregulated in *A. raveneliana* as were the prostaglandins Prostaglandin B_2 and 20-hydroxy-PGE2. Phospholipids are components of the cell membrane and their distribution helps to maintain stability of these membranes. Prostaglandins have many functions such as vasoconstriction, vasodilation, and immunomodulation. Based on principal component analysis of all significant metabolites there was a difference between species but not within each species (Figure 29).

Glycogen, a primary energy store in bivalves, was significantly lower in *A. raveneliana* at each site after 6 months and in *L. fasciola* after 6 and 12 months when compared with the baseline values (Figure 30). It was also lower in mussels held in the Little Tennessee River than mussels held in the Tuckasegee River. Amino acids, small peptides, and nucleotides along with glucocorticoids in both species were elevated above baseline, which suggested tissue catabolism. Essential fatty acid levels were lower compared to baselines. These results are consistent with a decrease in nutritional health seen in the sentinel animals during our study.

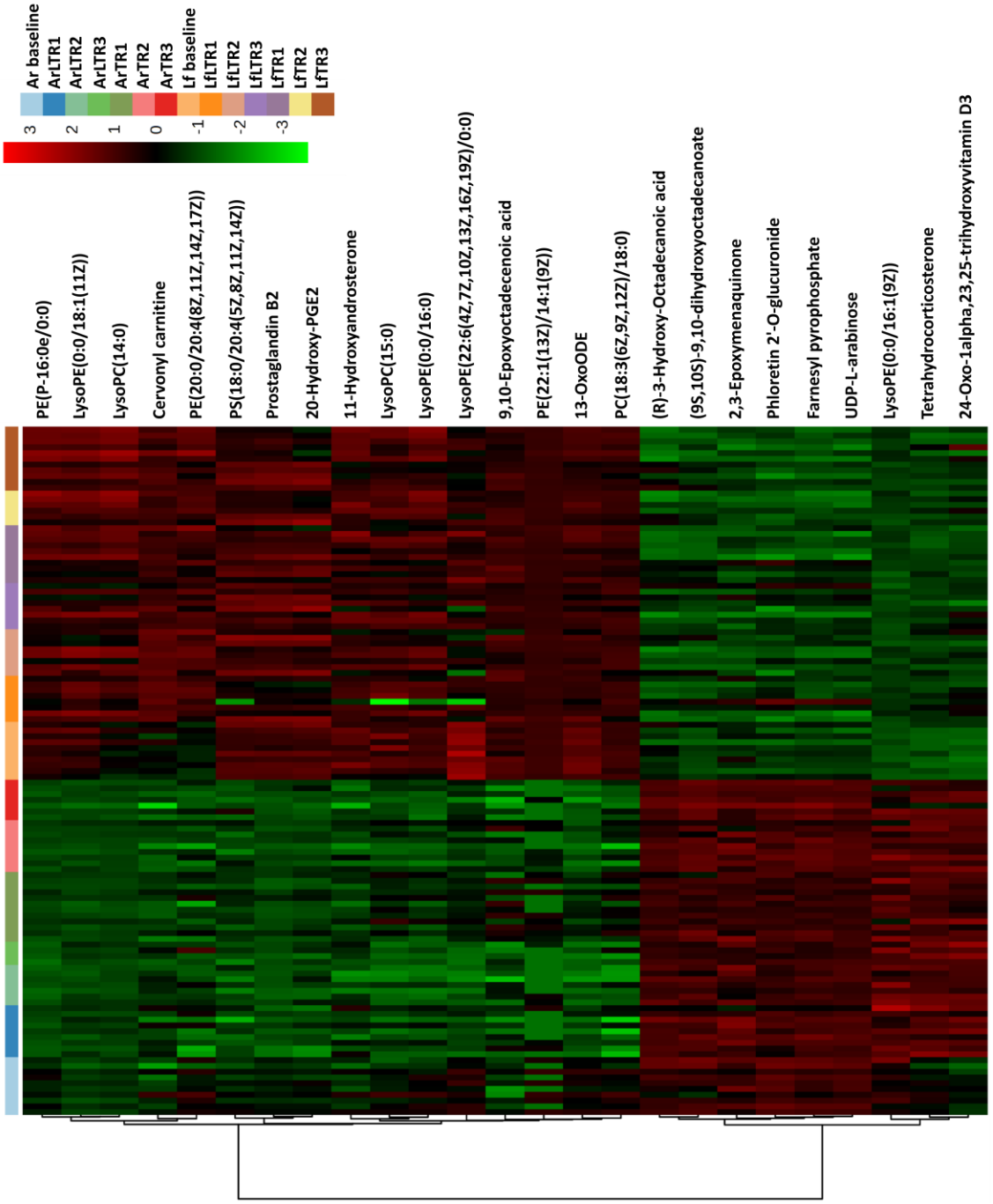

Figure 29: Metabolomic profiles of the 25 most significant metabolites identified from the digestive gland of *A. raveneliana* and *L. fasciola*. There are differences in the profiles between species but not within species between sites.

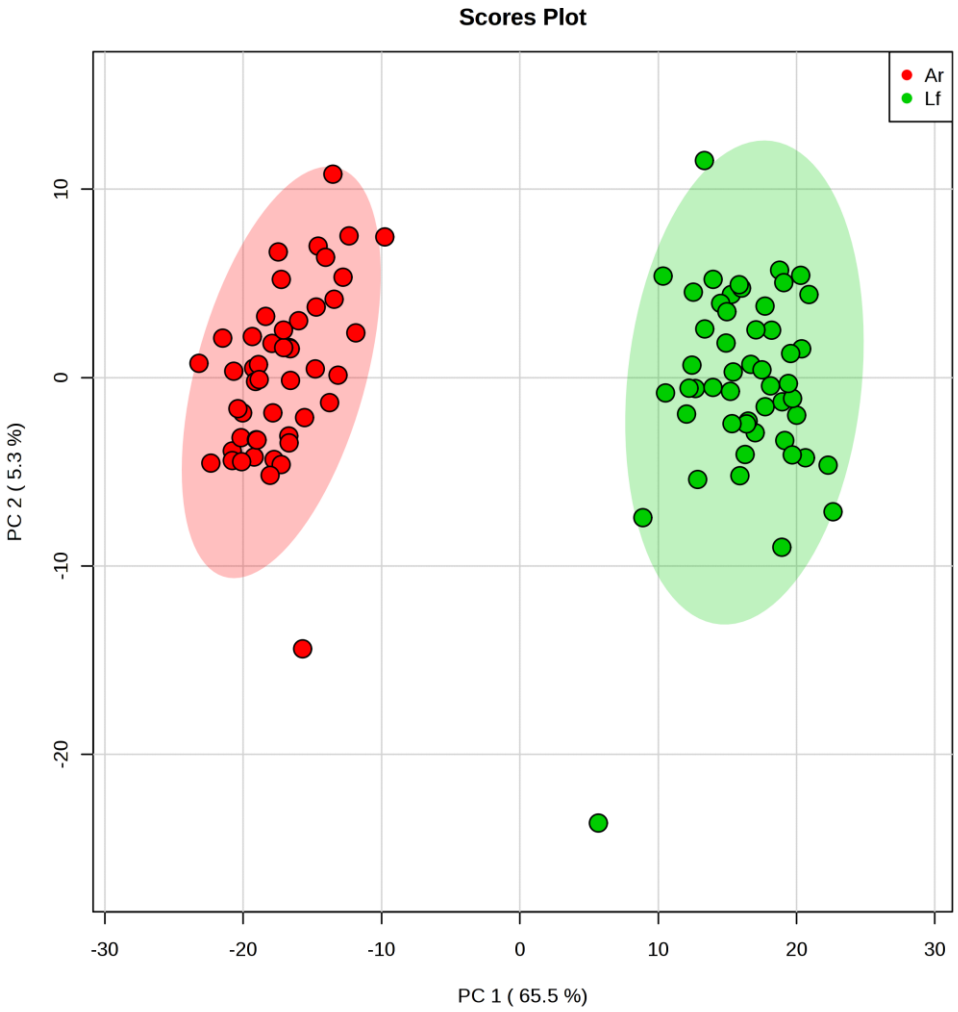

Figure 30: Principal components analysis of the relationship between compounds identified in *A. raveneliana* and *L. fasciola*.

A clustering of metabolomic compounds identified in *A. raveneliana* and *L. fasciola* baseline and site specific samples. Samples clustered within but not between species indicating that the metabolomic profiles are different between the two species.

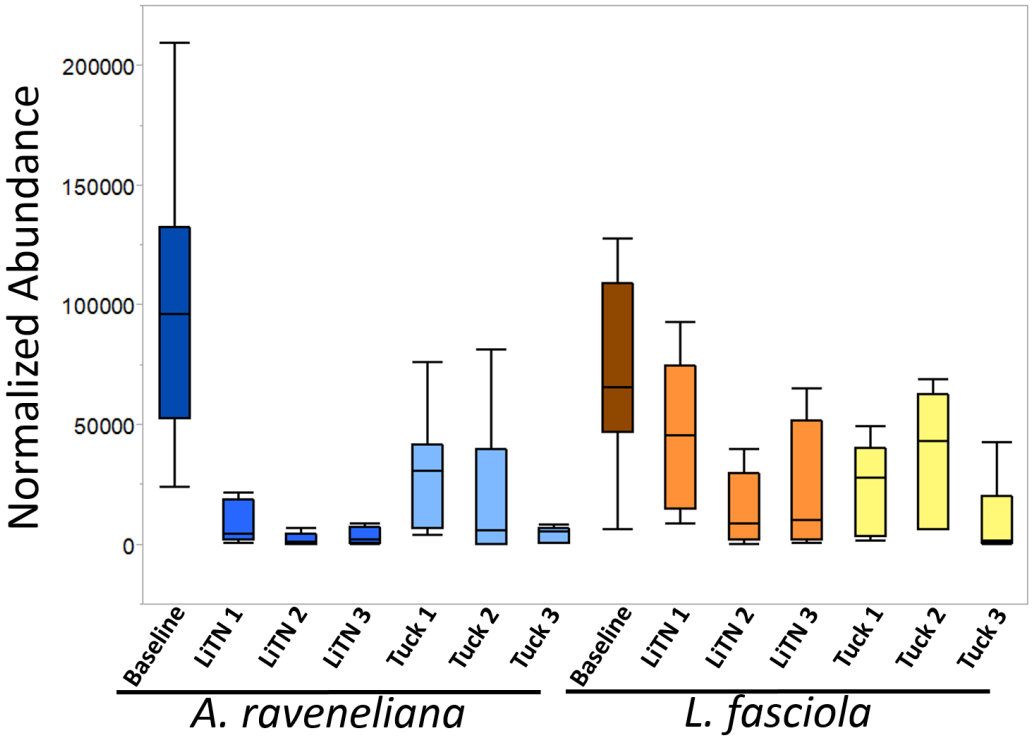

Figure 31: Glycogen abundances from mussel gill tissue. In both species, glycogen was decreased in sentinel animals from baseline, generally lower values further downstream in both rivers.

Chapter 3

Availability of Food-Web Resources in the Little Tennessee and Tuckasegee Rivers at Selected Study Sites

Efforts to study the diet of freshwater mussels have previously focused on their consumption of algae and detritus. However, recent studies have shown that their diet is more varied and that bacteria may play a large role in the nutritional health of native unionids (Nichols and Garling 2000). Fungi and other organisms may also be playing an as yet undefined role in their nutritional health. We conducted a comprehensive assessment of available food resources at the sites where the mussels are being held and a site without mussels in each river system.

Water samples were obtained during biweekly visits to assess mussel cage integrity and after 3 storm events. The samples were used to measure a suite of parameters that reflected the food resources available to freshwater mussels in the rivers. Particulate organic matter was measured biweekly, and after 3 storm events as well. A 2-liter water sample was obtained at each site and placed in a cooler containing blue ice for transport by courier to the laboratory. In the laboratory, the sample was fractioned into 1 L splits. Each liter was passed through a pre-cleaned 0.7 μm glass-fiber (GF/F) filter. One split was used to assess particulate organic matter (e.g. Fine and dissolved) concentration, measured by mass. A second split was extracted in 90% acetone:water (v/v) for chlorophyll-*a* and other pigments, measured by spectrophotometry. The final 2 L split was filtered and then archived for stable isotope and other analyses.

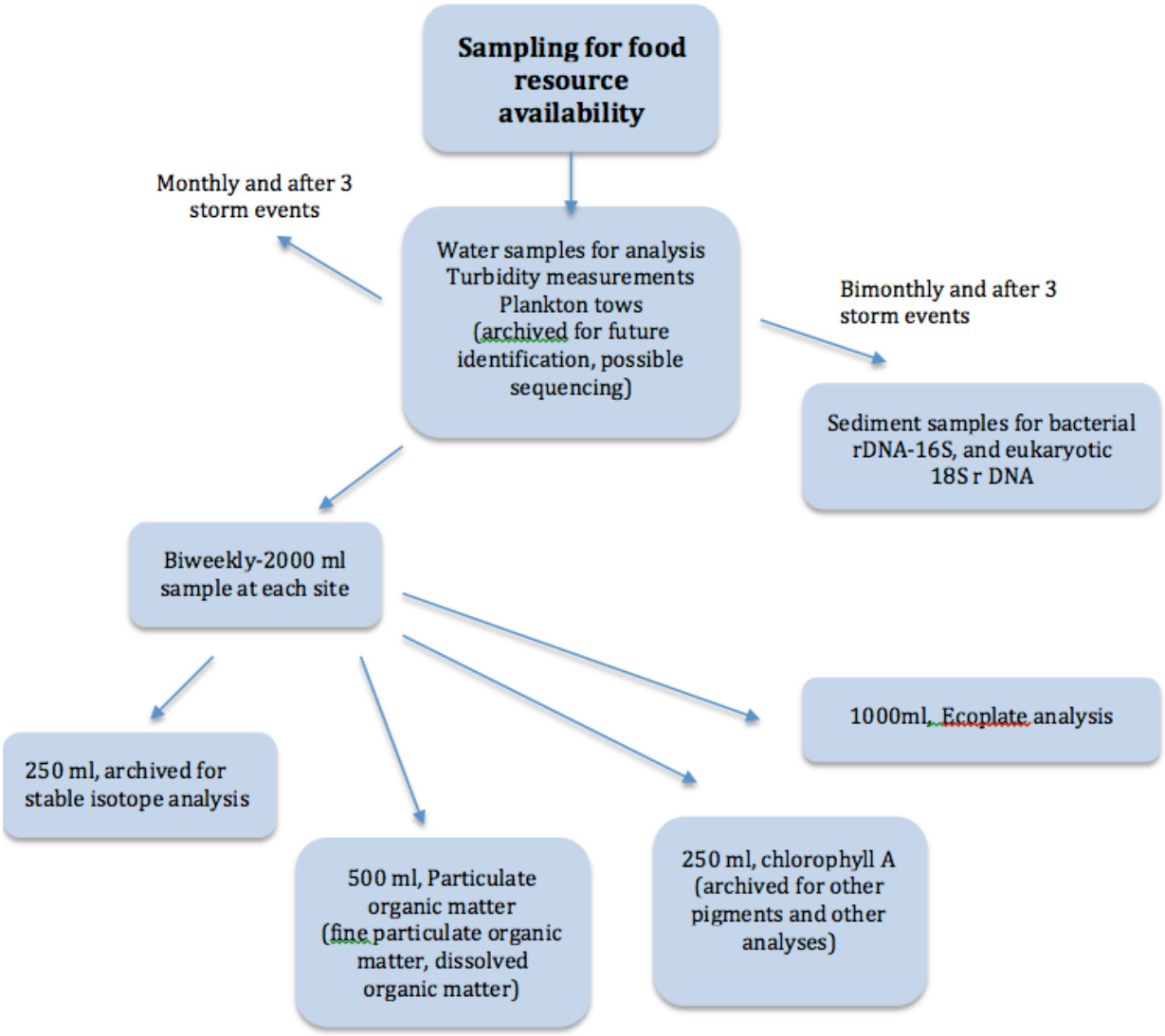

Figure 32: Sample collection and analysis plan for food resource availability.

Results

Dissolved organic matter (DOM) in the Little Tennessee and Tuckasegee Rivers from synoptic samplings and in experiments was based on absorption and measured on a Varian Cary 300 spectrophotometer. Dissolved organic matter absorption has been shown to be a key water quality indicator of humic substance transport and light environment in rivers (e.g., Spencer et al. 2007; Saraceno et al. 2009) and DOM excitation-emission matrix (EEM) fluorescence, measured on a Varian Cary Eclipse fluorometer (Stedmon et al. 2003). The EEMs were decomposed by a statistical method (PARAFAC; Stedmon & Bro 2008) into fluorescent components specific to DOM origin and reactivity. This technique has proved useful in determining how water quality varies as a function of land use-land cover (Williams et al. 2010).

Chlorophyll-*a* concentration was quantified by spectrophotometery (Lorenzen 1967) and pigment EEM fluorescence was measured to evaluate presence of cyanophytes (Welschmeyer 1994; Moberg et al. 2001).

Plankton tows were conducted monthly when the sites were visited to harvest zooplankton (e.g. daphia, holopedium, copopods) and phytoplankton for later analysis. The samples were stored in alcohol and archived for future morphologic assessment as well as genetic sequencing. Assessment will facilitate evaluation of this valuable resource if initial study findings warrant their examination and funds are available to support their analysis.

Water chemistry parameters for organic matter – principally dissolved organic carbon (DOC) – were measured to determine any differences between the Tuckasegee and Little Tennessee rivers as well as the three sampling sites in each stream. The results from each stream are summarized in Tables 5 and 6. Repeated measures ANOVA tests were used to look for differences in water chemistry results among streams and sites. TSS is the total suspended solids, a measure of stream turbidity.

DOC and Stable carbon isotopes ($\delta^{13}C$)

The stable carbon isotope ($\delta^{13}C$) value of DOC was measured along with DOC concentration. Mean DOC was higher in the Tuckasegee River compared to the Little Tennessee River though the differences were not significant ($p>0.05$). The DOC concentrations were similar to those reported by Yamashita et al. (2011) for streams in the Coweeta Hydrologic Laboratory, NC. However, Tuckasegee site 1 did have roughly 30% more DOC than did Little Tennessee site 1 (ANOVA; $P<0.05$; $N=24$). Tuckasegee site 1 also had more DOC than Tuckasegee sites 2 or 3 though the differences were not significant. The $\delta^{13}C$ values of the streams also overlap, but generally fall into the range expected for streams (-23 to -28‰). The values largely reflected soil inputs.

DOM Absorption

Absorption at 254 nm (a_{254}) is used as a proxy for DOC concentration as it is the primary absorption for aromatic ring carbon (Weishaar et al. 2003). S_R, the slope ratio, and $SUVA_{254}$ are qualitative measures of DOM. S_R is the ratio of spectral slopes fit to dissolved absorption spectra over 275-295 nm and 350-400 nm (Helms et al. 2008). S_R values in freshwater tend to range from 0.6 for humic water (aka "black water" streams that are hydrologically connected to swamps) up to 2-3 for groundwaters and oligotrophic streams and seawater. $SUVA_{254}$ is an index for aromatic carbon and should positively correlate with more terrestrial (soil-derived) DOC entering the streams. $SUVA_{254}$ values typically fall between 1 and 4 with lower values reflecting low DOC stream water and high values common to black water streams.

For the most part the streams exhibited very similar characteristics with respect to aromatic carbon quality. No clear differences between the streams or between sites were apparent with respect to S_R or $SUVA_{254}$.

Table 5. Summary statistics for organic carbon properties of Little Tennessee River Sites 1-3.

	DOC (mg/L)	*$\delta^{13}C$ (‰)*	*TSS (mg/L)*	*a_{254} (m^{-1})*	*S_R*	*$SUVA_{254}$*	*C1*	*C2*	*C3*	*C4*	*C5*
Mean	0.96	-25.67	13.75	7.17	0.75	3.38	3.77	2.32	2.43	1.50	1.41
S. D.	0.42	1.16	15.75	4.54	0.11	1.57	1.64	1.01	1.26	0.64	0.58
Min	0.39	-28.24	1.84	1.90	0.29	0.84	0.99	1.07	0.73	0.57	0.64
Max	3.02	-22.64	95.20	24.89	1.09	6.18	11.06	6.85	5.88	4.12	3.35
Count	77	77	69	78	78	77	75	75	75	75	75

Table 6. Summary statistics for organic carbon properties of Tuckaseegee River Sites 1-3.

	DOC (mg/L)	*$\delta^{13}C$ (‰)*	*TSS (mg/L)*	*a_{254} (m^{-1})*	*S_R*	*$SUVA_{254}$*	*C1*	*C2*	*C3*	*C4*	*C5*
Mean	1.22	-25.49	23.40	8.62	0.77	3.10	4.99	2.92	2.77	1.77	1.41
S. D.	0.51	0.97	57.01	4.49	0.12	1.27	1.81	0.90	1.32	0.64	0.75
Min	0.58	-27.27	1.10	1.92	0.24	1.17	0.86	1.63	1.29	0.99	0.70
Max	3.92	-22.20	375.60	25.79	0.98	5.36	11.36	5.69	6.52	4.34	4.97
Count	77	77	74	76	76	75	75	75	75	75	75

EEM-PARAFAC of DOM fluorescence

DOM fluorescence was measured as excitation-emission matrices (EEMs). These are 3-way data (excitation wavelength, emission wavelength, intensity) which can be decomposed by parallel factor analysis (PARAFAC; Murphy et al. 2013). C1-C5 represent components from a PARAFAC model fit to DOM fluorescence (Figure 33). The components were tested against the OpenFluor database (http://www.openfluor.org; Murphy et al. 2014), an online library of PARAFAC models from a variety of aquatic ecosystems, against which components from a model may be queried. The library provides matches that are ostensibly 95% similar to the queried components.

C1 is a common aquatic fulvic acid fluorescent component found in many surface water systems. Notable matches are to NC streams in the Coweeta Hydrologic Laboratory and to the Neuse River (Yamashita et al. 2011; Osburn et al. 2012) as well European headwater streams (Graeber et al. 2012). C2 resembled soil organic matter leachate and was enriched in the Neuse River estuary after strong precipitation occurring during Hurricane Irene in 2011 (Osburn et al. 2012). C3 fluorescence was common to microbially-produced humic substances resulting from biological activity in stream waters ranging from temperate (Graeber et al. 2012) to Arctic (Walker et al. 2014) to tropical (Yamashita et al. 2010) environments. C4 matched with several models though the origin of this component is uncertain. It shares fluorescence properties with components from models that have a planktonic signature and likely is related to ubiquinone (Brym et al 2014).

Repeated measures ANOVA showed only a significant ($p<0.05$) difference between the two streams for Little Tennessee site 3 which had less C4 fluorescence than did Tuckasegee site 2 or Tuckasegee site 3.

Differences between streams were apparent when PARAFAC values for each fluorescent component were subjected to principle components analysis (PCA; Fig. 34). In the top panel, Tuckasegee scores cluster mainly negative on PC1 showing some separation between the streams. Note that the total variance explained is low (about 25%) but do highlight subtle differences in DOM quality between the streams. Negative scores on PC1 are influenced by loadings for C1 and C2 (Fig. 35). Summer and Fall showed the clearest separation in the PCA scores (Fig. 34, bottom). Summer scores were mainly negative on PC1 and positive on PC2, whereas Fall scores were mainly positive on PC1 and negative on PC2. The loadings for C3 were positive on PC1 and negative on PC2 suggesting that Fall DOM quality was influenced by microbial humic substances. This result could indicate degradation of DOM contributed to each stream during Spring and Summer either via the watershed during precipitation events or perhaps from in-stream primary production.

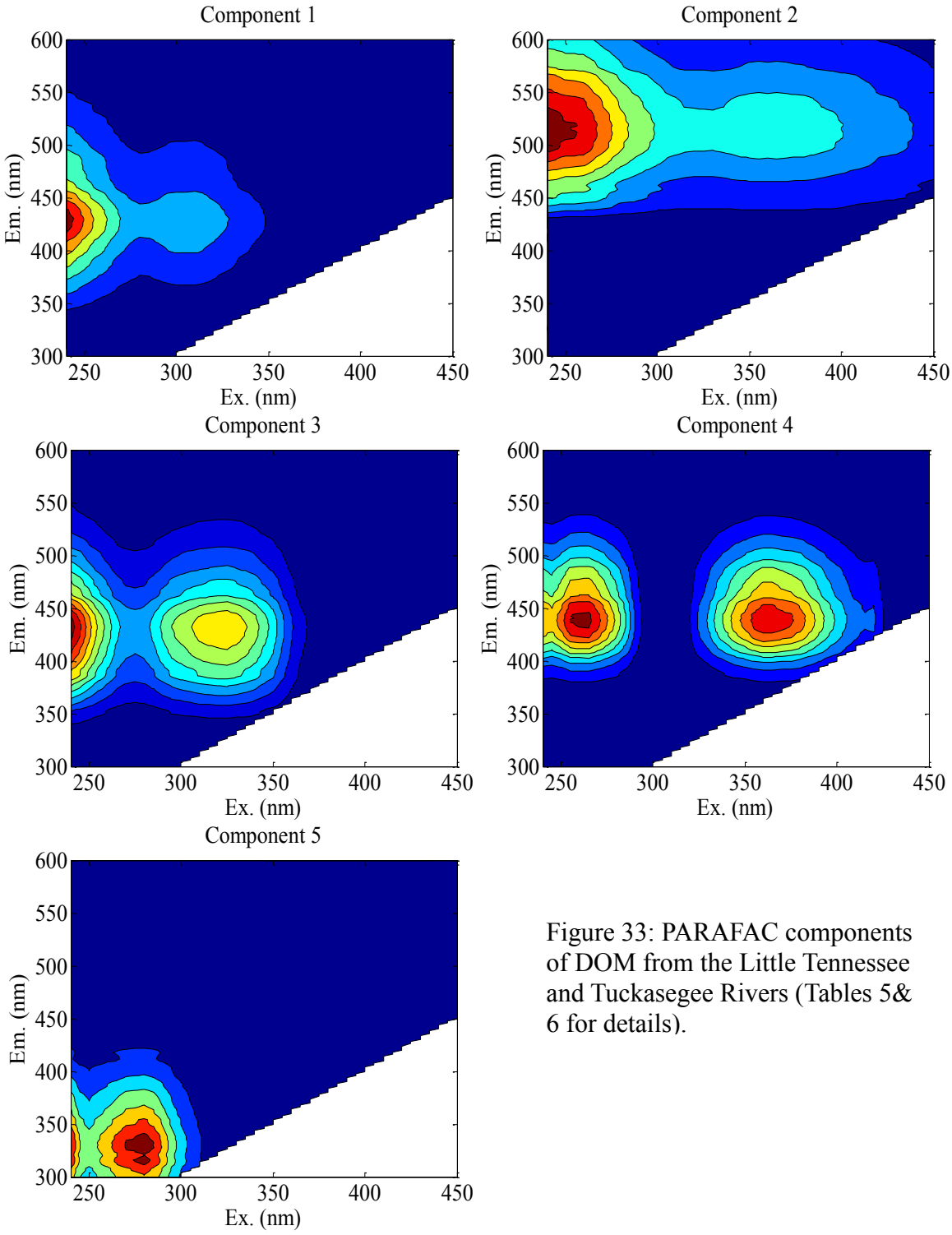

Figure 33: PARAFAC components of DOM from the Little Tennessee and Tuckasegee Rivers (Tables 5& 6 for details).

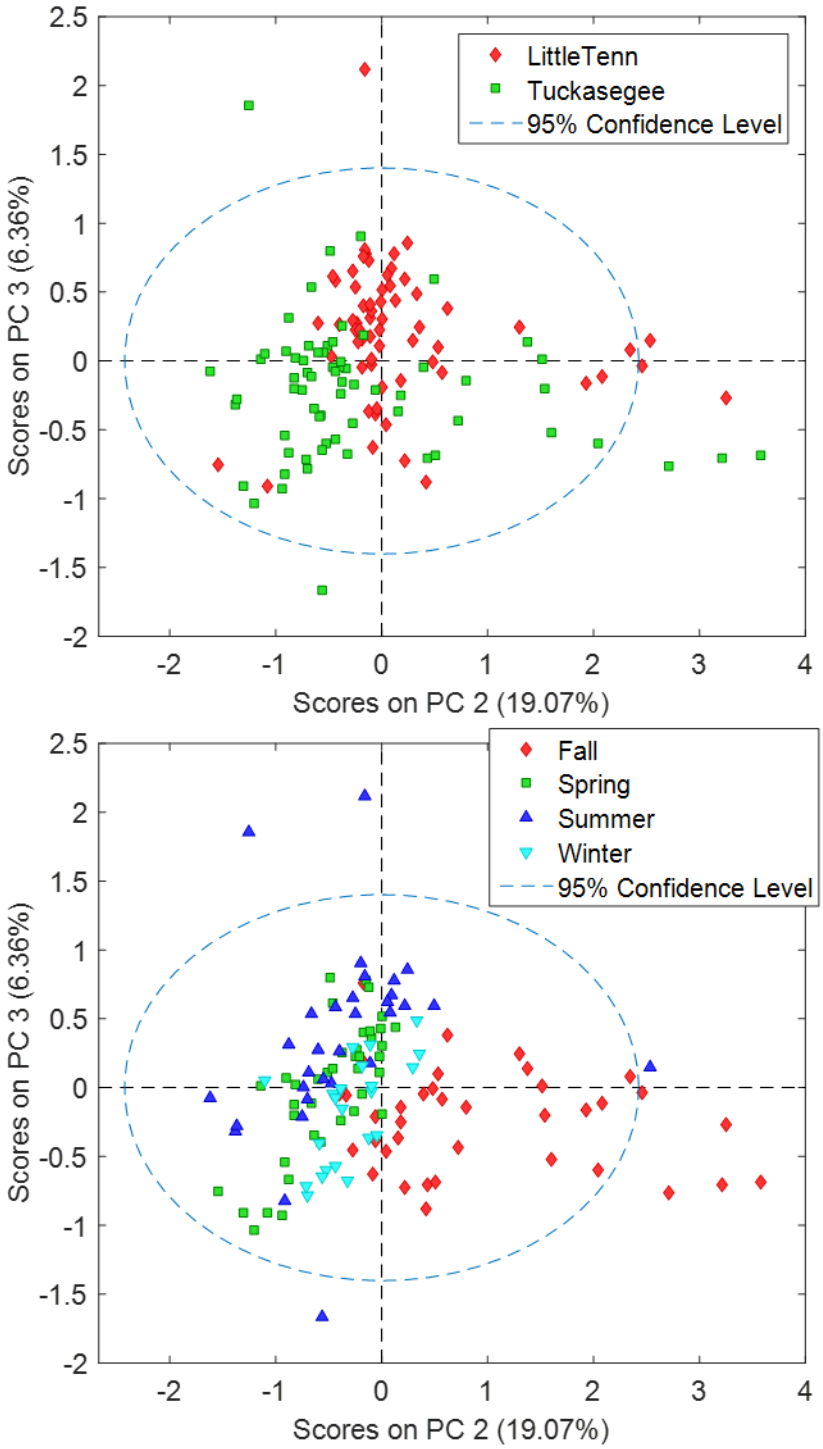

Figure 34: PCA conducted on PARAFAC results show differences between the Tuckasegee River and Little Tennessee River (TOP) as well as differences between seasons (BOTTOM).

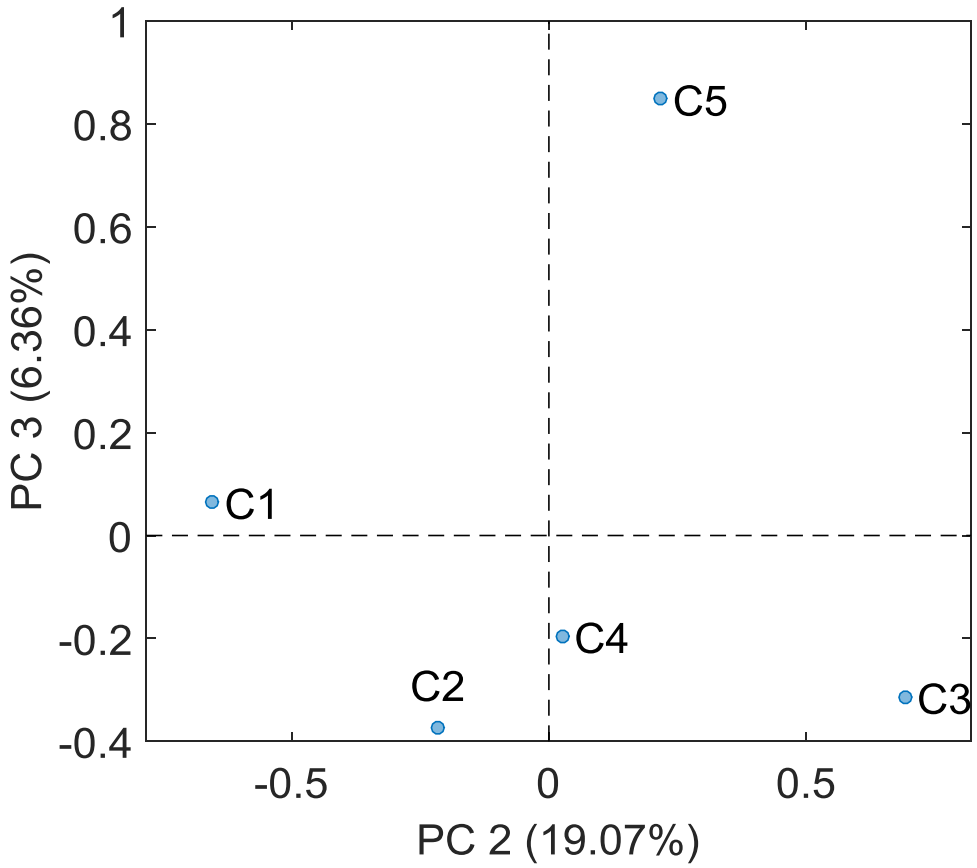

Figure 35. Loadings plot of PCA showing distribution of components. C1=aquatic fulvic acid-type; C2 = soil fulvic acid-type; C3 = microbial humic-type; C4 = ubiquinone-type; C5 = protein-type.

Chapter 4

Develop a Microbial Profile of the Little Tennessee and Tuckasegee River Sediment at the Selected Study Sites

Community Metabolic Profiles

Biolog EcoPlates® have been successfully employed in the study of spatial and temporal changes in environmental bacterial communities (Garland et. al. 1991). This technique uses substrate dependent dye reduction to measure cellular metabolism of 31 ecologically relevant carbon sources. Reduction of the tetrazolium dye by cellular metabolism results in a colorimetric change that is detected using an automated spectrophotometer. The differential utilization of substrates provides a community-level metabolic profile. Since their creation, Biolog EcoPlates have provided functional physiologic profiles of bacterial communities in various environments like soils (Gorlenko and Kozhevin, 1994; Winding, 1994; Zak et al., 1994; Bossio and Scow, 1995; Stephan et al., 2000; Muller et al., 2001; Widmer et al., 2001), freshwater (Garland and Mills, 1991), sediments (Fredrickson et al., 1991), activated sludge and seawater (Hollibaugh, 1994). In this study we used Biolog EcoPlates® to: 1) characterize the microbial communities in the sediment of the Tuckasegee and compare these profiles with sediment samples from the Little Tennessee River; 2) assess for temporal changes in the microbial community profiles by repeated sampling and 3) assess alterations in microbial community profiles following adverse events (e.g. introduction of waste water run-off following heavy rains).

Community Microbial Profiles

Bacteria apparently play a more prominent role in the diets of freshwater mussels than previously recognized. Although algae are the traditional diet fed to unionids in captivity, Allen (1921) found unionids ingest bacteria and protozoa. As noted above, stable isotope studies have confirmed the potential role of bacteria in the diets of some unionids (Nichols and Garling 2000). Bacteria, fungi and other microbes play an important role in the basic metabolic processes that help sustain the health of aquatic ecosystems.

Metagenomic profiles were sequenced on an Illumina MiSeq platform to assess spatial as well as temporal differences in microbial communities in water, sediment and gastrointestinal tracts of the mussels at the study sites.

Amplification of prokaryotic and eukaryotic rDNA:
Primers were designed to amplify the V3 and V4 regions of 16S rDNA or the V9 region of 18S rDNA compatible with the Illumina index and sequencing adapters and allowed for double indexing to increase the accuracy of the multiplexed reads. Prokaryotic 16S rDNA sequences were amplified from genomic DNA using Round1F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG) and Round1R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGTCAATTCMTTTRAGT) primer set. Eukaryotic 18S rDNA sequences were amplified from genomic DNA using EukRound1F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGTGCATGGCCGTTCTTAGT) and EukRound1R1 (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGATCCTTCTGCAGGTTCACCTAC) primer set (Amaral-Zettler et al. 2009). Polymerase Chain Reaction (PCR) conditions for

the amplification step were: 1 cycle of 95°C for 3 min; 25 cycles each of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 1 cycle of 72°C for 5 min; and hold at 4°C.

An 8 bp index was used to multiplex samples for sequencing in lanes of the Illumina MiSeq benchtop sequencer, two lanes per sample to increase the number of reads. PCR conditions for the index step were: 1 cycle of 95°C for 3 min; 8 cycles each of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 1 cycle of 72°C for 5 min; and hold at 4°C.

16S rDNA amplicon analysis: The resulting MiSeq reads were demultiplexed and sorted to the proper samples. The demultiplexed reads were joined and processed using the QIIME 1.8.0 metagenomics package (Caporaso et al. 2010). Uclust operational taxonomic unit (out) picking (Edgar 2010) was performed to search the Greengenes 13_8 reference database (DeSantis et al. 2006). Alpha-diversity and β-diversity was performed to determine relationships within and between individual samples, respectively.

18S rDNA amplicon analysis: Following demultiplexing of sequences, the same steps were used as above with the exception of the database used for OTU picking which was the SILVA release 119 reference database (Pruesse et al 2007, Quast et al 2013, Yilmaz et al 2014).

Results

Community Metabolic Profiling

Monthly water samples collected at each of the 6 sites were analyzed to develop community-level metabolic profiles. The samples were processed to the Biolog manufacturer's specifications and substrate utilization patterns were compared using Principle Components Analysis (PCA) of average well color development (Firestone et. al. 1997, Glimm et. al. 1997, Garland et. al. 1997).

Community metabolic profiles of the sites were compared between sites and overtime. In addition, since no mortality was observed at the site 1 in the Tuckasegee River we compared metabolic activity at the Tuckasegee 1 site with the metabolic activity at all the other sites. When Tuckasegee 1 was compared with all the sites there was a statistically significant difference in two metabolites in the winter, one in the summer, and five throughout the year. These metabolic differences reflect changes in the bacterial processing of stream nutrients and reflect the overall health of the stream ecosystem.

Compared to all other sites, the microbial community at Tuckasegee site 1 demonstrated increased utilization of 6 substrates and decreased utilization of 2 substrates. Of these, 5 substrates had increased metabolism in Tuckasegee 1 throughout the year: pyruvic acid methyl ester, d-galacturonic acid, l-asparagine, i-erythritol, and d-mannitol. In summer, significantly increased metabolism of 4-hydroxybenzoic acid was observed. Interestingly, in winter decreased metabolism of l-serine and itaconic acid were observed. This pattern of substrate utilization may be useful for identifying healthy streams for mussel population augmentation.

Microbial Community Profiles

The water, sediment, and mussel gastrointestinal samples analyzed had a large diversity of 16S rDNA present. The 16S small ribosomal subunit is found primarily in prokaryotes such as archaea and bacteria allowing us to determine their populations by amplifying nonconserved regions of their ribosomal DNA. In our study, we saw a large proportion of bacteria in the phyla Proteobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria, and Acidobacteria in all samples sequenced. These are ubiquitous, but highly diverse groups of bacteria encompassing species that metabolize carbon compounds, are symbionts of other organisms, are photosynthetic, and some that, in the correct conditions, are pathogenic.

The proportional profiles of OTUs identified from the sediment sample sequences throughout the study varied little between sites (Figure 36) but within the water column variation occurred between site with a decreasing proportion of Proteobacteria and an increasing proportion of Bacteroidetes moving from the upstream to downstream sites (Figure 37). Proteobacteria are a group of bacteria that include free-living organisms with some that fix nitrogen. The Bacteroidetes include Flavobacteria, which are ubiquitous in many freshwater streams and lakes. Sediment samples were dominated by the Proteobacteria and Acidobacteria, which are ubiquitous in soil. A larger proportion of unknown organisms were found in *L. fasciola* after being placed in the rivers (Figure 38). The proportions and types of bacteria found in *A. raveneliana* and *C. fluminea* were similar, with only 46 and 41 different species, respectively, found. *A. raveneliana* baseline animals were removed from the Tuckasegee River prior to placement of sentinel animals into the cages at their respective sites. *L. fasciola* baselines, on the other hand, were raised at the Marion Conservation Aquaculture Center, Marion, NC, which sources its water from the Catawba River, which could explain the different bacterial profiles between *L. fasciola* baseline animals and sentinel animals. The dominant bacterial groups found in the guts of the mussels were from the Proteobacteria, Firmicutes (include Bacillus and Clostridia which are common gut colonizers of many organisms), and Cyanobacteria (Figures 39, 40).

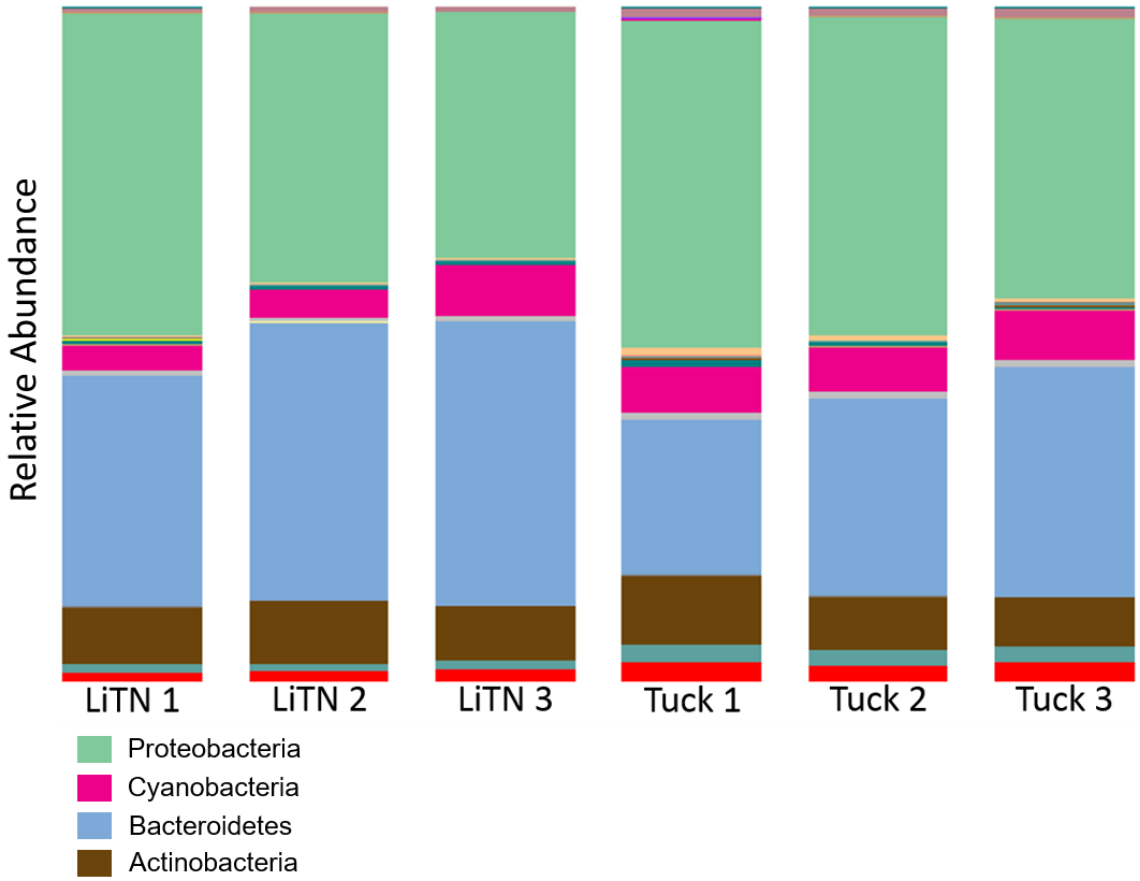

Figure 36: Proportional abundance of prokaryotic rDNA present in water samples from the study sites.

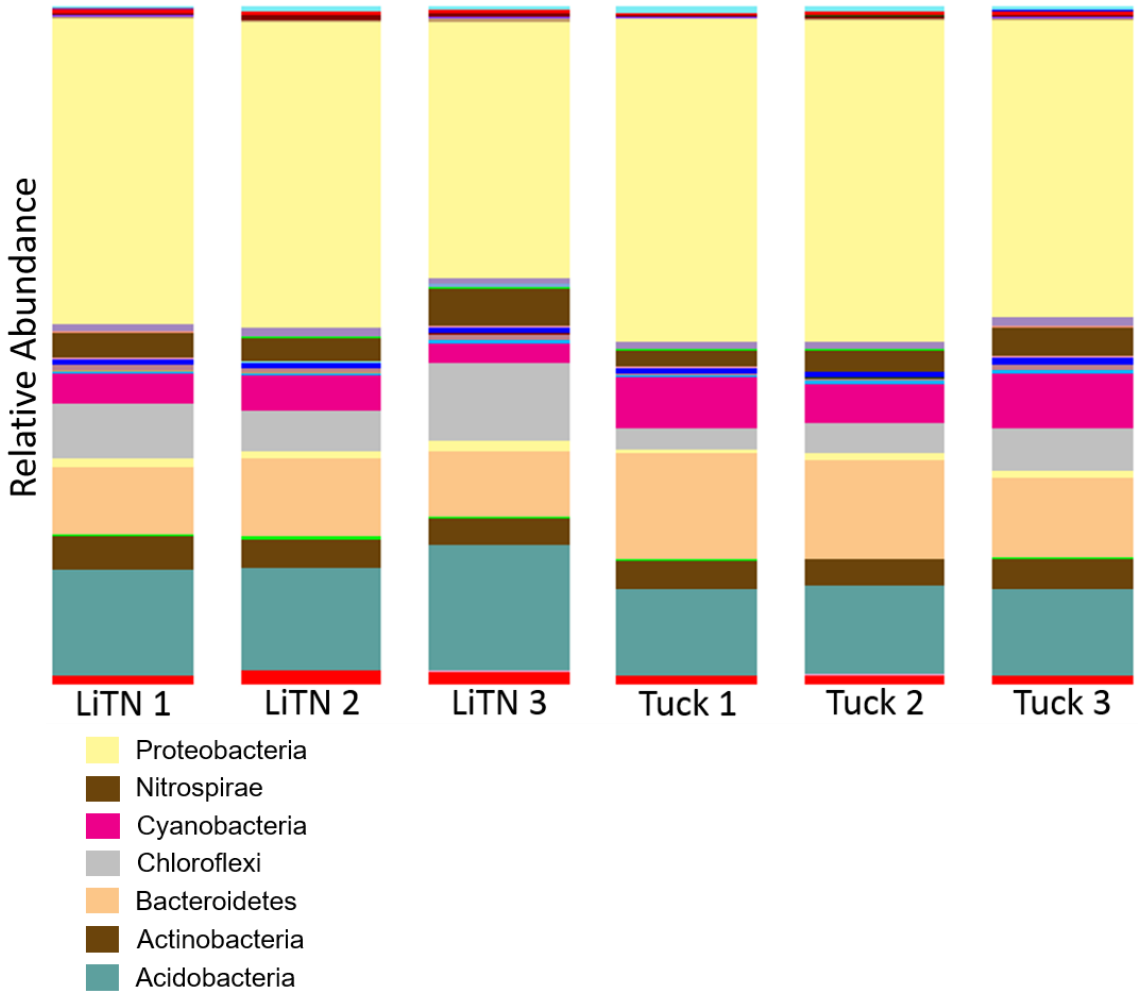

Figure 37: Proportional abundance of prokaryotic rDNA present in sediment samples from the study sites.

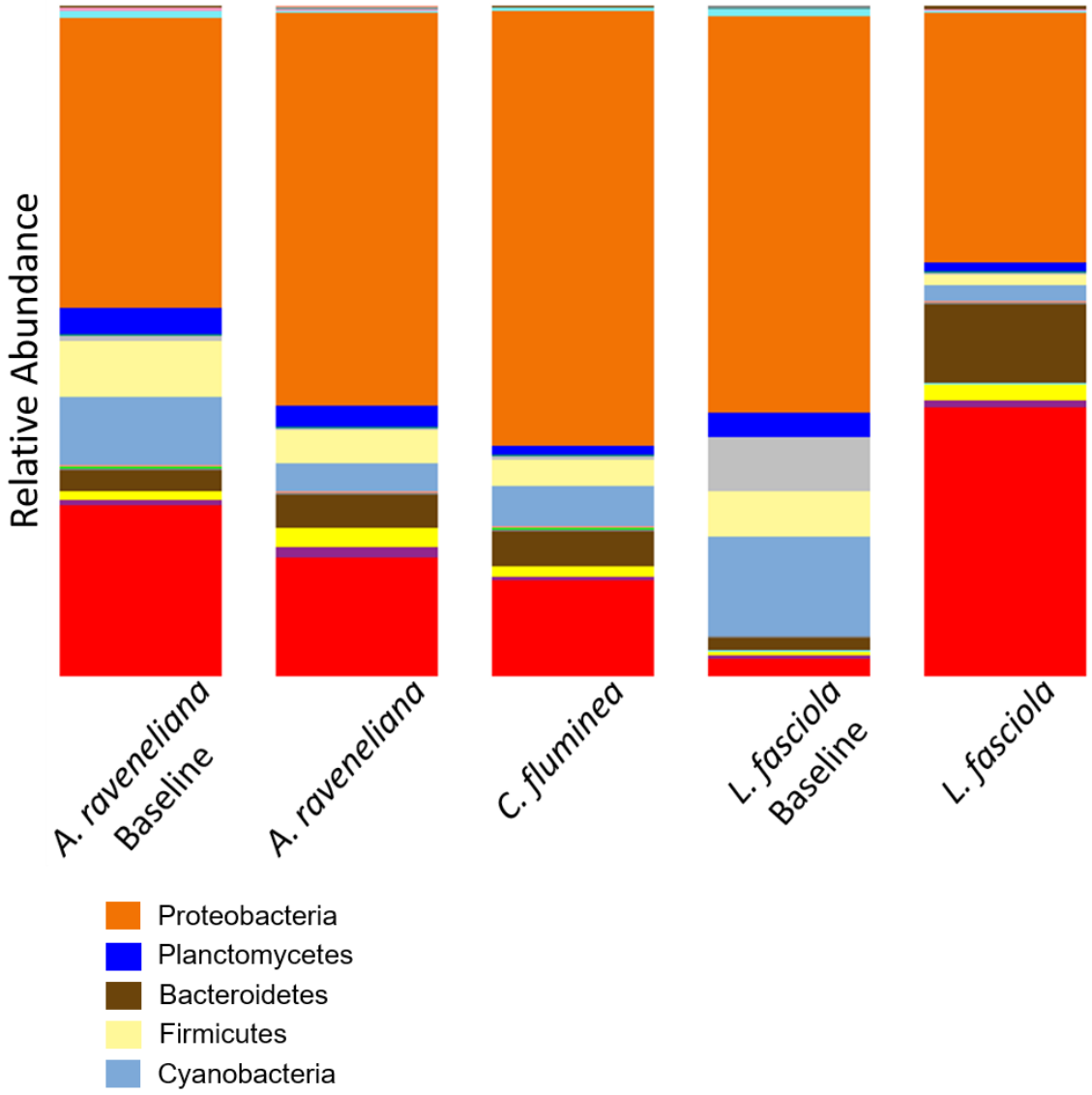

Figure 38: Proportional abundance of prokaryotic rDNA present in mussel gastrointestinal samples separated by species and site.

The eukaryotic profiles from the water and sediment samples were dominated by the SAR (Stramenopiles, Alveolates, and Rhizaria), Opisthokonta, Cryptophyceae, and Archaeplastida groups. The SAR group is dominated by species from Classes Oomycota (water moulds), Ochrophyta (diatoms), and Ciliophora (ciliated protozoans), while the group Opisthokonta are dominated by Fungi and Metazoans. Organisms of the SAR group were proportionally higher in the water column at Little Tennessee 3 than at the other two sites on that river. Proportions of dinoflagellates were higher in the water at Tuckasegee site 1 than at any of the other sites (Figure 390. In the sediment samples, Oomycota and Fungi were proportionally higher at Little Tennessee site 1 than the other sites while the Ochrophytes were higher in proportion at Tuckasegee sites 2 and 3 but not site 1 (Figure 40).

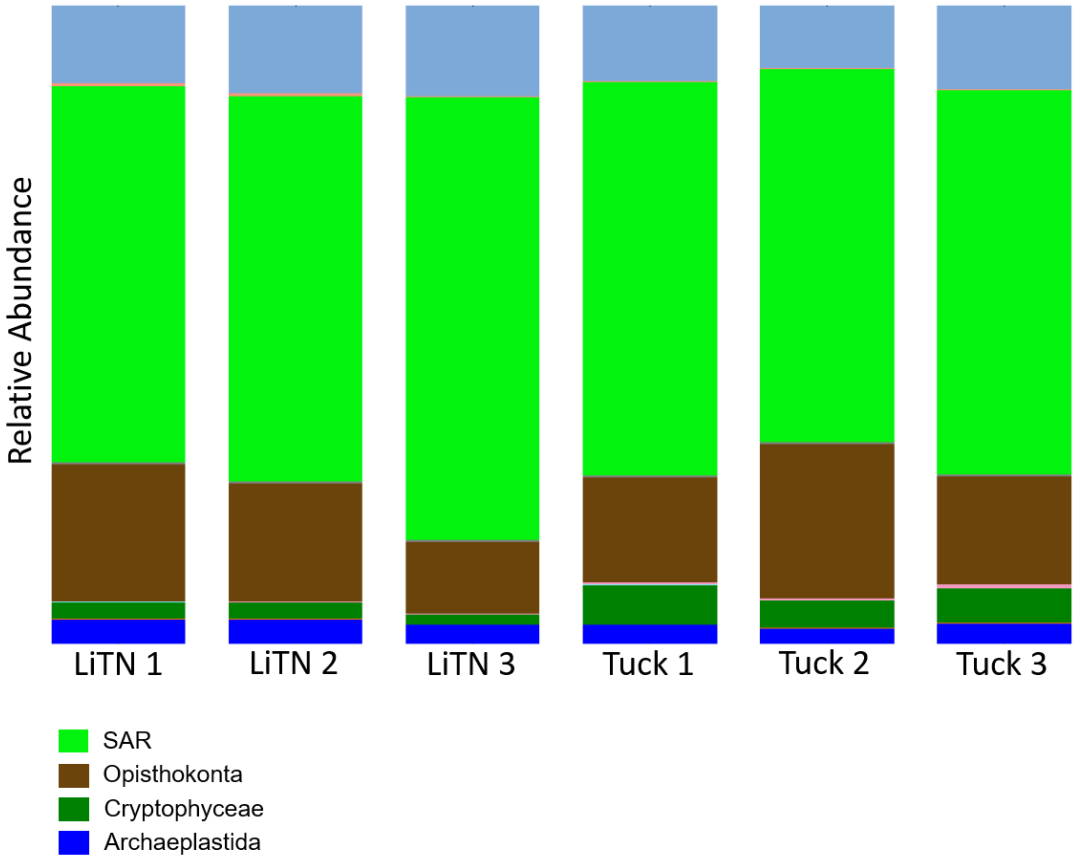

Figure 39: Proportional abundance of eukaryotic rDNA present in water samples from the study sites.

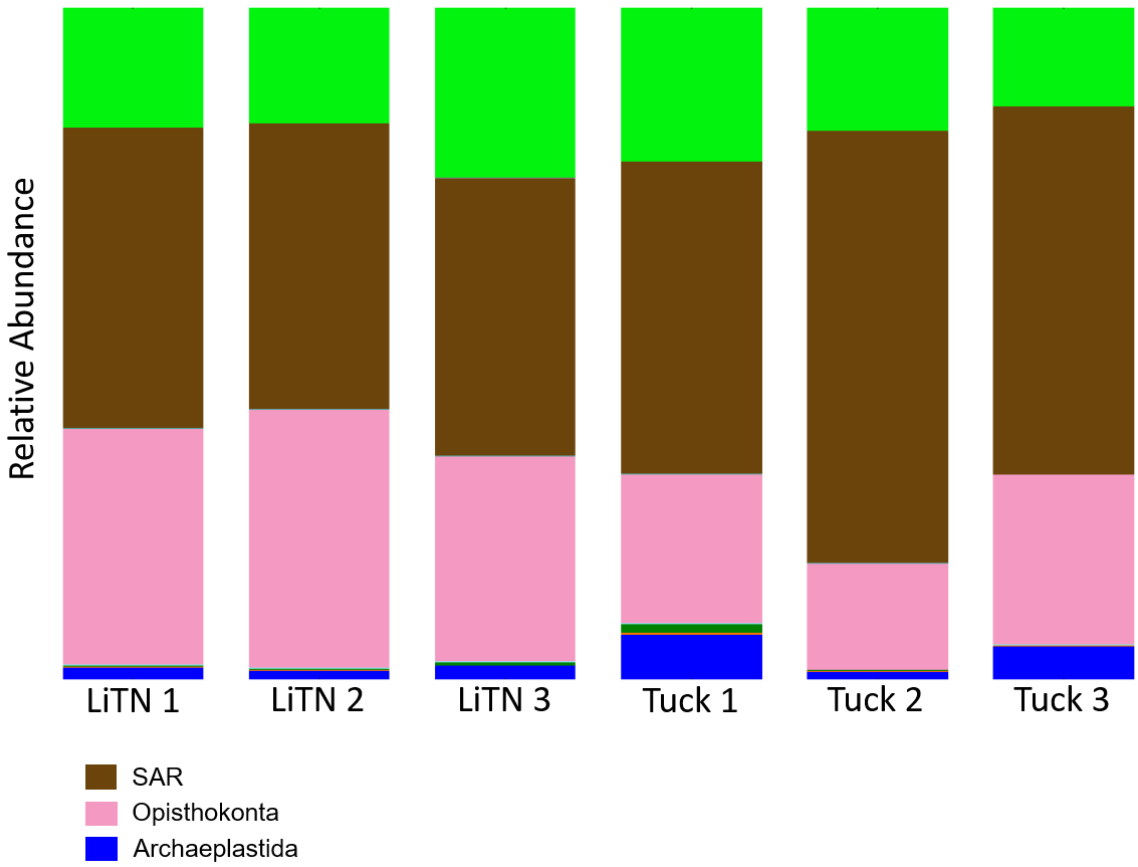

Figure 40: Proportional abundance of eukaryotic rDNA present in sediment samples from the study sites.

Alpha-diversity was measured by the Shannon Index metric as a measure of diversity within each site and mussel species. Prokaryotic diversity was higher in the Tuckasegee River water samples compared to the Little Tennessee River water samples but both rivers had similar diversity in the sediment samples. Prokaryotic diversity within the gastrointestinal tracts of all three species were similar (Table 7). Eukaryotic diversity at Little Tennessee site 3 was lowest in the water samples but highest in the sediment samples than any of the other sites during our sampling period (Table 8).

Table 7. Shannon index of diversity for all prokaryotic samples.

	Shannon Index
Mussel	
Ar baseline	4.76
ArLTR1	5.27
ArLTR2	4.61
ArLTR3	4.60
ArTR1	4.48
ArTR2	4.29
ArTR3	6.02
Lf baseline	4.01
LfLTR1	5.92
LfLTR2	4.63
LfLTR3	3.68
LfTR1	3.80
LfTR2	5.43
LfTR3	6.69
CfLTR1	4.97
CfLTR2	6.00
CfLTR3	6.09
CfTR1	3.32
CfTR2	3.94
CfTR3	5.51
Water	
LTR1	8.74
LTR2	8.47
LTR3	8.23
TR1	9.47
TR2	9.54
TR3	10.12
Sediment	
LTR1	10.71
LTR2	10.09
LTR3	10.41
TR1	9.73
TR2	10.33
TR3	10.06

Table 8. Shannon index of diversity for all eukaryotic samples.

	Shannon Index
Water	
LTR1	7.40
LTR2	7.49
LTR3	6.65
TR1	7.51
TR2	6.82
TR3	7.05
Sediment	
LTR1	5.59
LTR2	5.05
LTR3	6.16
TR1	5.63
TR2	4.46
TR3	4.65

Chapter 5

Use Of Fine Particulate Organic Matter, Bacteria, and Algae By Juvenile *A. Raveneliana* And *Corbicula fluminea*

An increase in *Corbicula* distribution and density in the Little Tennessee River that was coincident with the onset of the decline *A. raveneliana* populations in the Little Tennessee River is what prompted these field and studies. We hypothesized that *Corbicula* may be out-competing *A. raveneliana* for available food resources. These laboratory studies were initiated to determine if these bivalve species are consuming the same size particles, which would suggest they may directly compete for available food resources in the Little Tennessee River. To examine the particle sizes consumed by each of these species we held them in jars with aeration in the laboratory and added a commercial shellfish diet to the jars. We then followed the change in particle size within the jars.

Corbicula fluminea

Thirteen 1 L Ball jars (10 treatment, 3 control) with 500 mL of sodium thiosulfate conditioned FW were set up with airstones for circulation. *C. fluminea* were not fed for two days prior to the experiment to limit the amount of pseudofeces produced at the onset of the trial. 60 µL of Shellfish Diet (4-20 µm particles) and 6.7 µL of Nanno were measured into each jar (3.1 x 10^4 cells/µL). 200 µL samples were removed from each of the jars once completely mixed for initial measurement of algae concentration and sizes. Cell concentration and size were measured using a Cellometer Auto X4 cell viability counter (Nexcelom Bioscience, Lawrence, MA). One *C. fluminea* individual (1.733 g-3.485 g) was placed into each of 10 jars, the other 3 jars did not include *C. fluminea* and served as no animal controls. Cell concentration and sizes were measured every 30 min for 4 h after addition of animals.

Alasmidonata raveneliana

Thirteen 1 L Ball jars (10 treatment, 3 control) with 500 mL of sodium thiosulfate conditioned FW were set up with airstones for circulation. *A. raveneliana* were not fed for two days prior to the experiment to limit the amount of pseudofeces produced at the onset of the trial. 60 µL of Shellfish Diet (4-20 µm particles) and 6.7 µL of Nanno were measured into each jar (9.0 x 10^4 cells/µL). 200 µL samples were removed from each of the jars once completely mixed for initial measurement of algae concentration and sizes. Cell concentration and size were measured using a Cellometer Auto X4. Two *A. raveneliana* individuals (0.062 g-0.178 g) were placed into each of 10 jars, the other 3 jars did not include *A. raveneliana* and served as no animal controls. Cell concentration and sizes were measured every 30 min for 4 h after addition of animals.

Lampsilis fasciola

Thirteen 3.5 L rectangular tanks (10 treatment, 3 control) with 3 L of sodium thiosulfate conditioned FW were set up with airstones for circulation. *L. fasciola* were not fed for two days prior to the experiment to limit the amount of pseudofeces produced at the onset of the trial. 200 µL of Shellfish Diet (4-20 µm particles) and 16.7 µL of Nanno were measured into each tank (4.7 x 10^4 cells/µL). 200 µL samples were removed from each of the tanks once completely mixed for initial measurement of algae concentration and sizes. Cell concentration and size were measured using a Cellometer Auto X4. One *L. fasciola* individual (11.692 g-18.160 g) was placed into each of 10 tanks, the other 3 tanks did not include *L. fasciola* and served as no animal

controls. Cell concentration and sizes were measured every 30 min for 4 h after addition of animals.

Results

The goal of the experiment was to compare the change in distribution of particle sizes potentially being consumed by each species. We hypothesized that the preferred food particle size for *C. fluminea* and *A. raveneliana* would overlap, and thus we would see the relative proportion of that particle size decrease over time in the tanks of both species. To compare between species, we first compared the distribution of particle sizes in the tanks with organisms to the control tanks. Without clear differences between control replicates and organism replicates, however, interspecies comparison was confounded by overall particle settling. These studies could be informative if particle settling can be either negated or quantified. The studies should be repeated once appropriate techniques for reducing particle settling are developed.

Figures 41 shows the distributions of particle sizes in control and organism tanks from the beginning and end of the feeding time. The plot shows the changes in the particle size distribution in the organism tanks congruent with the control tanks. We could not distinguish between changes in distribution due to organism uptake and general settling. We examined this problem from multiple angles including using all time points of the study and matching control tanks to organism tanks. In each case, we could not confidently differentiate effects.

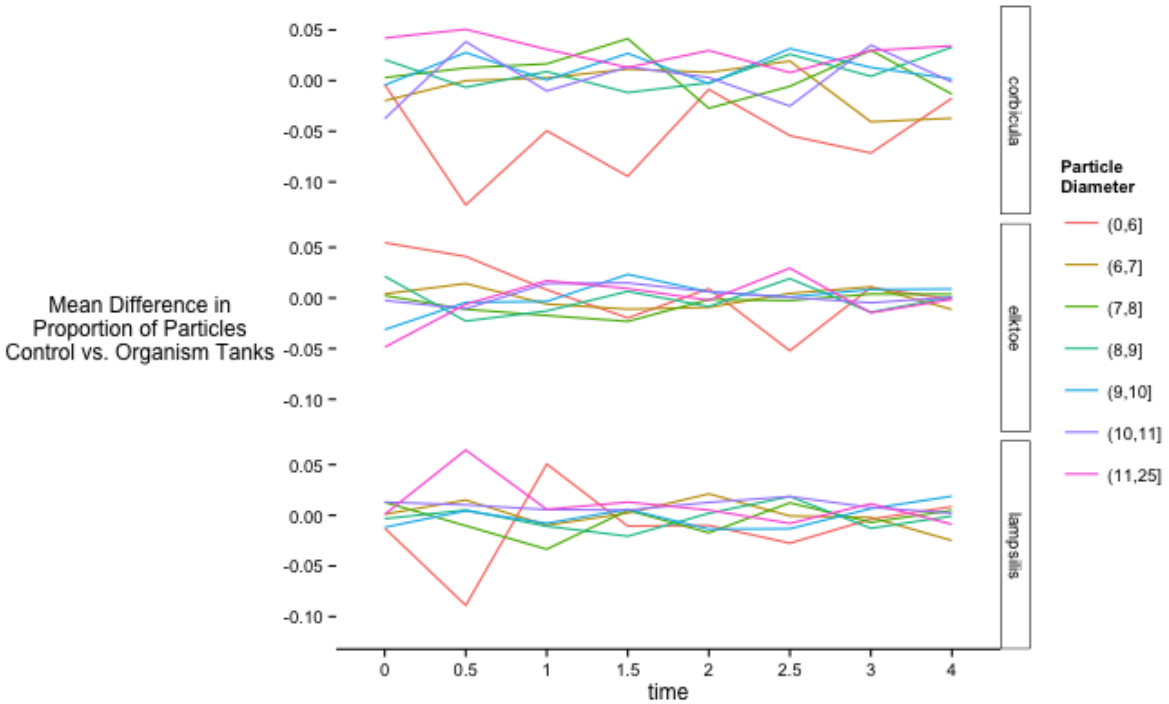

Figure 41: Mean difference in proportion of particles in control and bivalve treatment jars.

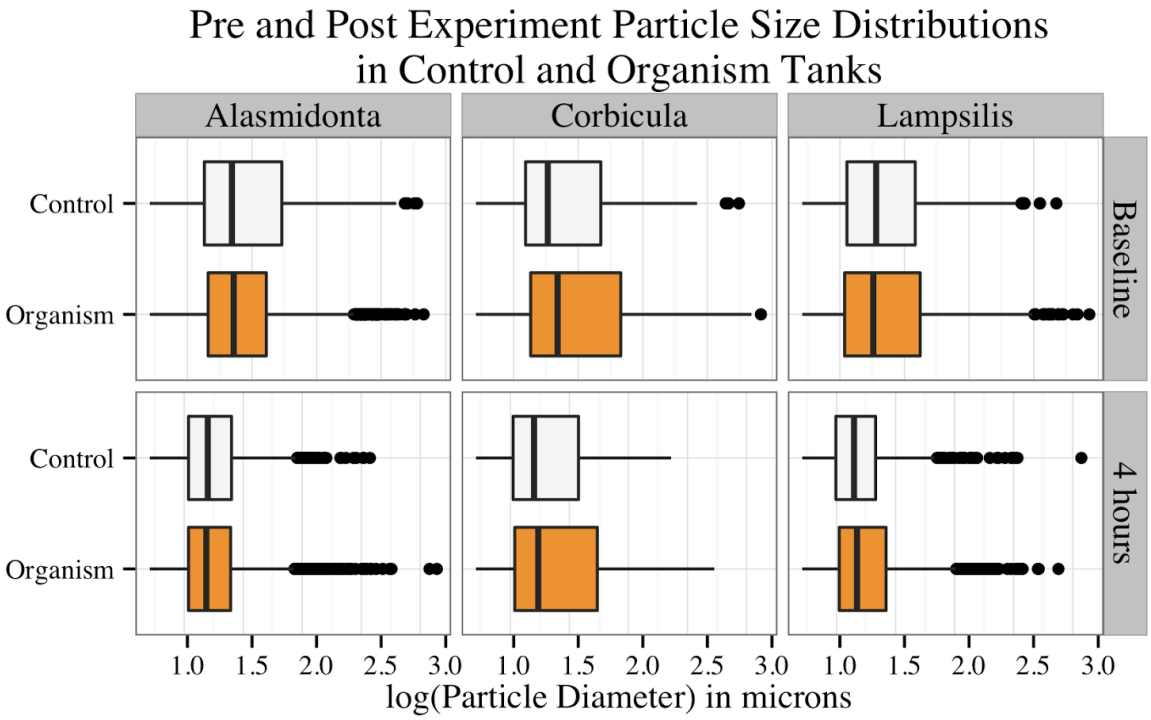

Figure 42: Pre and post experiment particles size distributions.

Chapter 6

Summary of Study Findings, Conclusions and Recommendations for the Conservation of *Alasmidonta raveneliana* in the Little Tennessee Watershed

A small advisory group of biologists including the project's investigators and colleagues with the US Fish and Wildlife Service, the NC Department of Transportation, and the NC Wildlife Resources Commission served as advisors to the project (Table 6).

Table 9: Project investigators and advisors

Luke Borst	Assistant Professor	Department of Population Health and Pathobiology	North Carolina State University
Steve Fraley	Western Aquatic Nongame Coordinator	Division Inland Fisheries, Habitat Conservation	NC Wildlife Resources Commission
John Fridell	Conservation biologist	Asheville, Office	US Fish and Wildlife Service (Retired)
Mike Gangloff	Assistant Professor	Department of Biology	Appalachian State University
Karen Kandl	Professor	Department of Biology	Western Carolina University
Mac Law	Professor of Pathology	Department of Population Health and Pathobiology	North Carolina State University
Jay Levine	Professor of Epidemiology and Public Health	Department of Population Health and Pathobiology	North Carolina State University
Jay Mays	Conservation biologist	Asheville, Office	US Fish and Wildlife Service
Chris Osburn	Associate Professor	Department of Marine, Earth and Atmospheric Sciences	North Carolina State University
Mike Sanderson	Ecosystem assessment	Office of Natural Environment	NC Department of Transportation

Conference calls were held to seek the advice of these advisors during the project. This report will be provided for their review and a follow-up conference call will be held to discuss potential recommendations for and a consensus statement for review by the NC Mollusk Advisory Council.

IMPLEMENTATION AND TECHNOLOGY TRANSFER PLAN

Research Products

The proposed field and laboratory studies documented the poor nutritional status of *A. raveneliana* in both the Little Tennessee River and the Tuckasegee River. Although our initial hypothesis that the decline has been associated with the increase in the density of *Corbicula* in the Little Tennessee River was not confirmed, we documented the poor nutritional health status of the *A. raveneliana* in the Little Tennessee River and demonstrated that mortality is also occurring in the Tuckasegee River. These studies have improved our understanding of freshwater mussel food resource availability in these Rivers and serves as a example of how "next-generation" molecular techniques can be used study river ecosystem health and freshwater mussel health. A rich trove of data has been developed during these studies that will serve as a baseline for future efforts. It demonstrated the utility in conducting similar studies over-time and how these technologies can be functionally used to monitor the health of our rivers, lakes and streams. They provide a means of measuring not just the direct impact of land-use by also the secondary cumulative impact of land-use changes. Construction activity is often the first target when health problems are observed in natural populations, but at times it not the construction but natural or other anthropogenic factors that are contributing to the decline.

Who at DOT will use This Report

The results of these studies published in the anticipated peer-reviewed publications will be of value to the DOT Natural Environment Section that is tasked with environmental assessments prior to proposed crossing structure construction, repair, reconstruction. The data provided on the project web site are available for review as needed to inform future studies.

How these products could be used

The primary products derived from these studies will be reflected in the peer-reviewed publications that result from the publication of study results and the specific recommendations provided below. Although no single factor has been identified that is contributing to the decline Appalachian Elktoe in the Little Tennessee River the body of data generated has provided some clear targets for additional study that should be pursued. This project also explored the use of several novel technologies to study the health of the Appalachian Elktoe population and the Little Tennessee and Tuckasegee Rivers. There was a clear indication that the nutritional health of the sentinel animals that were held at five of the six sites was impaired and it was the use of these technologies that facilitated this conclusion.

Department of Transportation biologists should consider adapting these technologies to obtain baseline values for stream reaches prior to new crossing structure construction or revitalization. If adapted as a component of a long-term routine environmental monitoring program they could provide an environmental warning system that could serve as an early indicator of declining habit quality. In this manner, problems could be detected before they have a dire health effect on remaining freshwater mussel and other aquatic species populations. In particular, routine Biolog ecoplate analysis in conjunction with 16s microbial and 18s eukaryotic

community analysis could provide a means of identifying changes in community structure or a loss of diversity and a potentially significant health risk to aquatic species before their decline. Shell micronutrient analysis could be used to identify contaminants at markedly reduced cost and could potentially play a role as a forensic tool for identify a time-sequence of contaminant introduction. The effective use of any of these technologies, however, would require the investment needed to conduct this baseline data overtime. These baseline signals would be needed to identify relative changes that reflect potential risks to aquatic community health. Concerns about the environmental impact of road construction projects often extends the proposal process and delays construction. Indeed a primary concern is the potential secondary nonconstruction effects of a project. These potential secondary effects are generally poorly defined. Documented mortality in an aquatic species is far too late of an indicator of declining ecosystem health. Having a long-term monitoring program in place using these technologies could enhance public confidence that a problem will be detected early prior to the loss of an aquatic species. In addition, they may effectively indicate that it is not road construction that is contributing to the decline but another unrelated natural or anthropogenic factor.

Training Needed

The studies conducted during the course of this project adapted a number of novel technologies that have not routinely been used to assess the health of freshwater mussel populations. Conducting and interpreting the results of these next-generation technologies require substantial expertise. A number of these tools could be used for environmental assessment prior to the construction of new crossing structures or the maintenance and reconstruction of existing structures. Although DOT could rely on contractual agreements with other agencies to conduct the analysis, DOT would benefit from having staff onboard that can interpret the data and make informed decisions based on the data collected.

Conclusions and Recommendations

Our basic hypothesis was that *A. raveneliana* populations in the Little Tennessee River were declining due to diminished food resource availability and poor nutritional health. Our metabolomics studies documented that the *A. raveneliana* are were in a state of negative nitrogen balance and experiencing marked protein catabolic change. In addition, there was substantial mortality noted at several of the study sites. In contrast, all the *L. fasciola* survived throughout the entire study period, grew and displayed little evidence that they were experiencing protein catabolic change. We had hypothesized that the health of *A. raveneliana* were suffering the impact of an expanding *C. fluminea* population. We examined *C. fulminea* density at the sentinel study sites, where they would be in direct competition with the cage mussels. Based on the documented density of *C. fulmenia* at our study sites in the Tennessee River and the Tuckasegee river there was no clear relationship between *C. fulminea* density and *A. raveneliana* mortality. However, the decline in *A. raveneliana* populations was coincident with the heightened density of *C. fulminea* in the Little T River (Steve Fraley, pers. comm.). Alternatively the density at the study site may not be the most appropriate indicator of the impact of *C. fulminea* on *A. raveneliana*. There could be a threshold number above which you can anticipate a decline, or it could be the totality of the river population that drives the availability of food resources to other species. Studies by other investigators, however, have documented that it native mussels often

out compete sympatric *C. fluminea*. If *Corbicula* competition was detrimental to *A. raveneliana* growth, it could be equally detrimental to the survival and growth of *L. fasciola*.
If food resource limitation is the primary factor contributing to the decline of *A. raveneliana*, why are the *C. fluminea* and *L. fasciola* thriving. We saw no *L. fasciola* mortality and they grew throughout the course of these studies. Unfortunately our feeding trials conducted to discern the comparative diets of these three species were inconclusive. Something else may indeed be driving the heightened mortality being observed.

Could it possibly be the biofilm noted in our scanning electron microscopy studies? Although it was observed on the cilia of both species, appeared to be more heavily affecting the cilia of *A. raveneliana*. One additional hypothesis is that the biofilm coating the gills of *A. ravenelian*a may actually be produced by *C. fluminea* or a one of its byproducts. The composition and the origin of the biofilm is unknown and warrants additional study.

The *A. raveneliana* population in the Little Tennessee River is at risk of extirpation. Studies conducted by NC Wildlife Resource Commission aquatic biologists have noted a marked increase in the time it takes to identify individual *A. raveneliana* in the River (Steve Fraley, pers. Comm.). We observed marked mortality at five of six sentinel animal study sites, three in the Little Tennessee River and at two of three study sites in the Tuckasegee River. No mortality was observed at the sixth site and this provided some key clues that informed our ability to interpret the data collected. We noted both differences in metabolic condition and stream metabolism when we compared this site with the other five.

3. Define the use of ecosystem health assessment techniques

One of the primary benefits of this work has been the clear demonstration of the utility of conducting both animal and stream-based metabolomic studies and water column and sediment measures of the availability of food resources. It emphasizes the need to invest in the development of baseline studies while populations are healthy and the long-term iterative studies needed to effectively monitor population overtime. Surveys to identify stream fauna are effective tools for assessing population changes overtime but they are temporally insensitive, major population declines may not be rapidly identified. Early hazard identification enhances the likelihood that a problem can be mitigated before major losses occur. Metabolomic profiling can provide a more sensitive tool for the long-term monitoring of populations that will help identify problems in a population before major losses have occurred. When used in conjunction water column and sediment microbiome analysis, community biochemical profiling and measurement of stream particulate matter they can serve as indicators of ecosystem health. However, substantial additional work is needed to make effective use of these technologies. Long-term monitoring programs need to be established using these tools so that early relative changes can be detected. Specific thresholds that warrant concern need to be established and aquatic biologists need to develop the expertise needed to effectively interpret the results.

4. Develop a specific protocol for identifying sites for potential population augmentation.

Although we have not definitely identified a single factor that has led to the precipitous decline of the Appalachian Elktoe we have documented that their nutritional health is impaired.

In addition, we documented substantial mortality at one of our study sites in the Tuckasegee River, a river that was thought to support a robust population of *A. raveneliana.* Recent reports from colleagues with the NC Wildlife commission suggest that *A. raveneliana* continues to decline and is at risk of extirpation from the Little Tennessee River. Captive propagation efforts have been underway, some supported by NCDOT to propagate *A. raveneliana* in captivity and potentially augment remaining populations. Neither the Little Tennessee River nor the Tuckasegee River appears to be viable sites for efforts to augment remaining *A. raveneliana* populations. These studies indicate that stream targets for augmentation must be thoroughly evaluated before restocking is attempted. Mussel biologists should convene a working group to develop specific criteria for selected streams or specific reaches for potential population augmentation. The working group should consider the use of several of the technologies used during the course of these studies as components of the stream selection process. However, additional studies are needed to refine the use of the ecosystem health tools (e.g. Biolog monitoring) used in these studies before they can be effectively integrated into a routine stream assessment protocol.

5. Work with State and Federal Agencies to Establish a Rapid Response Team

This project reflected the strong collaborative relationship that exists between North Carolina's freshwater biologists, the NC Wildlife Commission, NC Department of Transportation and the US Fish and Wildlife Service and NCUS. Numerous freshwater mussel species in NC are state or federally listed as endangered or threatened. The specific reasons for these declines are not always readily apparent. In some instances these declines are related to land-use practices, but other factors such as invasive species and contaminant input may also be contributing to the loss of some species from specific river systems. Efforts like this study cannot be undertaken without the collective support of individuals with diverse and complementary expertise. The strength of North Carolina's efforts to preserve its natural heritage is dependent on this close working relationship and efforts like those conducted during these studies cannot be conducted without financial support. North Carolina has had Mollusk Advisory Council that has provided important guidance about the conservation measures that need to be taken to preserve remaining species. But another type of team is needed, we recommend that the NC Wildlife Commission and the NC Department of Transportation work with the US Fish and Wildlife Service to assemble a response team in cooperation with the State's universities that can support the efforts of these agencies to preserve the state's remaining aquatic fauna. Funds should be set aside to provide the team with the resources needed to use the technologies adapted during this project to respond to episodes that place our state's fauna at dire risk of extirpation or extinction. The value-added benefit to the NC DOT will supporting efforts to prevent remaining species from being state or federally listed by intervening and mitigating problems before a species is severely imperiled. This presumptive effort could have substantial financial benefits to DOT because it will reduce the likelihood that additional regulatory oversight and compliance will be triggered by the presence of a state or federally listed species that is endangered, threatened or of special concern. The presence of state or federally listed species has previously resulted in substantial project planning and implementation delays and heightened construction costs. Preventing species from being state or federally listed will diminish the likelihood that regulatory actions will be taken that might slow project implementation.

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APPENDICES

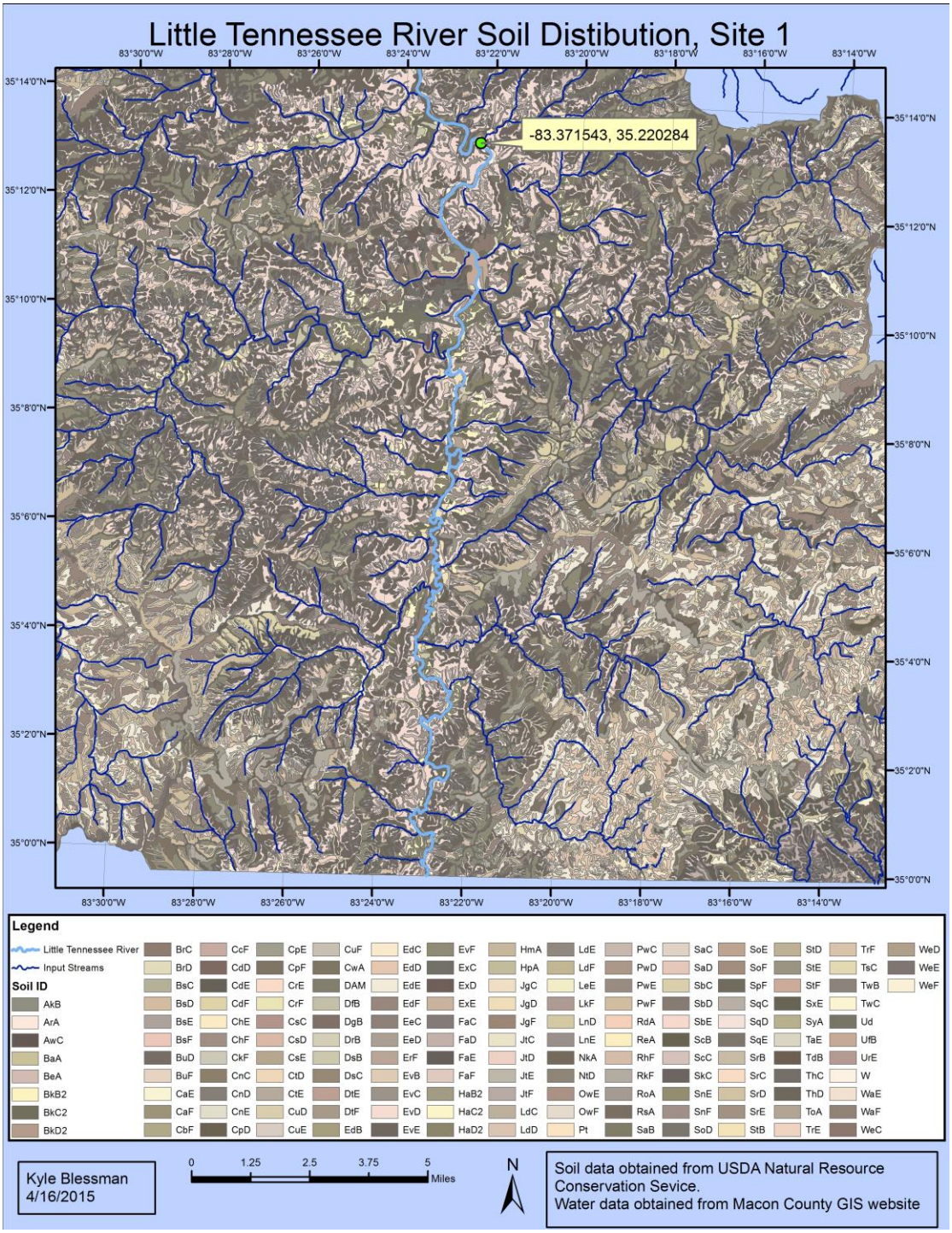
Appendix 1: Soil Codes for soil maps of the study sites.

Soil ID	Description
BaA	Biltmore sand, 0 to 3 percent slopes, frequently flooded
BkB2	Braddock clay loam, 2 to 8 percent slopes, eroded
BkC2	Braddock clay loam, 8 to 15 percent slopes, eroded
BkD2	Braddock clay loam, 15 to 30 percent slopes, eroded
BrC	Braddock-Urban land complex, 2 to 15 percent slopes,
BuD	Burton-Craggey-Rock outcrop complex, windswept, 8 to 30 percent slopes, stony
BuF	Burton-Craggey-Rock outcrop complex, windswept, 30 to 95 percent slopes, stony
CaC	Cashiers gravelly fine sandy loam, 8 to 15 percent slopes
CaD	Cashiers gravelly fine sandy loam, 15 to 30 percent slopes
CaE	Cashiers gravelly fine sandy loam, 30 to 50 percent slopes
CaF	Cashiers gravelly fine sandy loam, 50 to 95 percent slopes
CdC	Chandler gravelly fine sandy loam, 8 to 15 percent slopes
CdD	Chandler gravelly fine sandy loam, 15 to 30 percent slopes
CdE	Chandler gravelly fine sandy loam, 30 to 50 percent slopes
CdF	Chandler gravelly fine sandy loam, 50 to 95 percent slopes
CeC	Chandler gravelly fine sandy loam, 8 to 15 percent slopes, windswept
CeD	Chandler gravelly fine sandy loam, 15 to 30 percent slopes, windswept
CeE	Chandler gravelly fine sandy loam, 30 to 50 percent slopes, windswept
CeF	Chandler gravelly fine sandy loam, 50 to 95 percent slopes, windswept
ChE	Cheoah channery loam, 30 to 50 percent slopes
ChF	Cheoah channery loam, 50 to 95 percent slopes
CnC	Chestnut-Edneyville complex, windswept, 8 to 15 percent slopes, stony
CnD	Chestnut-Edneyville complex, windswept, 15 to 30 percent slopes, stony
CnE	Chestnut-Edneyville complex, windswept, 30 to 50 percent slopes, stony
CpD	Cleveland-Chestnut-Rock outcrop complex, windswept, 15 to 30 percent slopes
CpE	Cleveland-Chestnut-Rock outcrop complex, windswept, 30 to 50 percent slopes
CpF	Cleveland-Chestnut-Rock outcrop complex, windswept, 50 to 95 percent slopes
CrD	Cowee-Evard-Urban land complex, 15 to 30 percent slopes
CsD	Cullasaja very cobbly fine sandy loam, 15 to 30 percent slopes, extremely bouldery
CsE	Cullasaja very cobbly fine sandy loam, 30 to 50 percent slopes, extremely bouldery
CuC	Cullasaja-Tuckasegee complex, 8 to 15 percent slopes, stony
CuD	Cullasaja-Tuckasegee complex, 15 to 30 percent slopes, stony
CuE	Cullasaja-Tuckasegee complex, 30 to 50 percent slopes, stony
CuF	Cullasaja-Tuckasegee complex, 50 to 90 percent slopes, stony
CwA	Cullowhee fine sandy loam, 0 to 2 percent slopes, occasionally flooded
DfA	Dellwood gravelly fine sandy loam, 0 to 3 percent slopes, occasionally flooded
DrB	Dillard loam, 1 to 5 percent slopes, rarely flooded
DsB	Dillsboro loam, 2 to 8 percent slopes
DsC	Dillsboro loam, 8 to 15 percent slopes
EdC	Edneyville-Chestnut complex, 8 to 15 percent slopes, stony
EdD	Edneyville-Chestnut complex, 15 to 30 percent slopes, stony

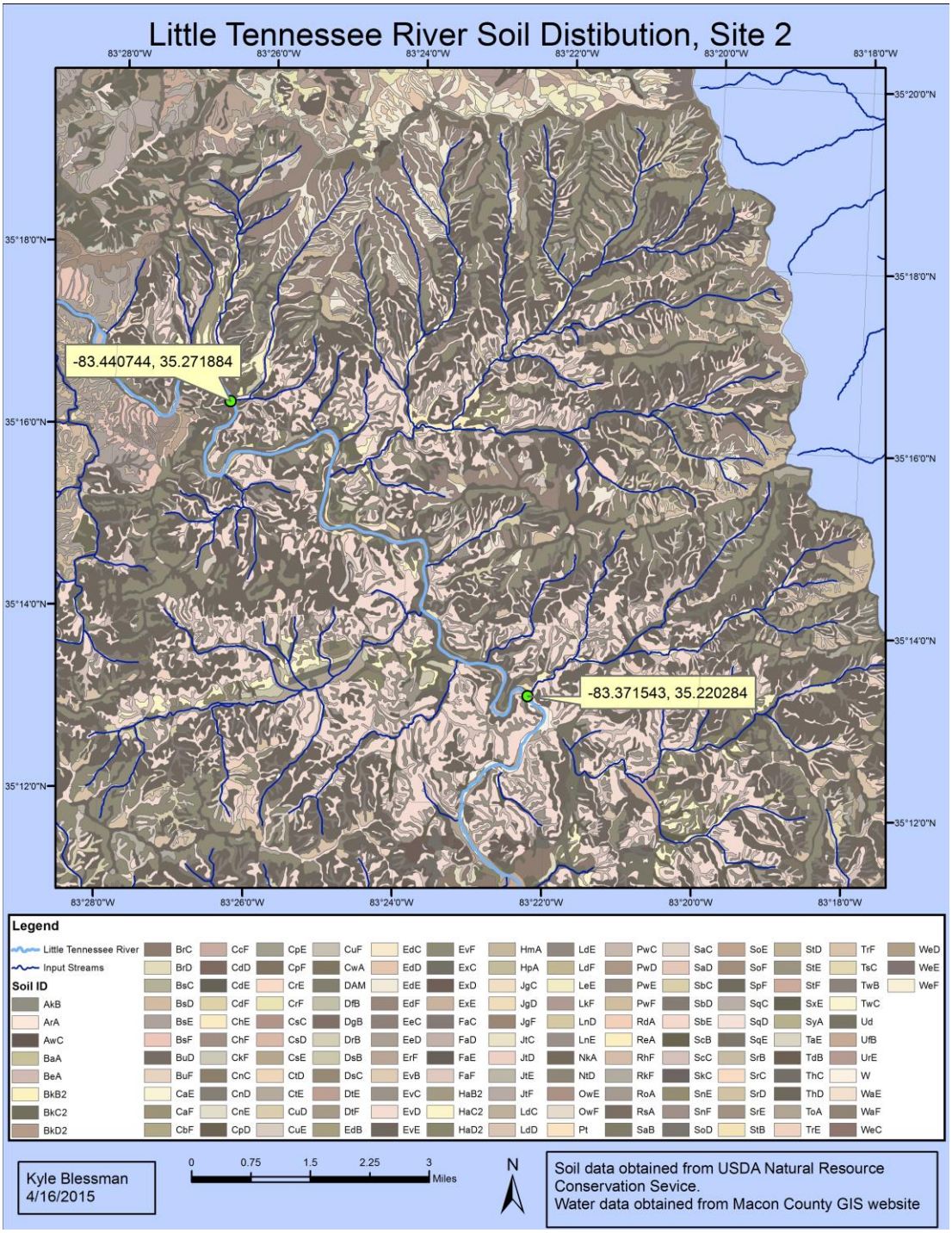
EdE	Edneyville-Chestnut complex, 30 to 50 percent slopes, stony
EdF	Edneyville-Chestnut complex, 50 to 95 percent slopes, stony
EgB2	Ellijay silty clay loam, 2 to 8 percent slopes, eroded
EgC2	Ellijay silty clay loam, 8 to 15 percent slopes, eroded
EgD2	Ellijay silty clay loam, 15 to 30 percent slopes, eroded
EvC	Evard-Cowee complex, 8 to 15 percent slopes
EvD	Evard-Cowee complex, 15 to 30 percent slopes
EvE	Evard-Cowee complex, 30 to 50 percent slopes
EvF	Evard-Cowee complex, 50 to 95 percent slopes, stony
FaC	Fannin fine sandy loam, 8 to 15 percent slopes
FaD	Fannin fine sandy loam, 15 to 30 percent slopes
FaE	Fannin fine sandy loam, 30 to 50 percent slopes
FaF	Fannin fine sandy loam, 50 to 95 percent slopes
HpA	Hemphill clay loam, 0 to 3 percent slopes, rarely flooded
JbD	Junaluska-Brasstown complex, 15 to 30 percent slopes
JbE	Junaluska-Brasstown complex, 30 to 50 percent slopes
JtD	Junaluska-Tsali complex, 15 to 30 percent slopes
JtE	Junaluska-Tsali complex, 30 to 50 percent slopes
JtF	Junaluska-Tsali complex, 50 to 95 percent slopes
NkA	Nikwasi fine sandy loam, 0 to 2 percent slopes, frequently flooded
OcD	Oconaluftee channery loam, 15 to 30 percent slopes
OcE	Oconaluftee channery loam, 30 to 50 percent slopes
OcF	Oconaluftee channery loam, 50 to 95 percent slopes
OwD	Oconaluftee channery loam, windswept, 15 to 30 percent slopes
OwE	Oconaluftee channery loam, windswept, 30 to 50 percent slopes
OwF	Oconaluftee channery loam, windswept, 50 to 95 percent slopes
Pt	Pits, quarries
PwD	Plott fine sandy loam, 15 to 30 percent slopes, stony
PwE	Plott fine sandy loam, 30 to 50 percent slopes, stony
PwF	Plott fine sandy loam, 50 to 95 percent slopes, stony
RdA	Reddies fine sandy loam, 0 to 2 percent slopes, occasionally flooded
RkF	Rock outcrop-Cleveland complex, windswept, 30 to 95 percent slopes
RoA	Rosman fine sandy loam, 0 to 2 percent slopes, occasionally flooded
SaB	Saunook gravelly loam, 2 to 8 percent slopes
SaC	Saunook gravelly loam, 8 to 15 percent slopes
SaD	Saunook gravelly loam, 15 to 30 percent slopes
SbD	Saunook gravelly loam, 15 to 30 percent slopes, stony
SoD	Soco-Stecoah complex, 15 to 30 percent slopes
SoE	Soco-Stecoah complex, 30 to 50 percent slopes
SoF	Soco-Stecoah complex, 50 to 95 percent slopes
SrD	Spivey-Santeetlah complex, 15 to 30 percent slopes, stony
SrE	Spivey-Santeetlah complex, 30 to 50 percent slopes, stony
SvB	Statler loam, 1 to 5 percent slopes, rarely flooded
SyA	Sylva-Whiteside complex, 0 to 2 percent slopes
TaC	Tanasee-Balsam complex, 8 to 15 percent slopes, stony
TaD	Tanasee-Balsam complex, 15 to 30 percent slopes, stony

TaE	Tanasee-Balsam complex, 30 to 50 percent slopes, stony
TrE	Trimont gravelly loam, 30 to 50 percent slopes, stony
TrF	Trimont gravelly loam, 50 to 95 percent slopes, stony
TwC	Tuckasegee-Whiteside complex, 8 to 15 percent slopes
Ud	Udorthents, loamy
UfB	Udorthents-Urban land complex, 0 to 5 percent slopes, rarely flooded
W	Water
WaD	Wayah sandy loam, 15 to 30 percent slopes, stony
WaE	Wayah sandy loam, 30 to 50 percent slopes, stony
WaF	Wayah sandy loam, 50 to 95 percent slopes, stony
WeC	Wayah sandy loam, windswept, 8 to 15 percent slopes, stony
WeD	Wayah sandy loam, windswept, 15 to 30 percent slopes, stony
WeE	Wayah sandy loam, windswept, 30 to 50 percent slopes, stony
WeF	Wayah sandy loam, windswept, 50 to 95 percent slopes, stony
WtB	Whiteside-Tuckasegee complex, 2 to 8 percent slopes

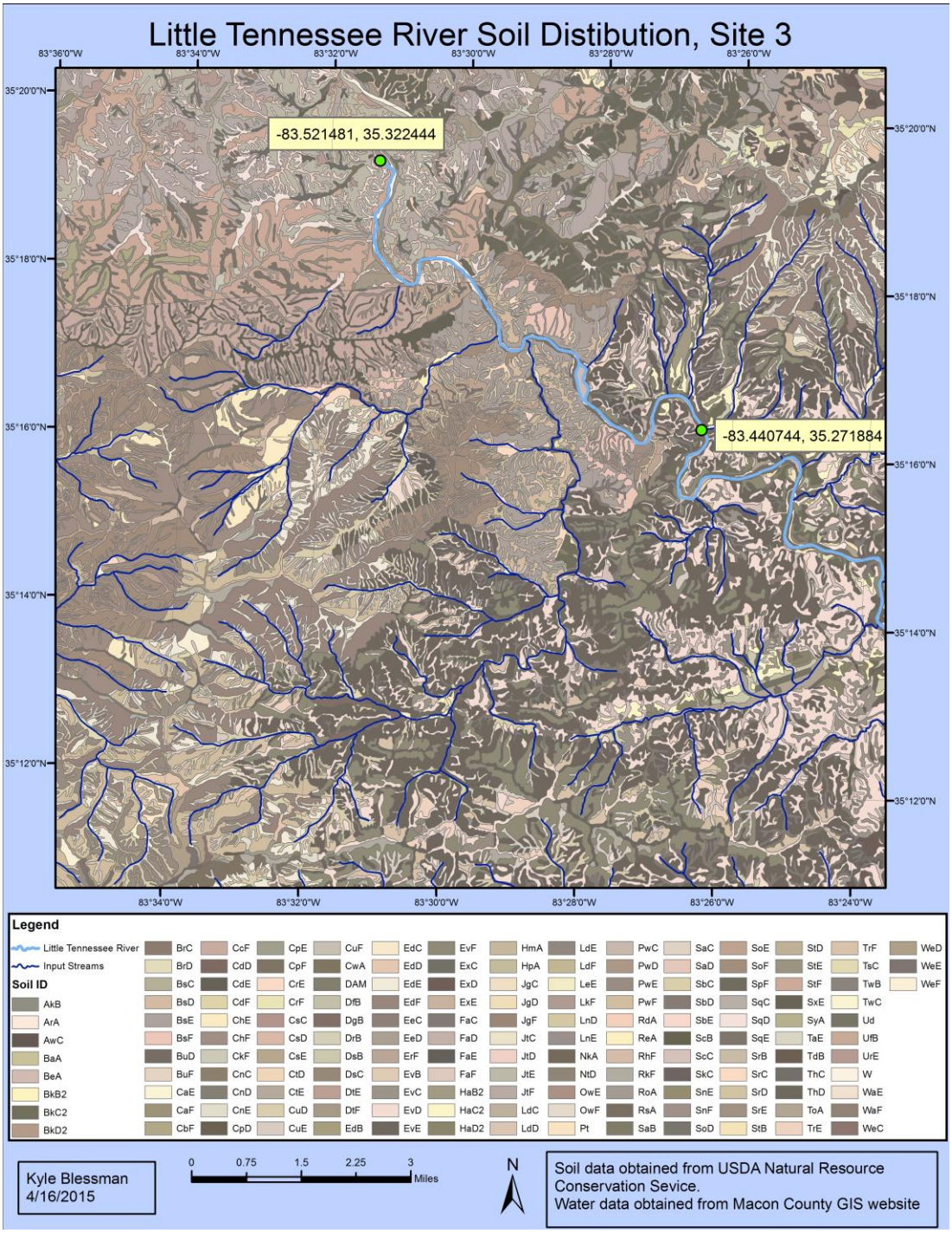
Appendix 2: Soil types distributed at Little Tennessee River Site 1.



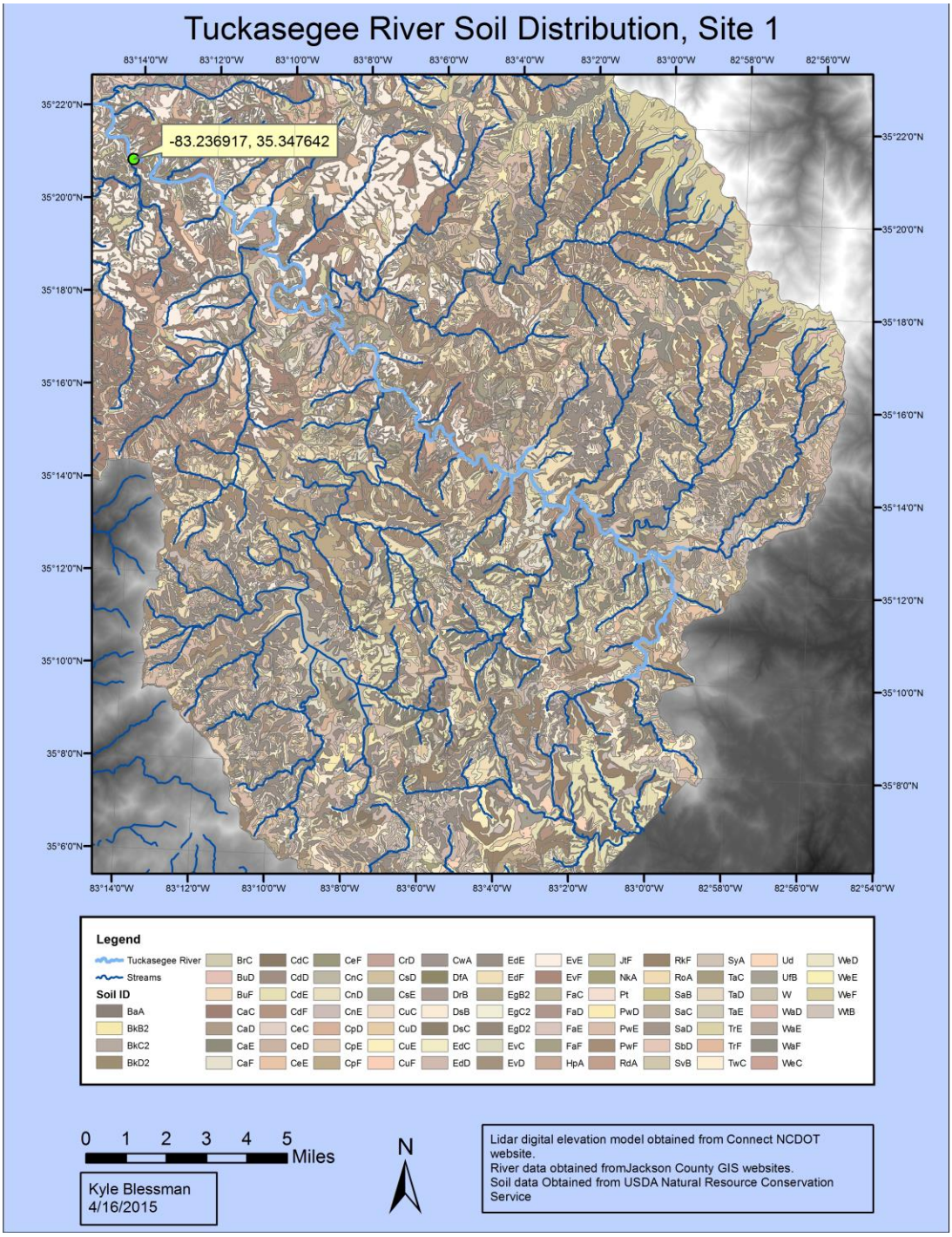
Appendix 3: Soil Types Distributed at Little Tennessee River Site 2.



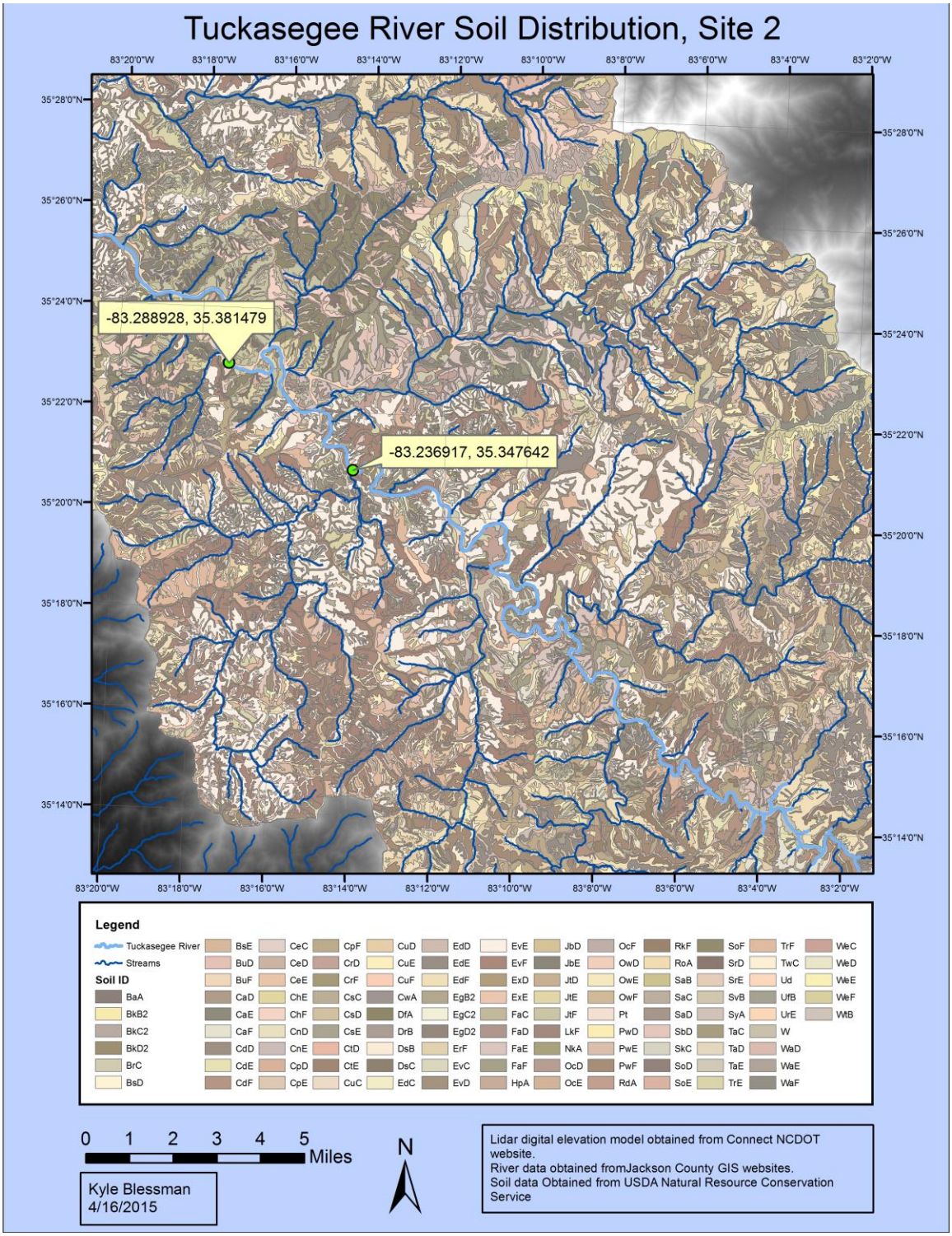
Appendix 4: Soil types distributed at Little Tennessee River Site 3.



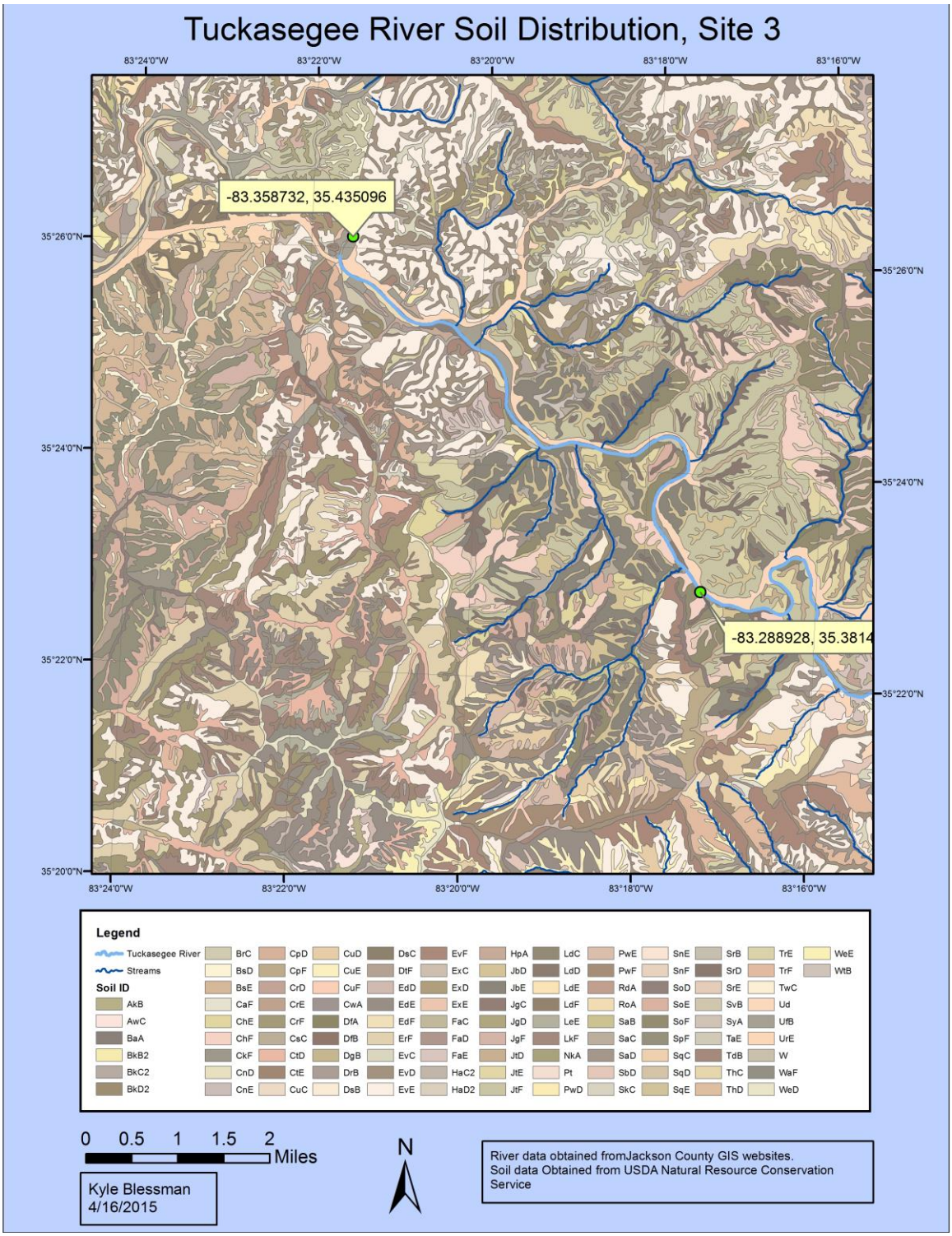
Appendix 5: Soil types distributed at Tuckasegee River Site 1.



Appendix 6: Soil types distributed at Tuckasegee River Site 2.



Appendix 7: Soil types distributed at Tuckasegee River Site 3.



Appendix 6: Soil types distributed at Tuckasegee River Site 2.

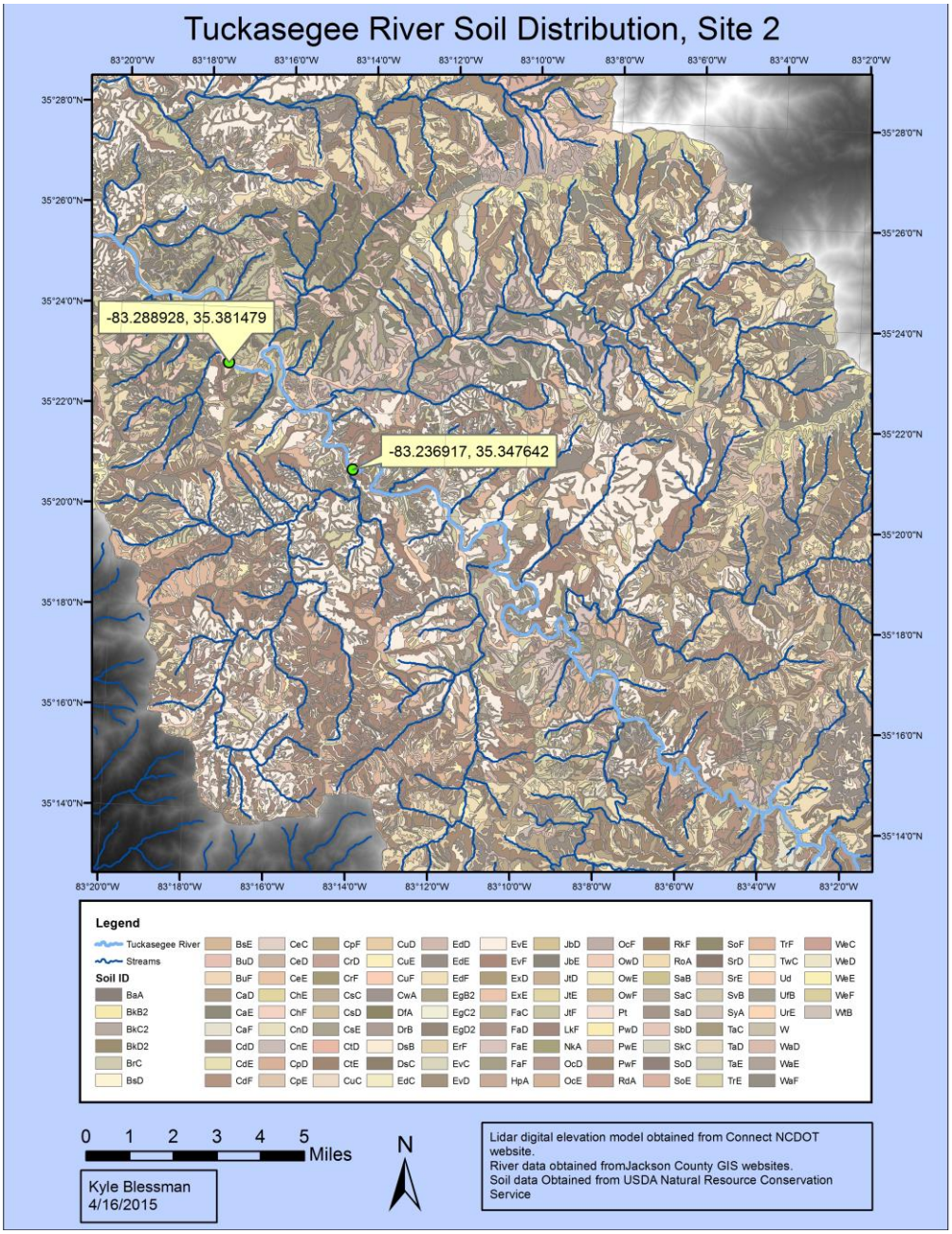
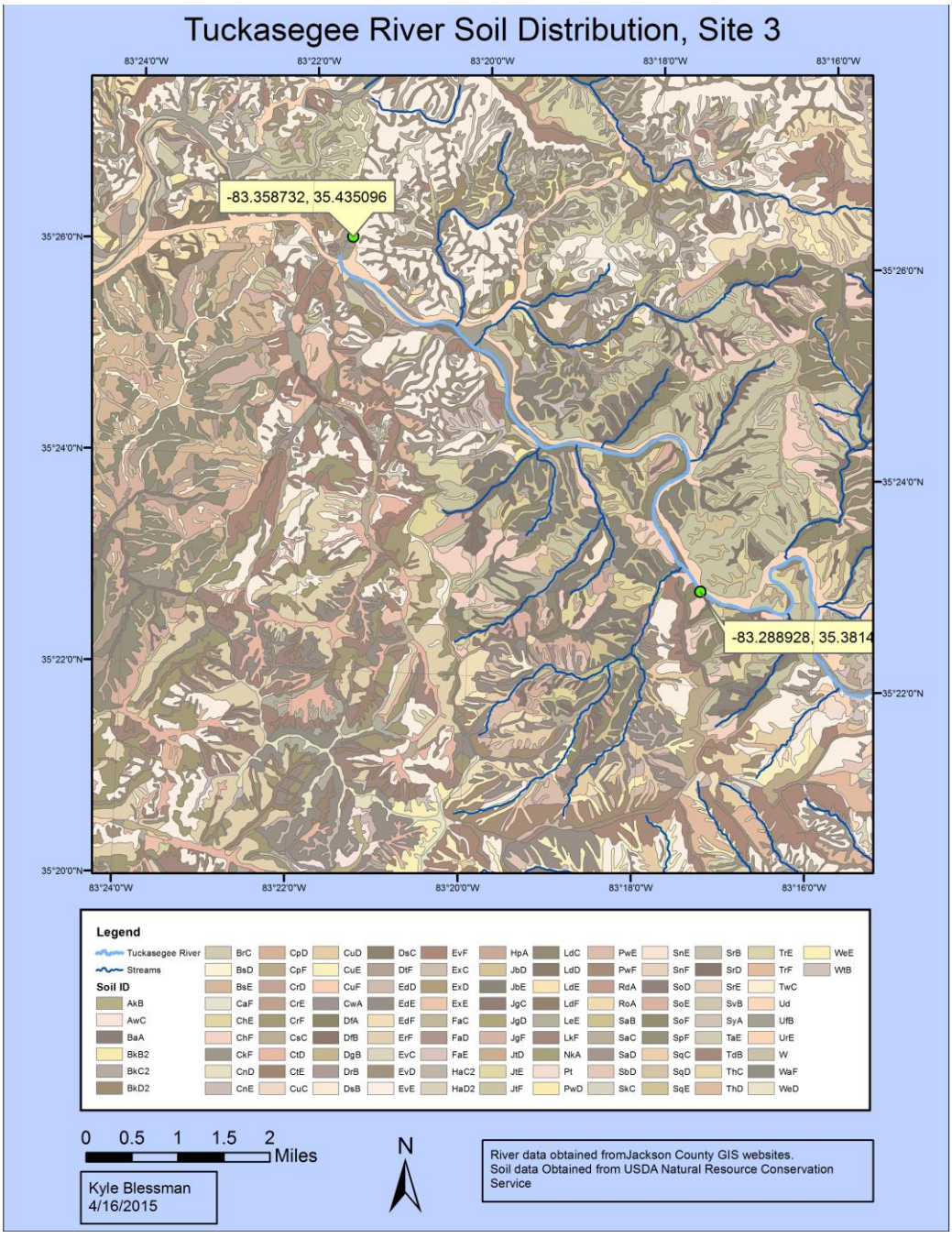


Figure 10: Tuckasegee River site 2 watershed soil types.


Tuckasegee River Soil Distribution, Site 3
83°24'0"W
83°22'0"W
83°20'0"W
83°18'0"W
83°16'0"W
35°26'0"N
35°24'0"N
35°22'0"N
35°20'0"N
-83.358732, 35.435096
-83.288928, 35.3814
Legend
Tuckasegee River
Streams
Soil ID
AkB
AwC
BaA
BkB2
BkC2
BkD2
BrC
BsD
BsE
CaF
ChE
ChF
CkF
CnD
CnE
CpD
CpF
CrD
CrE
CrF
CsC
CtD
CtE
CuC
CuD
CuE
CuF
CwA
DfA
DfB
DgB
DrB
DsB
DsC
DtF
EdD
EdE
EdF
ErF
EvC
EvD
EvE
EvF
ExC
ExD
ExE
FaC
FaD
FaE
HaC2
HaD2
HpA
JbD
JbE
JgC
JgD
JgF
JtD
JtE
JtF
LdC
LdD
LdE
LdF
LeE
LkF
NkA
Pt
PwD
PwE
PwF
RdA
RoA
SaB
SaC
SaD
SbD
SkC
SnE
SnF
SoD
SoE
SoF
SpF
SqC
SqD
SqE
SrB
SrD
SrE
SvB
SyA
TaE
TdB
ThC
ThD
TrE
TrF
TwC
Ud
UfB
UrE
W
WaF
WeD
WeE
WtB
0 0.5 1 1.5 2
Miles
N
Kyle Blessman
4/16/2015
River data obtained fromJackson County GIS websites.
Soil data Obtained from USDA Natural Resource Conservation Service