Studies on the digestive gland microbiome of freshwater mussels

by

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Abstract

Freshwater mussels are among the most imperiled aquatic invertebrates in North America. In the United States, Alabama has the highest diversity of freshwater mussels with 178 species. To address the conservation needs of Alabama's native freshwater mussels, ADCNR opened the Alabama Aquatic Biodiversity Center in 2005. The purpose of this facility is to lead captive propagation and reintroduction efforts of several unionid and other non-game species. However, understanding the role of bacterial communities ('microbiome') in mussel health has not yet been explored. The microbiome is defined as a community of symbiotic bacteria and their genes that provides the host with many benefits. These bacteria establish complex and dynamic interactions with their host. When the microbiome becomes imbalanced (e.g. the host experiences an environmental insult) those interactions are disrupted and could result in a pathological state for the host. The term dysbiosis is used to refer to an unbalanced microbiome. In these studies, I characterized the bacterial community associated with the digestive gland ('gut') of freshwater mussels using 16S rRNA gene sequencing. My results showed that the microbiomes from wild and cultured/captive mussels are significantly different from each other and that Tenericutes is the most abundant bacterial phylum in the microbiome of the mussels species analyzed to date. Tenericutes, specifically the bacterial class Mollicutes, are known intracellular parasites of humans, and their role in the microbiome of freshwater mussels is unknown. I also characterized the bacterial communities of water and sediment surrounding the mussels and found them diverse but very distinct from the digestive gland microbiome of

mussels. When mussels were challenged with environmental changes or chemical insults (e.g. rearing environment or antibiotics), freshwater mussels become dysbiotic a stage that was characterized by a loss of Tenericutes and an increase of Proteobacteria and Firmicutes. Overall, there was a significant change in bacterial diversity and a shift to a pathogen-dominated microbiome. I conclude that certain rearing environments modify the natural gut microbiome of freshwater mussels. This was particularly evident when wild mussels were relocated to laboratory facilities.

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List of Abbreviations

AABC	Alabama Aquatic Biodiversity Center
ACE	Abundance-based coverage estimation
ADCNR	Alabama Department of Conservation and Natural Resources
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
bp	Base pair
MDS	Multidimensional scaling
OTU	Operational taxonomic unit
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational multivariate analysis of variance
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PRIMER	Plymouth Routines in Multivariate
SIMPER	Similarity percentages
STAMP	Statistical Analysis of Metagenomic Profiles

I. CHAPTER 1. LITERATURE REVIEW

Freshwater mussels

Freshwater mussels belong to the second most diverse group of animals in the world, the phylum Mollusca (~100,000 species). Oysters, scallops, clams, and freshwater mussels belong to the class Bivalvia. Bivalves are animals with two calcium carbonate shells (valves) that enclose their soft tissue. The class Bivalvia consists of approximately 15,000 species, most of which are found in the marine environment.

Unionoida is a diverse and large order of freshwater mussels widely distributed across six continents (~679 species worldwide). Currently, there are six families of freshwater mussels that have been identified worldwide, two of which are found in Alabama: Margaritiferidae (2 species) and Unionidae (178 species). Unionidae is the most species rich family within the order Unionoida with 620 species and 142 genera (Bogan and Roe, 2008). Presently, there are three subfamily divisions within the Unionidae in North America: Anodontinae, Gonidaeinae, and Ambleminae (Williams et al., 2017). The subfamily Ambleminae further divides into five tribes: Amblemini, Lampsilini, Pleurobemini, and Quadrulini (Williams et al., 2017), and a recently recognized Mesoamerican clade – Popenaiadini (Pfeiffer et al., 2018).

Ecological and cultural significance of freshwater mussels

Freshwater mussels provide many ecosystem services to streams, rivers, and lakes (Vaughn, 2018). They are constantly filtering large volumes of water and ingesting bacteria, phytoplankton, detritus and pollutants (Vaughn et al., 2008), thereby removing particles from the water column and distributing it to the benthos (Vaughn and Hakenkamp, 2001). Their functional role in nutrient cycling makes them important biological indicators of water quality and water clarity (Chowdhury et al., 2016). Mussel shells also provide a suitable habitat for other

sessile organisms (Gutierrez et al., 2003), although the invasive species, *Dreissena polymorpha* (Zebra mussel) are also known to heavily colonize unionids, interfering with their feeding, respiration, excretion, and locomotion (Haag et al., 1993).

Freshwater mussels have also long been important to humans. Native Americans consumed a great amount of them and left piles of discarded shells (called shell middens) along riverbanks. They also used the shells for tools and ornamentation, jewelry and ceremonial objects. From the late-1800s to the mid-1900s, mussel shells were being harvested and commercially marketed as pearl buttons (Anthony and Downing, 2001). By 1899, sixty button factories lined the banks of the Mississippi River Valley near Muscatine, Iowa. However, the U.S. button industry began to decline as quickly as it rose, with the presence of Japanese competition and labor issues (Claassen, 1994).

Biology of freshwater mussels

Unionids feed by taking in water through their inhalant aperture. As water flows into the mantle cavity and through the gills, food particles are trapped by cilia. Particles become sorted in the gills and selected for ingestion. The digestive enzymes and the crystalline style (an organ with a rod against a hardened plate) work together to digest and assimilate food particles inside the stomach. Undigested particles are expelled as pseudofeces through the ventral margin or excurrent aperture. The deposited excess or unwanted particles on the habitat bottom provide nutrient rich detritus for other benthic organisms (Vaughn and Hakenkamp, 2001).

Freshwater mussels have a unique and complex life cycle involving a parasitic larval stage that uses a fish host (Barnhart et al., 2008). There are three basic life stages of freshwater mussels: larval (parasitic), juvenile, and adult. When water and other environmental conditions are right, males will expel sperm into the water. Females take up the sperm using their incurrent

siphon and fertilize their eggs. After fertilization, females brood the young from the egg to larval stage in their gills. The females will hold the larvae, called glochidia, within marsupial sacs (brood chambers) in the gills. Tankersley (1996) found that this reproductive strategy may have some effects to the feeding dynamics of female unionids. When the glochidia reach maturation, they are released into the water, where they swim and attach to the gills of a fish host to complete their metamorphosis to the juvenile stage, and then ultimately settle to the bottom.

Status of freshwater mussels in the southeastern U.S.

Freshwater mussels ('unionids') are among the most imperiled group of invertebrates in North America (Lopes-Lima et al., 2014) where there are currently 27 species of mussels extinct and 80 endangered species (IUCN, 2019). Causes to the decline of freshwater mussels has resulted from habitat loss, water pollution, construction of dams, siltation, and exotic species introduction (Williams et al., 1993;Neves et al., 1997).

The southeastern United States is considered a biodiversity hot spot for aquatic animals. Alabama has the highest diversity of mussels, snails, crayfish and aquatic turtles than any other state (Lydeard and Mayden, 1995). Freshwater mussels in Alabama represent approximately 60% of the total 298 species found in North America and Canada. The great mussel diversity of Alabama is in many ways attributed to the abundance of river and streams in the state. These river systems have been isolated for millions of years which allowed mussels in each river to evolve independently from one another, resulting in geographically distinct mussel assemblages in different drainages: Tennessee River drainage, Mobile Basin, Gulf Coast drainages and Apalachicola Basin (Williams et al., 2008).

Freshwater mussel propagation and reintroduction

Recent advances in rearing freshwater mussels in captivity have made juveniles available for release into the wild. ADCNR opened the AABC (established in 2006) to lead captive propagation and reintroduction efforts of aquatic animals. The AABC is the largest non-game culture facility in the United States. They have been successful in rearing and introducing several unionid species including *Villosa nebulosa,* one of the species targeted in this thesis.

Species Profiles

This thesis focuses on two species of freshwater mussels, both belonging to the subfamily Ambleminae: *Villosa nebulosa* and *Pleurobema cordatum*. Species are listed by tribe, then species and common name.

Tribe Lampsilini
Villosa nebulosa Alabama Rainbow (Conrad, 1834)

V. nebulosa is moderately thin and elliptically shaped. The interrupted greenish rays on its yellowish periostracum distinguish it from other *Villosa* species. *V. nebulosa* is a long-term brooder; females are gravid from late summer or autumn until the following summer. Females display a modified mantle margin in the form of a papillate fold to attract fish hosts. Haag and Warren (1997) identified four fish hosts for *V. nebulosa*: *Lepomis megalotis*, *Micropterus coosae*, *Micropterus punctulatus*, and *Micropterus salmoides*. *V. nebulosa* is endemic to the Mobile Basin in Alabama, and in some parts of Georgia and Tennessee. Historically, *V. nebulosa* was found in the Black Warrior, Cahaba, and Coosa River drainages above the Fall Line (Williams et al., 2008). The fall line in Alabama is the border between the Gulf Coastal Plain, which covers the southern half of the state, and in the other region is the Piedmont Plateau, the east central part of the state. *V. nebulosa* occurs in small streams in sand and gravel riffle areas in moderate water current. Currently, *V. nebulosa* is a conservation target at the AABC, where

reintroductions began several years ago in the Coosa and Black Warrior Basins with over 16,400 mussel reintroductions that occurred in 2018.

Tribe Pleurobemini
Pleurobema cordatum Ohio Pigtoe (Rafinesque, 1820)

P. cordatum can be distinguished from other *Pleurobema* by its triangular shape, median sulcus, and rounded anterior. *P. cordatum* is a short-term brooder, gravid from late April to mid-July. In laboratory trials, many species of fish have been identified as glochidial host of *P. cordatum*: *Lythrurus fasciolaris*, *Semotilus atromaculatus*, and *Culaea inconstans* (Watters and Kuehnl, 2004). Glochidia are packaged into white, oblong, flattened conglutinates with rounded ends and a straight line of holes down the center. *P. cordatum* is an inhabitant of large rivers and was once the most abundant mussel in the Tennessee River (Scruggs, 1960). Its historical range included the upper Mississippi River and St. Lawrence River drainages, covering western New York to Michigan, Wisconsin, Iowa, and Kansas, and in the south, from Arkansas to Alabama. The decline in *P. cordatum* numbers has warranted it a species of high conservation priority. Efforts to successfully hold these animals long-term in captivity has been difficult, thus rendering the need to understand their health.

Microbiome

The microbiome is defined as a collection of microorganisms and their genes that live in close association with a host (Mueller and Sachs, 2015;Huttenhower et al., 2012). It is sometimes referred to as our 'forgotten organ' (O'Hara and Shanahan, 2006). The core microbiome is composed of all the microbes that are shared across individuals or between groups and provide ecosystem functions in their environment (Shade and Handelsman, 2012;Turnbaugh and Gordon, 2009;Turnbaugh et al., 2009;Hernandez-Agreda et al., 2017). Before our recent advances in microbiology, Koch and Hill's fundamental postulate of "one microbe—one disease" set the

basis for clinical microbiology and defined a 'healthy host' as free of pathogens. Now we are beginning to understand that a majority of microbes are symbiotic and have co-evolved with their host since the beginning of time (Ruby et al., 2004). The discovery of these complex interactions between the host and its associated microbiome are now referred to as the holobiont (Rosenberg et al., 2007). Furthermore, many studies have found that these symbionts provide many important benefits for host physiological processes (Tuddenham and Sears, 2015), from boosting the immune system (Hooper et al., 2012;Stecher and Hardt, 2011) to producing metabolites for nutrient absorption (Nicholson et al., 2012) and organ development (Sommer and Bäckhed, 2013) to even controlling our moods (Sampson and Mazmanian, 2015) and behavior (Dinan et al., 2015).

Human microbiome

Until recently, the abundant community of human-associated microbes and the environments they inhabit remained largely understudied, rendering their effects on human health, development, physiology, immunity and nutrition largely unknown (Huttenhower et al., 2012). In 2008, the National Institutes of Health (NIH) launched the Human Microbiome Project with the mission of creating a foundation of microbial gene sequences found in and on humans. Sender et al. (2016) estimated that there are 3.8×10^{13} total bacterial cells in the colon, which is the organ that harbors the densest number of microbes. The human gastrointestinal (GI) tract is predominantly composed of three bacterial phyla: Firmicutes, Bacteroidetes and Actinobacteria (Tap et al., 2009).

Most of what we know about animal microbiomes (Bahrndorff et al., 2016) has been derived from humans (Clemente et al., 2012). More importantly, we know that the host regulates its gut microbiome largely to maintain homeostasis, and even favoring dominance of a single

genus (Byndloss et al., 2018). The development of our microbiomes from birth over the first few years of life is critical (Clemente et al., 2012). Typically, the gut microbiome starts with low species richness and high instability (i.e. can be altered easily). Overtime, the bacterial diversity increases and the gut community stabilizes. For example, in the highly oxidative environment of a newborn GI tract, early colonizers are facultative anaerobic bacteria (phylum proteobacteria), which adjust the gut environmental conditions by decreasing oxygen concentration, allowing for the booming colonization by anaerobic microbes, members of the genus *Bacteroides* and the phyla Actinobacteria and Firmicutes (Palmer et al., 2007). In channel catfish, drastic changes in their microbiome occur between different ontogenic stages until it finally stabilizes at ~90 days post hatch (Burgos et al. 2018).

Metagenomics

It has been estimated that we are only able to culture 1-10% of the bacteria found in nature (Amann et al., 1995). To address this challenge, researchers have shifted their efforts towards the use of metagenomics, that is, studying the genetic material of microbes taken from an environmental sample, and using bioinformatic tools to analyze those large datasets (Hiraoka et al., 2016). This allows researchers to study the genetic material of all microbes found in the microbiome, rather than just those that can only be cultured in a lab. One of the first steps in microbial ecology is to identify who is there and what is their diversity and distribution in a given environment (Robinson et al., 2010;Head et al., 1998). There are two approaches for microbiome analyses: shotgun metagenomics, non-targeted sampling of the all the genomes found in the sample, and 16S rRNA gene sequencing, broad-range bacterial amplification and sequencing (Zimmerman et al., 2014). The 16S rRNA gene is approximately 1,500 bp and has highly conserved regions, flanked by hypervariable regions (V1-V9) (Janda and Abbott,

2007;Woese et al., 1990). Bacterial genomes typically contain more than one copy of the 16S rRNA gene but numbers range from 1 to 15 copies per cell (Klappenbach et al., 2001;Veˇtrovsky and Baldrian, 2013). There are a number of different next-generation sequencing technologies that are used to study the host-associated microbiome (Tarnecki et al., 2017;Ghanbari et al., 2015) with Illumina MiSeq being the most popular platform in recent years. Briefly, bacterial community analysis based on 16S rRNA gene sequencing involves three steps: DNA extraction from any environmental sample, PCR amplification of the 16S rRNA gene pool, and sequencing of individual molecules of 16S rRNA gene using pyrosequencing. Each sequence (or 'read') is then compared to those already present in public databases such as the National Center for Biotechnology Information (Geer et al., 2009) or Greengenes (DeSantis et al., 2006) and ascribed to specific operational taxonomic units (OTUs), if there is > 97% sequence similarity. The potential function of 16S rRNA data can also be predicted using computational approaches (Langille et al., 2013).

Dysbiosis

It is important to characterize the microbiomes of healthy individuals and then compare them to individuals with different pathologies (Huttenhower et al., 2012). This will help our understanding of whether there are any relationships between altered microbiomes and diseases. There are multiple factors that cause the disruption of the microbiome e.g. the use of antibiotics, changes in diet, and many other environmental insults (DeGruttola et al., 2016). When the healthy, homeostatic state of the microbiome becomes imbalanced, it is referred to as dysbiosis. Dysbiosis is often characterized by 1) a loss of beneficial microbes 2) shift to a pathogen-dominated community 3) a loss of microbial diversity (Petersen and Round, 2014) and 4) increased susceptibility to disease (Sekirov et al., 2010). Recently, there has also been a

paradigm shift towards understanding the interactions of pathogens within a microbial community, known as the pathobiome concept (Vayssier-Taussat et al., 2014;Kamada et al., 2013). Nevertheless, the important role of the microbiome is to protect the host against the overgrowth of pathobionts (colonization resistance) (Stecher and Hardt, 2011). An imbalanced gut microbiome is often accompanied by an increase in the bacterial phylum Proteobacteria (Shin et al., 2015). Many members of this phylum are the facultative anaerobe Enterobacteriaceae (class Gammaproteobacteria), and their overall increase in the human gut during dysbiosis causes a consumption of available oxygen, favoring obligate anaerobes (phyla Bacteroidetes and Firmicutes) that were established a few days after birth (Rigottier-Gois, 2013). This increase in the bacterial family Enterobacteriaceae is likely caused by the production of host-derived nitrate during inflammation in the lower GI tract, because members of this family happen to be more likely to encode the enzymes for nitrate respiration (Winter and Bäumier, 2014b, 2014a).

Objectives

Prior to this study, bacterial communities associated with freshwater mussels were believed to reflect the bacterial communities in their surrounding habitats including water and sediments. Those bacterial communities were thought to be transient and the idea of mussels harboring their own community of bacterial symbionts that were beneficial, even critical, to their well-being had not been explored.

The goal of mussel restoration, besides assisting in the recovery of imperiled species, is to restore complete mussel assemblages and the ecological services that they provide. We hypothesized that the gut microbiome of freshwater mussels affects host health and development and that the restoration of healthy mussels requires them to have a healthy gut microbiome. The overall goal of my thesis research has been to study the digestive gland microbiome of freshwater mussels using next-generation sequencing technology. To that end, the objectives of these studies were to:

1. Characterize and compare the digestive gland microbiomes of wild and cultured/captive mussels, specifically the species *V. nebulosa* and *P. cordatum.*
    a. H_1: There will be significant differences in bacterial diversity and composition between wild and cultured/captive microbiomes.
2. Evaluate the influence (if any) of the surrounding habitat on the core microbiome of freshwater mussels.
    a. H_1: There will be a significant difference between the bacterial communities found in the surrounding water and sediment and mussel microbiomes.

3. Evaluate the effects of dysbiosis on the digestive gland microbiome of *V. nebulosa* using chemotherapeutic treatments and if it causes increased susceptibility to bacterial infection by a known opportunistic pathogen, *Aeromonas hydrophila*.

    a. H_1: Tetracycline dosed *V. nebulosa* will exhibit a dysbiotic microbiome (reduced abundance of Tenericutes).

    b. H_2: *V. nebulosa* challenged with *A. hydrophila* will be more susceptible to septicemia.

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Chapter 2. Description and characterization of the digestive gland microbiome in the freshwater mussel *Villosa nebulosa* (Bivalvia: Unionidae)

Abstract

The aim of this study was to characterize the digestive gland microbiome from wild and cultured (hatchery-reared) Alabama rainbows (*Villosa nebulosa*) using 16S rRNA gene pyrosequencing in order to understand the role of recovery efforts via propagation on microbial community structure in freshwater mussels. A total of 10 digestive glands from Alabama rainbows were analyzed in the study (wild = 5, hatchery = 4, and translocated = 1). Pyrosequencing yielded a total of 32,962 bacterial sequences and 387 OTUs. Tenericutes was the most dominant phylum from all samples analyzed (>87%), followed by Proteobacteria (4.6%), Fusobacteria (4.5%) and Bacteroidetes (1.4%). Digestive gland microbiomes were overwhelmingly dominated by OTUs related to the genus *Mycoplasma*. Interestingly, those *Mycoplasma*-like sequences could not be ascribed unequivocally ascribed to the genus *Mycoplasma* and probably represent new lineages within the class Mollicutes. Our study identified a core microbiome in the digestive gland microbiome of Alabama rainbows, with all individual mussels sharing 11 OTUs. However, the digestive gland microbiome from mussels collected from the wild versus those from hatchery-reared mussels were significantly different. These results collectively show that novel microbial communities exist within the digestive gland of freshwater mussels.

Introduction

Freshwater mussels (Bivalvia: Unionoida: Unionoidae) are filter-feeding parasitic bivalves that, once transformed from the fish-parasitic larval stage (glochidia) and residing as post-transformed juveniles and adults in sediment, consume or otherwise process bacteria, phytoplankton, detritus, and particulate organic matter from the water column (Silverman et al., 1995;Vaughn and Hakenkamp, 2001). They perform many important ecosystem services, e.g., turning over sediments (Vaughan, 2017;Gutierrez et al., 2003), filtering water and maintaining its quality (Naimo, 1995;McGregor and Garner, 2004;Atkinson et al., 2013), and translocating nutrients from the water column to the benthos thereby making resources available to organisms in other trophic levels (Atkinson et al., 2013;Howard and Cuffey, 2006;Vaughn and Hakenkamp, 2001). Historically, freshwater mussels dominated the benthos of rivers and lakes in eastern North America (Strayer, 2008;Vaughan, 2017). Now, they are the most imperiled North American faunal group (Bogan, 2008;Williams et al., 1993). Population declines and species extirpations have resulted from physical modification of riverine habitats, water quality degradation, and introduction of invasive species (Bogan, 2008;Williams et al., 1993).

Much of the literature on freshwater mussels has focused on feeding behavior (Vaughan, 2017) and diet (Silverman et al., 1995;Atkinson et al., 2013;Vaughn and Hakenkamp, 2001;Christian et al., 2004); however, the role of gut-bound bacterial symbionts is underexplored. This represents a significant gap in our knowledge of the biology of freshwater mussels because such bacteria in other metazoan lineages are likely critical for nutrient assimilation (Mueller et al., 2012). This lack of information also makes vulnerable attempts to culture and enhance stocks of imperiled freshwater mussels if culture conditions inadvertently destroy beneficial or requisite microbial communities that affect freshwater mussel nutrition,

growth, and propagation. This study is among the first to present evidence of microbial endosymbionts in freshwater mussels. It also evaluates the environmental effects on microbial community structure by contrasting the bacterial community composition between conspecific hatchery-reared and in-stream freshwater mussels.

The association between freshwater mussels and their gut microbes is generally attributed to the ingestion of bacteria (Harris 1993). This host-microbe interaction may be the direct result of the bivalve digesting and consuming microbes (Silverman et al., 1995;Nichols and Garling, 2000;Christian et al., 2004) or indirectly as transient or commensal bacteria in the gut (Harris, 1993). To date, most studies evaluating the microbial communities of freshwater mussels have focused on identifying potential pathogens (Grizzle and Brunners, 2009;Starliper et al., 2008). Starliper et al. (2008) investigated the normal microbiota of healthy freshwater mussels from the Holston and Clinch rivers in Virginia to identify potential bacterial pathogens. Similarly, Chittick et al. (2001) assessed the health status of a freshwater mussel, Eastern Elliptio (*Elliptio complanata*), from North Carolina by culturing the digestive gland as a means of assessing bacterial community diversity. Although these studies provided important baseline data for mussel health, they all employed culture-based methods, which routinely recover <1/10 of the total microbial diversity (Amann et al., 1995). Recent molecular -based techniques have characterized the microbial communities in several marine bivalve species, thus eradicating the need to isolate and cultivate specific microbes. Such molecular-based studies include characterizing the microbiome in the gill, stomach, gut and whole homogenate of oysters (Romero et al., 2002;Hernandez-Zarate and Olmos-Soto, 2006;King et al., 2012), estuarine mussels (*Brachidontes* sp.) from Indonesia (Cleary et al., 2015), abalone (Huang et al., 2010), and the freshwater zebra mussel (*Dreissena polymorpha*) (Winters et al., 2011). Because of its

value as seafood (Keithly and Diop, 2011) and because it carries human-pathogenic bacteria (Kelly and Dinuzzo, 1985), much effort has been placed on characterizing the microbiota of oysters (Lokmer and Wegner, 2015;Trabal et al., 2012)

A recent review (Bahrndorff et al., 2016) suggested that the microbiome could be important regarding conservation biology, especially for threatened or endangered species. Freshwater mussels are recognized as an important ecological species in river systems (Vaughan, 2017), and recovery efforts through propagation and reintroduction are ongoing across several Southeastern states (Barnhart, 2006b) One conservation target is the Alabama rainbow, *Villosa nebulosa* (Conrad, 1834), which has been petitioned for federal protection under the Endangered Species Act (Center for Biological Diversity Petition 2010). Research and recovery efforts for *V. nebulosa* are being led by the Alabama Aquatic Biodiversity Center (AABC), and reintroductions began several years ago. The Alabama rainbow is a Mobile River Basin endemic and its historic distribution included the upper Coosa, Cahaba, and Warrior river basins above the Fall Line (Williams et al., 1993).

In an effort to assist ongoing recovery efforts, we herein characterize and contrast the microbiome of the digestive gland from cultured and in-stream Alabama rainbows using 16S rRNA gene pyrosequencing. No previous study has determined the bacterial composition and diversity of any freshwater mussel's digestive gland using genomic methods. We hypothesize that significant differences exist between the gut microbiomes of cultured vs. in-stream Alabama rainbows. Characterization of these microbiomes could have profound implications for better understanding the fundamental feeding biology of the Alabama rainbow as well as other freshwater mussels as well as assist ongoing, propagation-based recovery activities.

Materials and Methods

Sample collection. Five (5) mature female Alabama rainbows were collected from Terrapin Creek, Cleburne Co., AL (N 33.861306° W -85.5225730°) on May 1, 2011. Additional Alabama rainbows from that locality also were collected and transported to Auburn University for analyses ('wild mussels'= DG4-DG10). The cultured Alabama rainbow studied herein were produced by transforming glochidia that infected the gill of Coosa bass, *Micropterus coosae* (Hubbs and Bailey, 1940), and rearing the newly-transformed juveniles in upwelling chambers (Barnhart, 2006a). Juvenile Alabama rainbow were fed a mix of commercially available *Nanochloropsis* spp. and shellfish diet (Reed Mariculture) added to hatchery pond surface water filtered to 120 µm. After 60-90 days post-transformation, juvenile Alabama rainbows were transferred to suspended upwelling systems (SUPSYS) deployed in an AABC rearing pond wherein they were kept for approximately 15 months ('hatchery mussels'= DG78-81) before being shipped alive to Auburn University. A separate cohort of Alabama rainbows that was transformed on July 2009 at the AABC was translocated to Choccolocco Creek, Talladega Co., AL (N 33.55166° W -86.10631°) where they were kept in an in-stream "silo" (container) for 3 months, before returning them to an AABC earthen pond wherein they remained for an additional 2 years. Four of the translocated Alabama rainbows were originally part of the study but only one yielded sufficient sequencing reads to be included in the present study ('translocated mussel'= DG84; Table 1).

DNA extraction. Approximately 25 mg of digestive gland tissue was aseptically collected from each Alabama rainbow. DNA extraction was carried out using DNeasy Blood & Tissue Kit (Qiagen CA, USA) following Gram-positive bacterial DNA extraction. Extracted DNA was quantified by photometry using the Nanodrop 2000 (Thermo Scientific, Rochester, USA) and the

quantities adjusted to 20 ng/µL. Samples were deemed sufficient for PCR amplification by using universal primers against the 16S rRNA gene (Larsen et al., 2015). Samples were kept at -20°C until sequencing. Roche titanium 454 sequencing was performed on 10 digestive gland samples (5 in-stream, 4 hatchery-reared, and 1 translocated Alabama rainbow) using individual barcodes and primer 27F (5'-AGRGTTTGATCMTGGCTCAG-3') amplifying the variable V1-V3 region of the 16S rRNA. PCR conditions were as follows: initial denaturation at 94°C for 3 min and 30 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, including a final elongation at 72°C for 5 min. Resulting sequences were processed with an exclusive analysis pipeline (MR DNA, Shallowater, TX) including removal of barcodes and primers as well as sequences of less than 200 base pairs, a base call error rate of less than 0.3% (Q<25), ambiguous base calls, and long (>6 base pairs) stretches of identical bases. Following denoising and chimera and singleton sequence removal, operational taxonomic units (OTUs) were defined in agreement with the current accepted prokaryotic species concept (>3% sequence agreement (Rossello-Mora and Amann, 2001)) and taxonomically identified using BLASTn against the Greengenes database (DeSantis et al., 2006).

Data analysis. The Alabama rainbow with the fewest number of total sequences (*n* = 291) was used for standardization for diversity analyses and rarefaction curves for the microbial communities identified from all other Alabama rainbows. Using Mothur v.1.33.3 software (Schloss et al., 2009), rarefaction curves, Good's coverage, abundance-based coverage estimation (ACE), Chao1, Shannon evenness, observed OTUs, and shared OTUs were generated. A one-way ANOVA was performed on all diversity indexes. An OTU abundance table was loaded into PRIMER v6 (Clarke and Warwick, 2001) to perform similarity percentages

(SIMPER) analysis in order to determine OTU differences between Alabama rainbow microbial communities. Cut-off for low contributions was set at the default of 90.

Results

Diversity analysis. Pyrosequencing of the 16S rRNA gene yielded a total of 32,962 bacterial sequences and 387 OTUs. After standardization, 291 sequences remained along with 247 OTUs. Sequence coverage was $\geq$ 89% for all Alabama rainbows sampled (Table 1). Total expected richness was calculated by ACE and Chao1 but no significant difference between mussel origins was found (note: only wild *versus* hatchery origins were compared; the translocated mussel was not included in the comparison since no replicated samples were available). Individual rarefaction curves displaying the sequence coverage of each digestive gland sample is shown in Figure 1.

Gut microbiome composition. Collectively, 16 bacterial phyla were identified from the digestive glands of the sampled Alabama rainbows (Figure 2) although only 4 phyla represented more than 1% of all OTUs. The phylum Tenericutes dominated all samples analyzed, having >87% of OTUs. OTUs of Proteobacteria were the second most common (4.6%), followed by those of Fusobacteria (4.5%) and Bacteroidetes (1.4%). Within the Proteobacteria, each Alabama rainbow microbiome comprised OTUs assigned to Gammaproteobacteria (4.5%) and Betaproteobacteria (2.9%). Less common phyla varied in abundance between Alabama rainbows, e.g., DG10 was unique by having no representative from Fusobacteria; DG6 lacked a representative from Bacteroides (data not shown).

At the genus level, microbial diversity of Alabama rainbow digestive gland microbiomes was dominated by OTUs that were similar to isolates of *Mycoplasma* spp. deposited in GenBank and GreenGenes; however, microbial OTUs from Alabama rainbows sampled were largely

unique, i.e., percent identities between our samples and microbial OTUs in GenBank and GreenGenes was low (74-92%). On average, those OTUs only shared 81% sequence identity with known sequences from *Mycoplasma*,sp. and we therefore refer to them herein as "*Mycoplasma*-like." However, they likely represent a species or group of species that should be assigned to a new genus. We detected OTUs of the *Mycoplasma*-like clade in all digestive glands sequenced (Figure 3). *Cetobacterium* dominated the microbiome in DG4 and DG6 only; however, OTUs of the *Mycoplasma*-like clade also were present. Interestingly, those same two individuals (DG4 and DG6) had nodular masses on their mantle and appeared emaciated (indicative of poor health). *Cetobacterium* (second most abundant genus in our sample) was present in the digestive gland of 6 of 10 Alabama rainbows. Other genera (Table 2) were present in only 1 Alabama rainbow but typically at low percentages. The genus *Xanthomonas* comprised > 22% of the community in DG78 but was absent from any other sample.

A multidimensional scaling (MDS) plot based on digestive gland OTU abundances was generated in Mothur for better visualization of clustering patterns based on the origin of the Alabama rainbows sampled (Figure 4). The MDS plot showed that bacterial composition was influenced by origin, with OTUs from hatchery-reared Alabama rainbows forming a tighter cluster than those collected from the wild. The clusters were supported by ANOSIM with a global R value of 0.724 ($p = 0.04$) for origin. Anecdotally, the microbial community of the translocated Alabama rainbow was most similar to that of the in-stream Alabama rainbows.

A total of 11 OTUs were shared between the wild and hatchery-reared Alabama rainbows, representing 6% of the total OTUs (Figure 5). Similarity percentage (SIMPER) analysis by OTUs revealed large differences in digestive gland bacterial communities between wild and hatchery-reared Alabama rainbows. Among the wild Alabama rainbows, the highest

contribution of similarity was OTU_6 and among hatchery-reared Alabama rainbows was OTU_47; both of which were *Mycoplasma*-like sequences. These results indicate OTU_47 contributes to the highest dissimilarity between wild and hatchery mussels, followed by OTU_6.

Discussion

The core microbiome is defined as the group of microbes that are present in all individuals of the same species regardless of the environment (Turnbaugh et al., 2007). Characterization of the core microbiome of freshwater mussels will facilitate malacologists' culture efforts not only to improve initial survivorship and production efforts, but eventually to identify 'normal' or 'healthy' core microbial communities. However, further understanding core microbiome structure and diversity across a large number of mussel species will be required before evaluation can infer mussel health. Additional core microbiome data could also facilitate evaluation for the loss of mussels during kill events or disease epizooties (Southwick and Loftus, 2003). Several studies have attempted to characterize the core gut microbiomes of commercially important fish species (Tarnecki et al., 2017) but few studies have focused on aquatic invertebrates. King et al. (2012) characterized the stomach and gut core microbiomes from the Eastern Oyster (*Crassostrea virginica*) from two localities. The authors reported the core gut and stomach microbiomes were different with the core stomach microbiome having less microbial diversity than the core gut that represented about 16% of all OTUs. Pierce et al. (2015) also supports the hypothesis that a core microbiome exists in *C. virginica* in a study suggested that seasonality had a stronger effect on the gut microbiome than locality. However, Trabal *et al.* (2012) have reported geographic location as the primary driver for sharing the oyster gut microbiome. In our study, we focused on a single Unionidae species reared under two completely different conditions. Although we found significant alpha-diversity between wild and

hatchery-reared Alabama rainbows, all individual shared 11 of OTUs, suggesting a core microbiome exists for *V. nebulosa*.

Our results showed no significant differences in terms of OTUs diversity and evenness between wild and hatchery-reared mussels, suggesting that cultured and wild Alabama rainbows had the same degree of bacterial diversity in their digestive glands although species composition varied significantly. Overall, both groups were dominated by OTUs ascribed to the phylum Tenericutes, and in specific to the class Mollicutes. Previous studies have identified Mollicutes as the dominant constituent of bacterial communities from a marine mussel, (*Brachiodontes* sp.,) from in Indonesia (Cleary et al., 2015). Mollicutes have also been reported as primary constituents of the digestive gland of the Sydney Rock Oysters (*Saccostrea glomerata*) and in the intestine of abalones (*Haliotis discus hannai*) (Tanaka et al., 2004;Green and Barnes, 2010). Our findings were surprising since Mollicutes comprised up to 98% of total sequences identified in some of the analyzed Alabama rainbows. These results were even more intriguing given that our Mollicutes-OTUs had strikingly low similarity to previously-sequenced microbial OTUs associated with marine molluscs. Kostanjsek at al. (2007) reported a similar problem when they characterized the gut microbial community of the terrestrial isopod *Porcellio scaber*. After an extensive microscopic characterization on the bacteria associated with the hindgut wall of the isopod, they proposed '*Candidatus* Bacilloplasma' as a new lineage within Mollicutes to accommodate their newly-identified sequences and reported that the average similarity between new and previously sequenced databases was below 82.6%. This finding is similar in variation to the results presented herein, i.e., our *Mycoplasma*-like OTUs share an average of 81% sequence similarity with those deposited in the databases. Our *Mycoplasma*-like OTUs could represent one or more novel lineages within the class Mollicutes; however, further phylogenetic studies and

ultrastructure characterization of these putatively new bacteria are required before a formal proposal for a new lineage is made.

The genus *Mycoplasma* comprises Gram-positive bacteria that are phylogenetically related to the *Bacillus*/*Clostridium* branch of the Firmicutes. Mycoplasmas lack a cell wall, have a low G+C content, and have the smallest genome of any known self-replicating organism. Phylogenetic analyses indicate that Mycoplasmas underwent multiple reductions in genome size (Joblin and Naylor, 2002). Because of their small genomes, they are unable to perform many basic metabolic functions and are considered obligate commensals or parasites (no free-living Mycoplasmas have been identified to date). Mycoplasmas are typically associated with respiratory or urogenital mucosae wherein they attach to the host eukaryotic cell through their tip organelle. In some cases, they become intracellular pathogens but under appropriate environmental conditions most remain a benign member of the host's microbiome (Brown et al., 2005). Some are associated with chronic illnesses in humans whereas others are well-known pathogens, e.g., *M. pneumonia* and *M. gallisepticum.* Because of the large number of *Mycoplasma*-like OTUs identified herein, it is tempting to speculate that they confer some benefit to their host. Wang et al., (2016) assembled two draft genomes of Mycoplasmas found in the stomach of the deep-sea isopod *Bathynomus giganteus*, performed a comparative genome analyses with four previously sequenced Mycoplasma genomes, including *Candidatus* Hepatoplasma crinochetorum isolated from the terrestrial isopod *P. scaber* (Leclerq et al., 2014), and found sialic acid lyase genes that can block attachment of pathogenic bacteria to the stomach wall and thereby protect the host from invading pathogens. In addition, Wang et al. (2016) found multiple copies of genes related to proteolysis and oligosaccharide degradation and speculated that these genes may help the host survive under low-nutrient conditions.

This is the first study to evaluate the microbiome of a unionid species using next generation sequencing. Our results revealed that the phylum Tenericutes, specifically, the class Mollicutes, dominated the gut microbiome. The only two exceptions were the two wild Alabama rainbows that appeared emaciated but further study is required to explore this aspect. Future studies are ongoing to further characterize the Mycoplasmas found in Alabama rainbow and to explore the gut microbiome of other species of freshwater mussels in streams and hatchery settings. These initial data indicate a much greater diversity of Mycoplasma-like bacteria in the gut of a freshwater mussel than that reported from the gut of an isopod. Further evaluation of this gut microbiome will require a much more powerful whole-genome sequence approach.

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Table 2-1. Mussel origin and diversity indexes as calculated by MOTHUR software (ver. 1.33.3).

Sample ID	Group	# Observed OTUs	Good's coverage	# Predicted OTUs		Shannon evenness
				ACE	Chao1	
DG4	Wild	64	0.8969	133.38	100.25	0.7938
DG6	Wild	68	0.8935	109.16	101.21	0.8131
DG7	Wild	39	0.9416	84.07	51.36	0.7268
DG8	Wild	43	0.9278	109.09	78.00	0.7277
DG10	Wild	29	0.9553	65.58	107.00	0.7409
DG78	Hatchery	42	0.9175	131.18	88.00	0.6874
DG79	Hatchery	53	0.9141	85.16	86.33	0.7295
DG80	Hatchery	41	0.9381	77.69	62.86	0.7003
DG81	Hatchery	50	0.9210	106.46	73.00	0.7482
DG84	Translocated	35	0.9519	70.58	45.11	0.7202

Table 2-2. Percent abundance of bacterial genera found in mussel digestive glands (only top five genera from each individual mussel are listed).

DG4 -Wild		**DG6 – Wild**	
Cetobacterium	62.28%	*Cetobacterium*	92.10%
Mycoplasma-like	27.98%	*Aeromonas*	3.44%
Lactobacillus	3.61%	*Mycoplasma*-like	3.10%
Ralstonia	1.10%	*Shewanella*	0.69%
Dysgonomona	1.00%	*Klebsiella*	0.34%
		Parabacteroides	0.34%

DG7 – Wild		**DG8 – Wild**	
Mycoplasma-like	95.82%	*Mycoplasma*-like	93.36%
Acidovorax	1.94%	*Acinetobacter*	0.84%
Acinetobacter	1.08%	*Prevotella*	0.81%
Chryseobacterium	0.26%	*Acidovorax*	0.66%
Hyphomicrobium	0.10%	*Akkermansia*	0.47%

DG10 – Wild		**DG78 – Hatchery**	
Mycoplasma-like	98.26%	*Mycoplasma*-like	73.74%
Acidovorax	0.51%	*Xanthomonas*	22.84%
Acinetobacter	0.51%	*Flavobacterium*	0.69%
Spiroplasma	0.28%	NC10 (Candidate division)	0.46%
Edaphobacter	0.11%	*Ureaplasma*	0.42%
Pseudomonas	0.11%		

DG79 – Hatchery		**DG80 – Hatchery**	
Mycoplasma-like	89.51%	*Mycoplasma*-like	65.53%
Cetobacterium	1.53%	*Cetobacterium*	10.11%
Flavobacterium	1.31%	*Flavobacterium*	5.19%
Microbacterium	0.98%	*Acinetobacter*	4.87%
Sphingomonas	0.95%	*Pseudomonas*	3.73%

DG81 – Hatchery		**DG84 – Translocated**	
Mycoplasma-like	88.53%	*Mycoplasma*-like	94.56%
Cetobacterium	2.80%	*Rickettsia*	1.72%
Staphylococcus	1.44%	*Cetobacterium*	1.48%
Fusobacterium	1.41%	*Fusobacterium*	0.62%
Flavobacterium	0.74%	*Flavobacterium*	0.52%

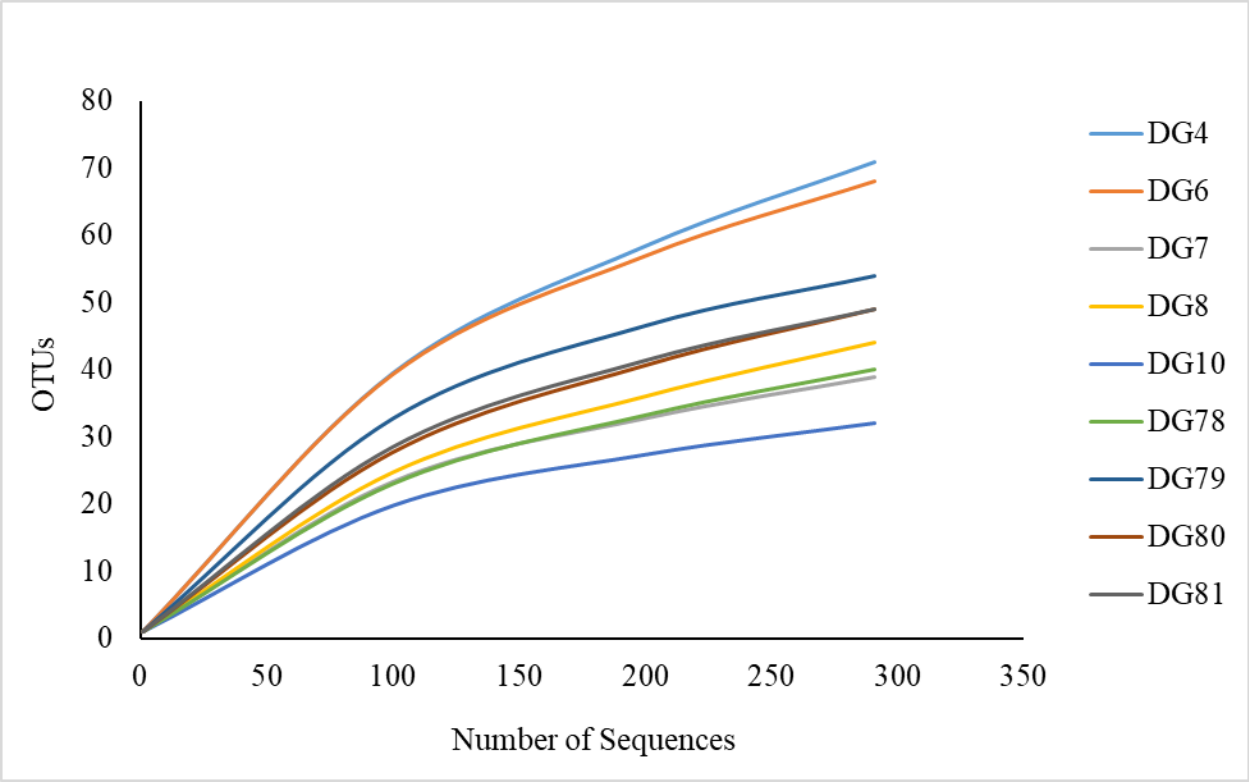

Figure 2-1. Rarefaction curves of individual *Villosa nebulosa* analyzed in the study. Sequences were standardized to equal sequencing sizes for direct comparisons. Wild mussels: DG4-10 and hatchery-reared musses: DG78-81.

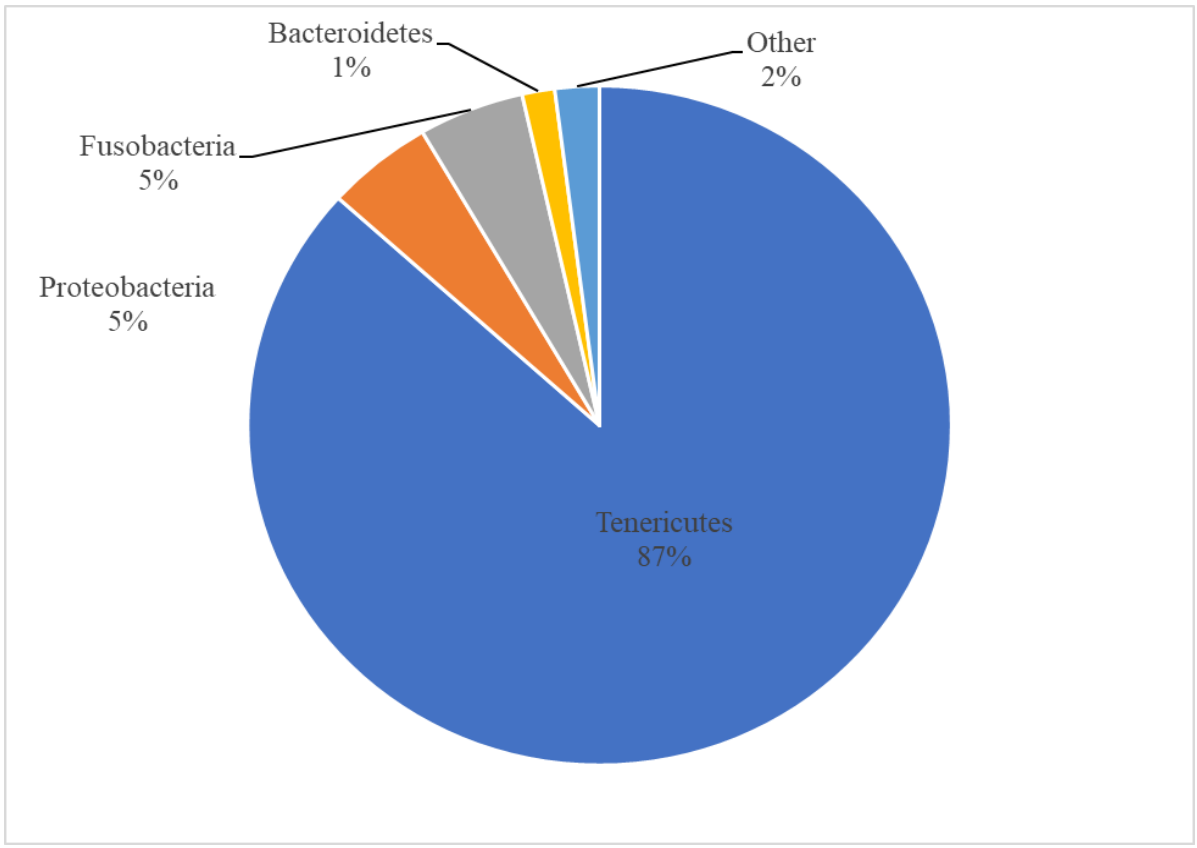

Figure 2-2. Phyla composition of the digestive gland microbiome of all *Villosa nebulosa* samples.

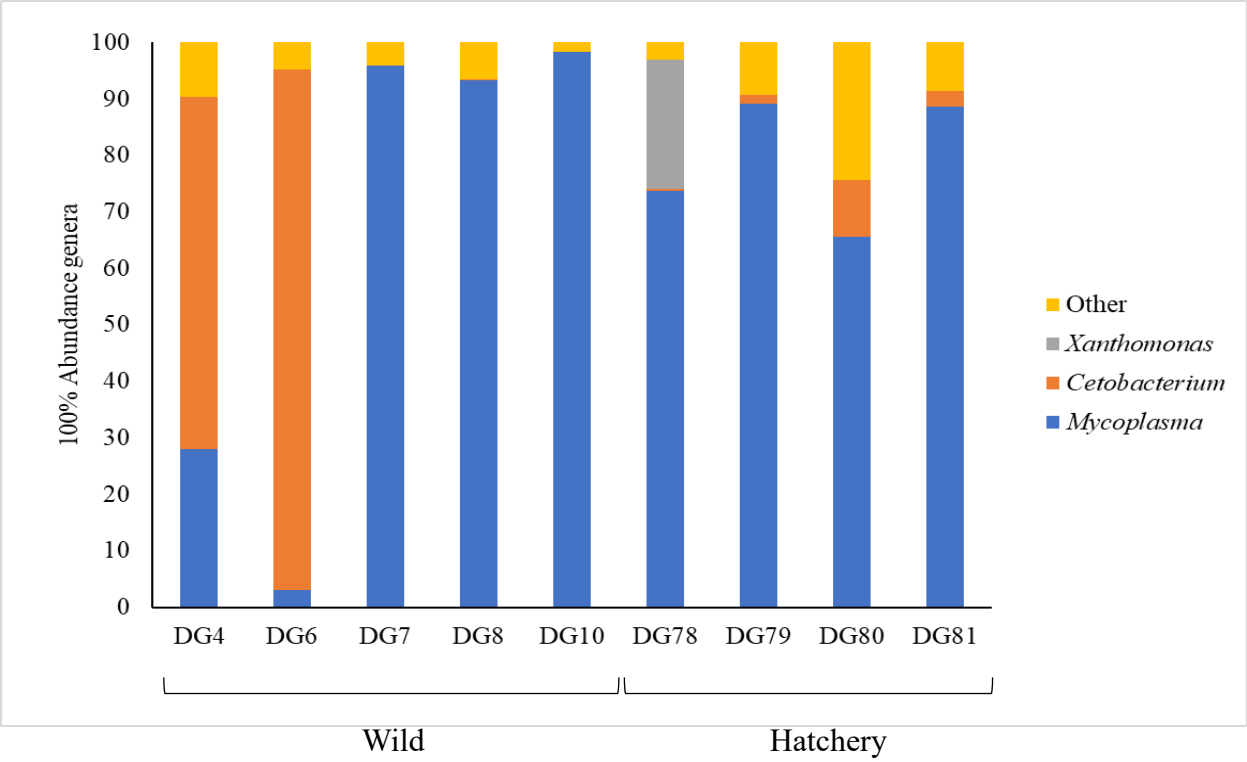

Figure 2-3. Distribution of predominant genera in digestive gland microbiome of each individual *Villosa nebulosa*.

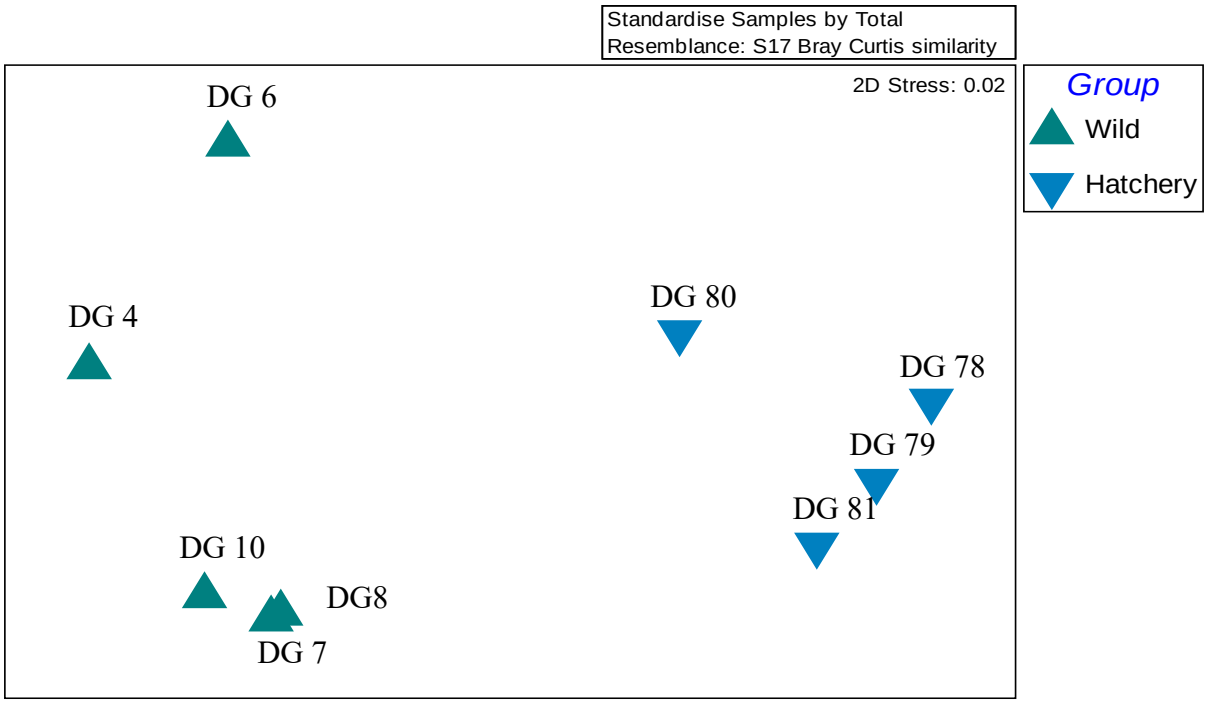

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Figure 2-4. Multidimensional scaling of digestive gland samples according to mussel origin, based on percent similarity in OTU abundances.

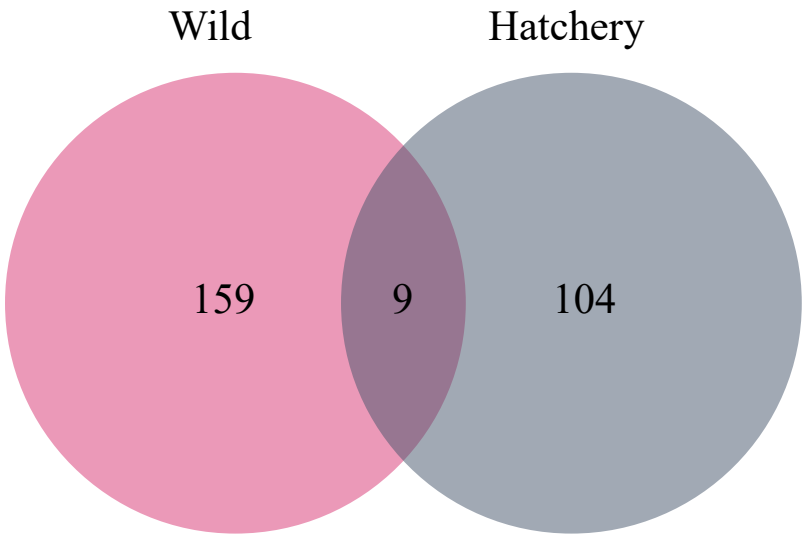

Figure 2-5. Venn diagram showing the number of shared and unique OTUs in the digestive gland microbiome of wild and hatchery-reared *V. nebulosa*.

Chapter 3. Contribution of surrounding habitat to the digestive gland microbiome of the freshwater mussel *Villosa nebulosa* (Alabama Rainbow)

Abstract

Freshwater mussels (Bivalvia: Unionidae) are the most imperiled faunal group in North America. The state of Alabama harbors the highest biodiversity of freshwater mussels in the world and currently leads restoration efforts across the Southeast. Freshwater mussel propagation is challenging due to their unique life cycle and only a few species can be cultured in captivity. It is well known that gut microbiomes play a key role in the nutrition and metabolism of their host. The objective of this study was to compare the digestive gland ('gut') microbiome of *Villosa nebulosa* (of the few freshwater mussels species that can be cultured) raised under culture conditions and those collected from the wild. We analyzed 15*V. nebulosa* specimens including animals reared in captivity that derived from two different wild stocks: Shoal Creek (n = 5) and Flannigan Creek (n = 5), as well as, wild mussels collected from Shoal Creek (n = 5). Overall, the microbial communities between cultured and wild mussels were significantly different. Wild Shoal Creek mussels had a higher abundance of Tenericutes (46.9%) and Chlamydiae (25.6%), whereas, Tenericutes (43.3%) and Proteobacteria (25.3%) were the predominant phyla in cultured mussels. Our results do indicate that microbial communities found in sediment samples, although unique to the locality (Shoal Creek and hatchery), were similar in terms of microbial diversity and composition. Conversely, microbial communities found in water were quite

different among the environments sampled. Our results have collectively demonstrated that *V. nebulosa* harbors a unique microbiome than that from their surrounding habitat.

Introduction

Freshwater mussels (Bivalvia: Unionoidae) are among the most imperiled group of aquatic invertebrates in North America (Bogan, 2008; Williams et al, 1993). Representing over 60% of North America's species richness, Alabama has the highest diversity of freshwater mussels. Freshwater mussels perform many important ecological services (Vaughan, 2017), e.g., ecosystem engineers (Vaughan, 2017;Gutierrez et al., 2003), acting as bio-indicators of water quality (Naimo, 1995;McGregor and Garner, 2004;Atkinson et al., 2013), and nutrient cycling (Atkinson et al., 2013;Howard and Cuffey, 2006;Vaughn and Hakenkamp, 2001). Current threats to population declines and species extirpations of freshwater mussels have resulted from habitat modifications, water pollution, and invasive species introduction (Bogan, 2008;Williams et al., 1993). However, the need for research and conservation status for these imperiled species remains ongoing (Lopes-Lima et al., 2018; Haag and Williams, 2014).

Studies on the association between freshwater mussels and their symbiotic microbes using molecular-based techniques has recently been explored (Aceves et al., 2018). While the gut microbiome is a complex community of bacteria that provides many benefits for host health and development (Tuddenham and Sears, 2015;Kamada et al., 2013;Stecher and Hardt, 2011), it is generally unknown how these symbionts benefit bivalves other than in nutrition or immunity (Harris, 1993). It has been suggested that the evolutionary process of the host and its' symbiotic microbes is important for host fitness and survival (Rosenberg et al., 2007). This becomes especially important for conservation targeted species that are held in captivity before being released back into the wild (Bahrndorff et al., 2016;West et al., 2019;Hird, 2017). The symbiotic nature of the gut microbiome allows researchers to understand the selective process the host undergoes where they tend to keep the microbes that benefit their lifestyle. Furthermore, recent

studies have began to explore the differences between captive and wild microbiomes of animals in order to understand the influence of their surrounding habitat (Lavoie et al., 2018;Roeselers et al., 2011;Kohl et al., 2017;Wan et al., 2016).

Previous research has focused on identifying potential pathogens of wild freshwater mussels using culture-dependent methods (Starliper et al., 2008;Starliper et al., 2011;Chittick et al., 2001). While these data provide a baseline on bacteria that can be cultured from whole tissue homogenates and the digestive gland from different species of mussels, there remains a gap in the literature on characterizing the gut microbiome of cultured mussels and the animals' surrounding habitat using metagemonics. Many studies have shown that the gut microbiota of fish can be shaped by salinity, trophic level, and habitat use although host species remains the main driver for bacterial colonization (Roeselers et al., 2011;Sullam et al., 2012;Larsen et al., 2013;Wu et al., 2012). Fewer studies have characterized how invertebrates are colonized by bacteria therefore analyzing the microbial community of water and sediments surrounding freshwater mussels could shed light on how their gut microbiomes are shaped. Zhang et al. (2016) found that the gills and gut bacterial community of Chinese mitten crab were distinctly different from their surrounding water, suggesting that the relationship between the host and its' microbes may be evolutionary, not ecologically. Interestingly, they also reported a high abundance of Tenericutes in the gut, which has similarly been characterized in freshwater mussels (Aceves et al., 2018).

In the previous study, Aceves et al. (2018) characterized the gut microbiome of cultured and wild Alabama Rainbow (*Villosa nebulosa*), a conservation targeted species that is endemic to the Mobile River Basin in Alabama (Williams et al., 1993). Herein, we expand upon our previous study by comparing two populations of cultured *V. nebulosa* and investigating the influence of

water and sediment on their core microbiomes. Our objectives were to 1) compare the gut microbiomes of two populations of cultured *V. nebulosa* 2) compare cultured *V. nebulosa* to their wild counterparts, and 3) evaluate if the microbial community of water and sediment influence the microbiomes of *V. nebulosa* by rearing environment.

Materials and Methods

Sample collection and DNA extraction. Ten Alabama Rainbows ('cultured'), water, and sediment were collected from the Alabama Aquatic Biodiversity Center (AABC) on April 12, 2017 in 22.3°C water, 5.7 pH and 7.8 ppm. For more information on culturing conditions of mussels, see Aceves et al. 2018. Five Alabama Rainbows ('wild'), water, and sediment samples were collected from Shoal Creek (N33°43.160' W85°35.273') on May 8, 2017 in 22°C water, 6.1 pH and 7.2 ppm. Samples were transported at ~ 10°C to Auburn University (Auburn, Alabama) where they were processed for microbial community analysis.

Approximately 25 mg of digestive gland tissue was aseptically removed from each Alabama rainbow. The DNeasy Blood & Tissue Kit (Qiagen CA, USA), following Gram-positive bacterial DNA extraction, was performed for mussels. Water samples (100 ml) were centrifuged at 2,250 rpm for 5 minutes at 4˚C, supernatant was discarded, and the pellet was filtered through an autoclaved funnel onto a 0.22 µm polycarbonate membrane. The filter was put into a 5 ml PowerWater Bead tube, and stored at -80˚C. Using sterilized tweezers the filter was cut into pieces and then DNA extraction was performed (PowerWater DNA isolation kit). Sediment samples were stored at -80˚C until DNA extraction was performed using approximately 25 mg of sediment (PowerSoil DNA isolation kit). The concentration of extracted DNA was quantified using spectrophotometry (Nanodrop 2000, Thermo Scientific, Rochester,

USA) and their purity assessed using the absorbance ratios at 260 nm and 280 nm (OD 260/OD 280).

PCR amplification and sequencing. Samples were deemed free of PCR inhibitors by amplifying a conserved region of the 16S rDNA using universal primers 63V and 1387R (Larsen et al., 2015). PCR conditions were as follows: initial denaturation at 95°C for 5 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and final elongation step at 72°C for 10 min. Quantities were adjusted to 20 ng/µL before sequencing. A total of 15 mussels (AABC = 10 and in-stream Shoal Creek = 5), 8 water samples (AABC = 3 and in-stream Shoal Creek = 5), and 6 sediment samples (AABC = 3 and Shoal Creek = 3) were submitted to MR DNA (Shallowater, TX) for PCR amplification and Next Generation sequencing using the Illumina MiSeq platform. The primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') was used, targeting the V4 variable region of the 16S rRNA gene. All samples were sequenced as pair end reads (2 x 300) following the manufacturer's protocol. Resulting sequences were processed using MR DNA's proprietary pipeline. Briefly, sequencing data were joined, barcodes and primers were removed, followed by the removal of sequences <150 bp and ambiguous base calls. After denoising, chimeras were removed, and operational taxonomic units (OTUs) were generated as proxies for bacteria species, defined by the current prokaryotic species concept ($\geq$ 97% 16S rRNA sequence similarity (Rossello-Mora and Amann, 2001)). Taxonomic classifications were obtained using BLASTn against a curated database derived from the Ribosomal Database Project II (Cole et al. 2014) and National Center for Biotechnology Information (Geer et al. 2009).

Data analysis. The sample with the fewest number of total sequences was used for standardization for diversity analyses and rarefaction curves for the microbial communities

identified from all samples. Using Mothur v.1.35.1 software (Schloss et al., 2009), rarefaction curves, diversity indices (Good's coverage, abundance-based coverage estimation (ACE), Chao1, Simpson's diversity index, and Shannon evenness), observed Operational Taxonomic Units (OTUs), and shared OTUs (Venn diagrams) were generated. A one-way ANOVA was performed on all diversity indexes. An OTU abundance table was deposited into PRIMER/PERMANOVA + (Clarke and Warwick, 2001) to generate principal coordinate analysis (PCoA) plots to visualize the differences between samples and permutational multivariate analysis of variance (PERMANOVA) to test whether there were significant differences between different groups. Additionally, similarity percentages (SIMPER) analysis was used to define the taxon for causes in the dissimilarity between samples and their relative abundances. Cut-off for low contributions was set at the default of 90.

To determine predictive metagenome functions of the gut microbiome of Alabama rainbows, we used PICRUSt (Langille et al., 2013). OTUs with a 97% sequence similarity to those deposited in the Greengenes database (version 13.5.) were picked and a biom file was created (MR DNA) as required by the PICRUSt Galaxy tool (Afgan et al., 2018) http://huttenhower.sph.harvard.edu/galaxy/. Briefly, data were normalized by copy number, and metagenomic function in the form of functional orthology determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology was predicted. The resulting table was collapsed to a KEGG pathway hierarchy level of three. Predicted functions were compared between groups using Statistical Analysis of Taxonomic and Functional Profiles (STAMP) version 2.1.3 (Parks et al., 2014).

Results

Alpha diversity. A total of 1,319,604 bacterial sequences representing 17,709 OTUs were recovered from all combined samples in the study, after an abundance cutoff of < 0.000151%. After standardization, 12,992 bacterial sequences remained in the analysis comprising of 13,006 OTUs. Sequence coverage was $\geq$ 82 % for all samples analyzed (based on Good's coverage, data not shown). Overall, the microbial community from cultured mussels exhibited the highest average diversity, with the microbial community from wild Shoal Creek mussels revealing individual variability (Figure 1). Predicted OTUs as calculated by Chao1 revealed a significant difference between the mussels from the two localities. AABC sediment exhibited the highest richness and Shoal Creek sediment showing specific species dominance, indicated by the highest Simpson's index (Figure 2). AABC water revealed higher species richness, with Shoal Creek water exhibiting more sample variability (Figure 3).

The digestive gland microbiome of AABC and in-stream Shoal Creek mussels contained nine bacterial phyla > 1% abundance (Figure 4). The most abundant bacterial phylum from AABC mussels was Tenericutes (43.3%), followed by, Proteobacteria (25.3%) and Firmicutes (12.6%) (Figure 4A). In-stream Shoal Creek mussels, Tenericutes (46.9%) was the most abundant phylum, followed by, Chlamydiae (25.6%), and Proteobacteria (10.6%) (Figure 4D).

The microbial community from the sediments was diverse with 12 phyla represented from the AABC sediment and 13 phyla represented from Shoal Creek sediment > 1% abundance (Figures 4B & E). Overall, the compositions of predominant phyla were distinctly different between water groups. Water microbial communities were less diverse than sediments with 7 phyla from AABC water and 8 phyla from Shoal Creek with abundances > 1% (Figures 4 C & F).

At the genus level, there was individual variability between mussels (Figure 5). For genera > 1% in abundance in cultured mussels, the top five were *Ureaplasma* (43.2%), followed by, *Pseudogulbenkian* (15.6%), *Geobacillus* (12.4%), *Spiroplasma* (11.3%), and *Synechococcus* (9.4%). In wild Shoal Creek mussels, *Spiroplasma* (30.3%), *Criblamydia* (29.6%), *Ureaplasma* (23.4%), *Owenweeksia* (4.2%), and *Geobacillus* (2.8%) were the top five genera in the digestive gland microbiome. One wild Shoal Creek mussel had a high abundance of *Spiroplasma*, which attributed to its overall high abundance. The genera *Pseudogulbenkia* (Proteobacteria) and *Synechococcus* (Cyanobacteria) that were abundant in cultured mussels were in fact only < 1% in wild Shoal Creek mussels.

Core microbiomes. Venn diagrams were generated to compare the number of distinct and shared OTUs between localities (data not shown). Microbial communities from mussels, water, and sediments shared 395 OTUs, representing 23.1% of the total OTUs. The in-stream Shoal Creek system shared 264 OTUs, representing 25.4% of the total OTUs. Sediment from AABC had the highest number of distinct OTUs (5, 935) than in-stream Shoal Creek (4,197).

Beta-diversity. PCo analysis and PERMANOVA results showed that the OTU composition between cultured mussels and in-stream mussels were statistically significant, with AABC mussels clustering together (data not shown), suggesting similar composition of dominant OTUs. The plot displays some overlap in microbial community distribution between AABC Shoal Creek and in-stream Shoal Creek mussels, but the p-value shows statistical significance (Table 1). When comparing OTU distribution between surrounding habitat from AABC and in-stream, there was a statistically significant difference, with hatchery water having some overlap with Shoal Creek water (Table 2). The PCo plot shows that sediments from all systems form a

tight cluster and PERMANOVA results indicate that microbial communities from all the groups are not statistically significant (Figure 5).

SIMPER analysis based on OTUs revealed large differences in digestive gland microbiomes between cultured and wild *V. nebulosa* (data not shown). The microbial structure from wild Shoal Creek mussels had a relatively distributed abundance of OTU_6 (23.97%) and OTU_8 (17.54%) (both OTUs were classified as *Ureaplasma*). However, cultured Shoal Creek mussels also had a higher abundance of OTU_9 (16.30%) than wild Shoal Creek mussels (1.21%), which was classified as *Pseudogulbenkiania* (class betaproteobacteria).

Predicted functions using PICRUSt. An error bar chart showed that 55 functional categories based on KEGG were significantly different between cultured and wild Shoal Creek *V. nebulosa* (Figure 7). Interestingly, there was 39 functional categories more enriched in cultured Shoal Creek mussels. When comparing the two populations of cultured *V. nebulosa*, Flannigan Creek had a higher proportion of sequences with functional genes involved in Biosynthesis of 12-,14-, and 16-membrane macrolides than Shoal Creek mussels (Welch's t-test, $p = 0.026$) (Figure 8).

Discussion

The gut microbiome of bivalves and other filter-feeding organisms has generally been attributed to be the ingestion of bacteria (Harris, 1993). Many host-bacteria relationships are specific, where animals may have the ability to selectively acquire for beneficial microbes. While the gut microbiome provides many known physiological benefits to humans, mammals, and fish (Bäckhed et al., 2012;Desselberger, 2018;Tarnecki et al., 2017), it remains unclear what functions microbes provide to freshwater mussels. However, recent studies have expanded beyond characterizing the gut microbiomes of aquatic invertebrates and are now revealing the

potential functions of those 16S rRNA gene profiles (Gao et al., 2019;Zeng et al., 2017;Hou et al., 2017) using computational approaches (Langille et al., 2013). This study aimed at expanding upon the characterization of the gut microbiome of *V. nebulosa* by including 1) two cultured and one wild population 2) functional profiling of the 16S rRNA genes from their digestive gland and 3) evaluating the influence of microbial communities from water and sediment to the digestive gland microbiomes of *V. nebulosa*.

Since freshwater mussels have a close association with their environment, it was expected that their digestive gland microbiomes reflect the microbial communities found in their surrounding habitat. Water and soil are reported to have a high abundance of proteobacteria (Pascault et al., 2014;Lauber et al., 2009). Proteobacteria is a major phylum of gram-negative bacteria that are physiologically, morphologically and ecologically diverse and are notorious for being the microbial signature of dysbiosis (Shin et al., 2015). Many well-known opportunistic pathogens that belong to Proteobacteria (namely, *Aeromonas* spp. and *Pseudomonas* spp.) have been isolated from freshwater mussels (Grizzle and Brunner, 2009;Carella et al., 2016;Starliper et al., 2008), establishing our hypothesis that mussels are concentrating bacteria via filter feeding. However, the presence of these genera do not necessarily represent most of the microbial community in freshwater mussels. Some studies have already established that Proteobacteria were also among the most predominant phyla in fish gut, gills, and skin (Larsen et al., 2013;Wu et al., 2012), shrimp intestines (Fan et al., 2019;Zeng et al., 2017;Hou et al., 2017), and the gut and gills of Pacific oysters *Crassostrea gigas* (Wegner et al., 2013). Many studies have also attempted to investigate the influence of the surrounding environment to the gut microbiota of fish (Sullam et al., 2012;Dehler et al., 2017;Roeselers et al., 2011), but Larsen et al. (2013) found fish gut microbiota to be species-specific. Burgos et al. (2018) revealed that at

the OTU level, there was no correlation between each ontogenic stage of Channel Catfish to their surrounding water and feed; however, water may have had some influence on the animals' bacterial communities before the first feeding.

In the present study, Tenericutes was the most abundant phylum found in cultured and wild Shoal Creek *V. nebulosa*. This finding is consistent with the previous study on characterizing the gut microbiome of wild Terrapin Creek and cultured *V. nebulosa* (Aceves et al., 2018) and wild *Pleurobema cordatum*, a large river inhabitant from the Tennessee River (refer to chapter 3 of this thesis). We found a low abundance of Tenericutes in AABC sediment (0.6%), AABC water (0.2%), Shoal Creek sediment (0.3%), and Shoal Creek water (0.2%). We expected to find that transient bacteria from the surrounding habitat were concentrating in mussel tissues, however, the microbial communities between groups was statistically significant from each other at the alpha and beta diversity levels. Zhang et al. (2016) reported a similar finding to ours in the Chinese mitten crab, where Tenericutes dominated their gut microbiota, but were in low abundances in their surrounding water and gills. OTUs ascribed to the genus *Mycoplasma* (Tenericutes) have also been consistently associated in the gut of oysters, marine mussels, and clams (King et al., 2012;Cleary et al., 2015;Lokmer et al., 2016b;Milan et al., 2018). Overall, the microbial communities from sediments from the different localities were overlapping and not statistically significant from each other; however, the water microbial community was significantly different between AABC and Shoal Creek.

There was a high abundance of Actinobacteria in AABC water (23%) and Shoal Creek water (31%). Fan et al. (2019) had reported a high abundance of Actinobacteria in shrimp intestine than in their surrounding sediment, suggesting their ability to enrich for Actinobacteria in their gut. Actinobacteria is a phylum of gram-positive bacteria with high G+C content and

found in the terrestrial or aquatic environments, where they play an important role in the decomposition and recycling of organic matter. They are also major producers of clinically important antibiotics, especially members of the genus *Streptomyces*, which are known for producing more than 50% of the total known microbial antibiotics (Shiylata and Satyanarayana, 2015). At genus level, we observed 13 genera >1.0% from all *V. nebulosa* samples. The genus *Synechoccus* (phylum Cyanobacteria) was more enriched in cultured mussels and AABC water than wild Shoal Creek groups. This genus is generally widespread in ocean regions (Honda et al., 1999) and its abundance in cultured mussels may be attributed to the Shellfish Diet (Reed Mariculture) feed during propagation. Interestingly, we detected a high abundance of the genus *Criblamydia* (phylum Chlamydiae) from one wild Shoal Creek *V. nebulosa*. The Chlamydiae phylum consists of members who are small, gram-negative obligate intracellular bacteria, ranging from pathogens of humans and animals to symbionts of ubiquitous protozoa. Chlamydia also have a unique physiological status, including, their biphasic lifestyle and reproduction requirement of replicating within a eukaryotic host.

To determine the predictive functional profiles of the gut microbiome of *V. nebulosa*, we used PICRUSt (Langille et al., 2013). The functional categories transporters and ABC transporters were more enriched in wild Shoal Creek mussels. In a later study comparing the gut microbiome of captive and wild *Pleurobema cordatum* (Ohio Pigtoe), ABC transporters were more enriched in captive *P. cordatum* (A.K. Aceves, unpublished data). ATP transporters function by transporting substrates across the cell membrane (Davidson et al., 2008;Horikoshi et al., 2010). When comparing the predictive functions of the two cultured populations of *V. nebulosa*, only one was significantly different: Biosynthesis of 12-, 14-, and 16-membered macrolides. Macrolides are a group of polyketides that have a lactone ring where sugars attach

and they can be used as antibiotics (Park et al., 2010), inhibiting bacterial protein synthesis by binding to the 50S subunit of the ribosome.

In conclusion, the microbiomes of *V. nebulosa* were unique and microbial communities from water and sediments were diverse. Individuals from the wild Shoal Creek population exhibited variability in the alpha diversity analysis and had a higher Simpson's index, showing dominance of a few OTUs. The significant differences between cultured and wild *V. nebulosa* could aid in future propagation efforts in understanding if their microbiomes can be influenced by their surrounding habitat if mussels are to become dysbiotic or relocated. This study further suggests that the host harbors a more specific and unique gut microbiome than its environment. Although we are still uncertain of the role that Mollicutes play in mussel health, as there are only a few examples on this class being symbionts in aquatic invertebrates (Zhang et al., 2016;Zhang et al., 2014;Chen et al., 2015;Neulinger et al., 2009;Kostanjsek et al., 2007), herein, we provided the potential functions of the 16S rRNA genes found in the gut microbiome of *V. nebulosa*.

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Table 3-1. Permutational multivariate analysis of variance between *Villosa nebulosa* groups (p-value < 0.05 shows significance).

Groups	t	p-value
Cultured Shoal Creek, Cultured Flannigan Creek	1.44	0.08
Cultured Shoal Creek, Wild Shoal Creek	1.51	0.02
Cultured Flannigan Creek, Wild Shoal Creek	1.37	0.07

Table 3-2. Permutational multivariate analysis of variance between water and sediment groups (p-value < 0.05 shows significance).

Groups	t	p-value
AABC sediment, AABC water	4.98	0.11
AABC sediment, Shoal Creek sediment	3.43	0.10
AABC water, Shoal Creek water	2.04	0.04
Shoal Creek water, Shoal Creek sediment	2.22	0.02

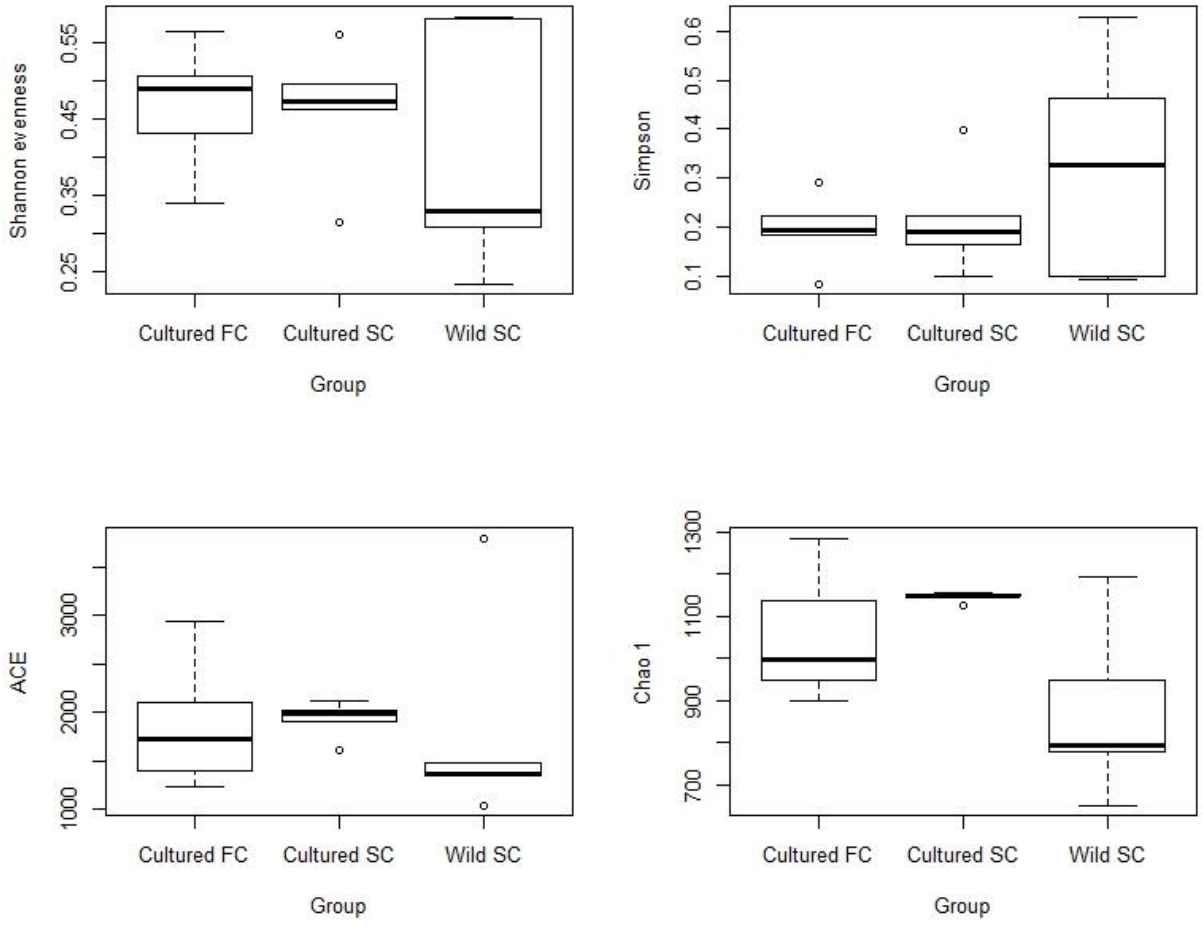

Figure 3-1. Diversity indices as boxplots of *Villosa nebulosa*.

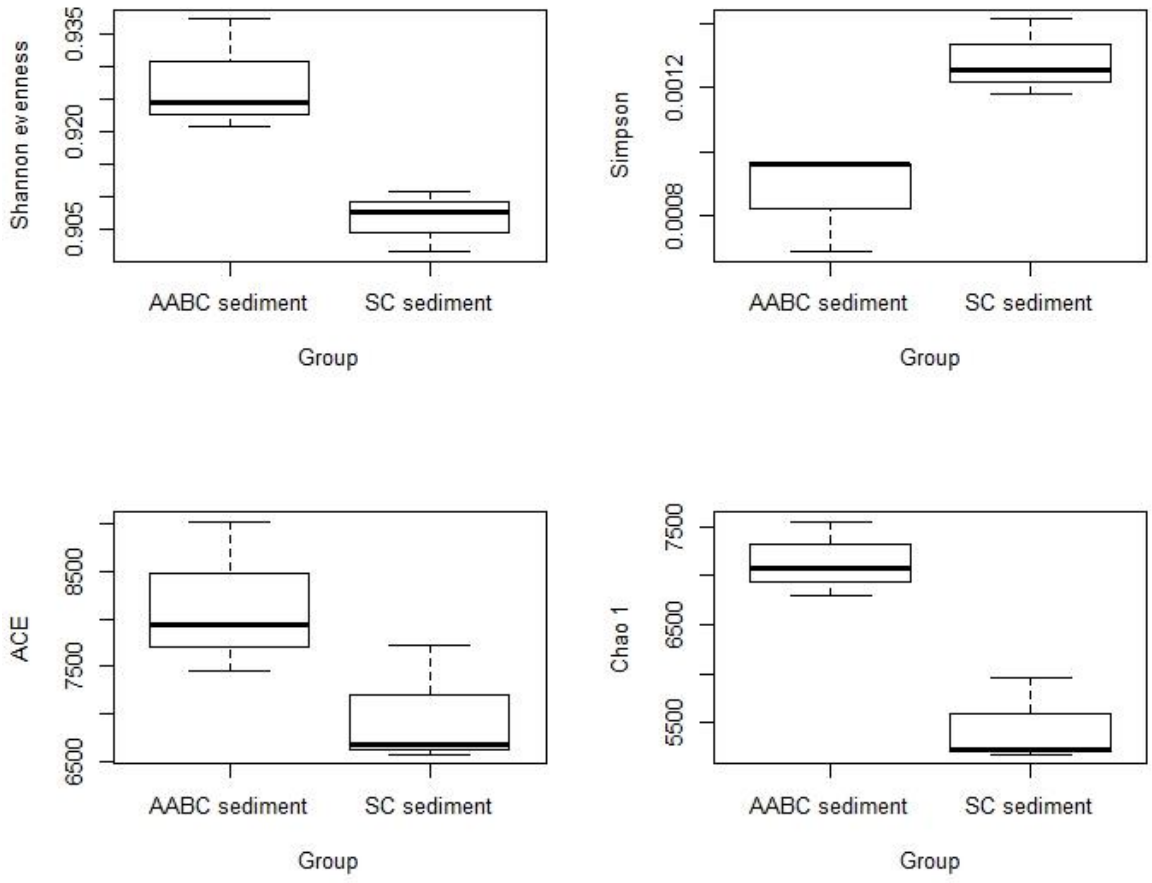

Figure 3-2. Diversity indices as boxplots of sediments.

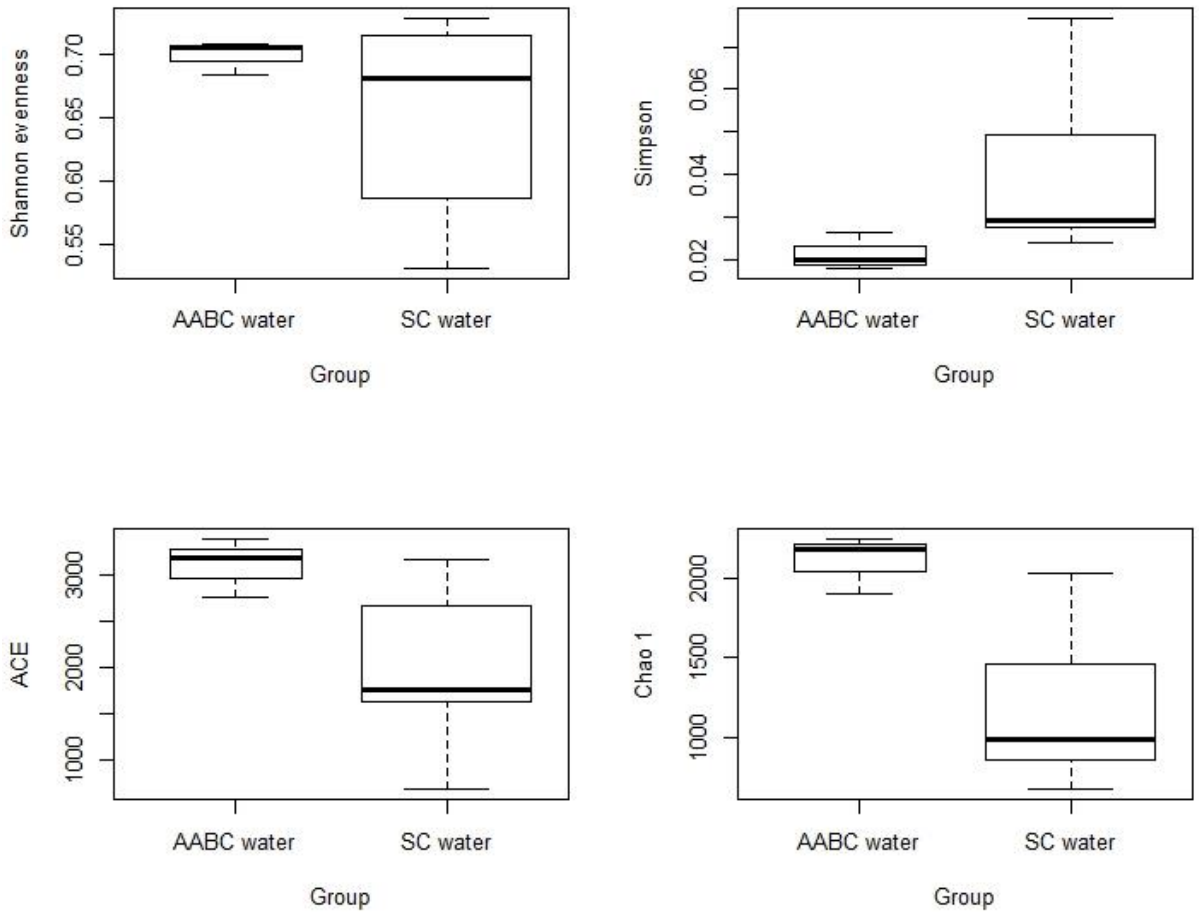

Figure 3-3. Diversity indices as boxplots of water.

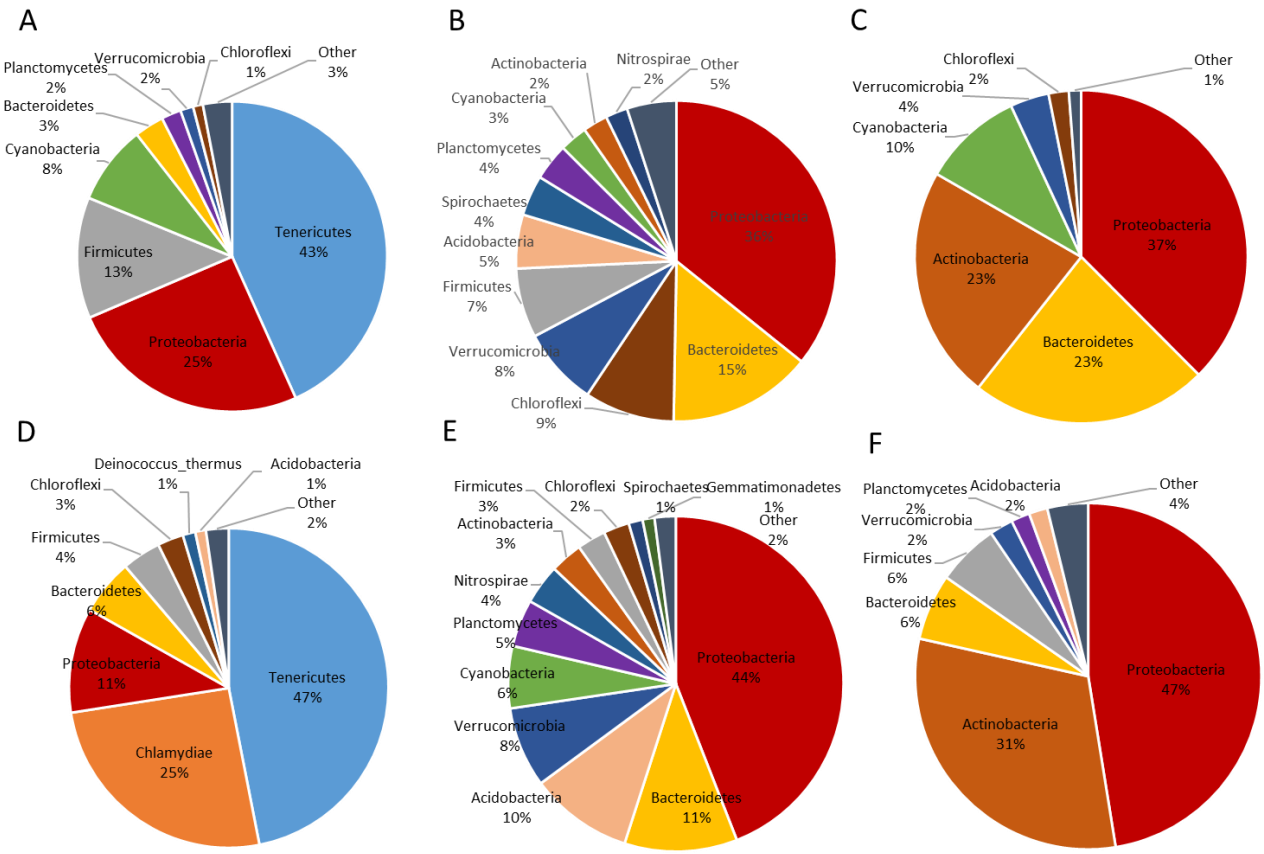

Figure 3-4. Bacterial phyla composition of (A) AABC *V. nebulosa* (B) AABC sediment (C) AABC water (D) Shoal Creek *V. nebulosa* (E) Shoal Creek sediment and (F) Shoal Creek water.

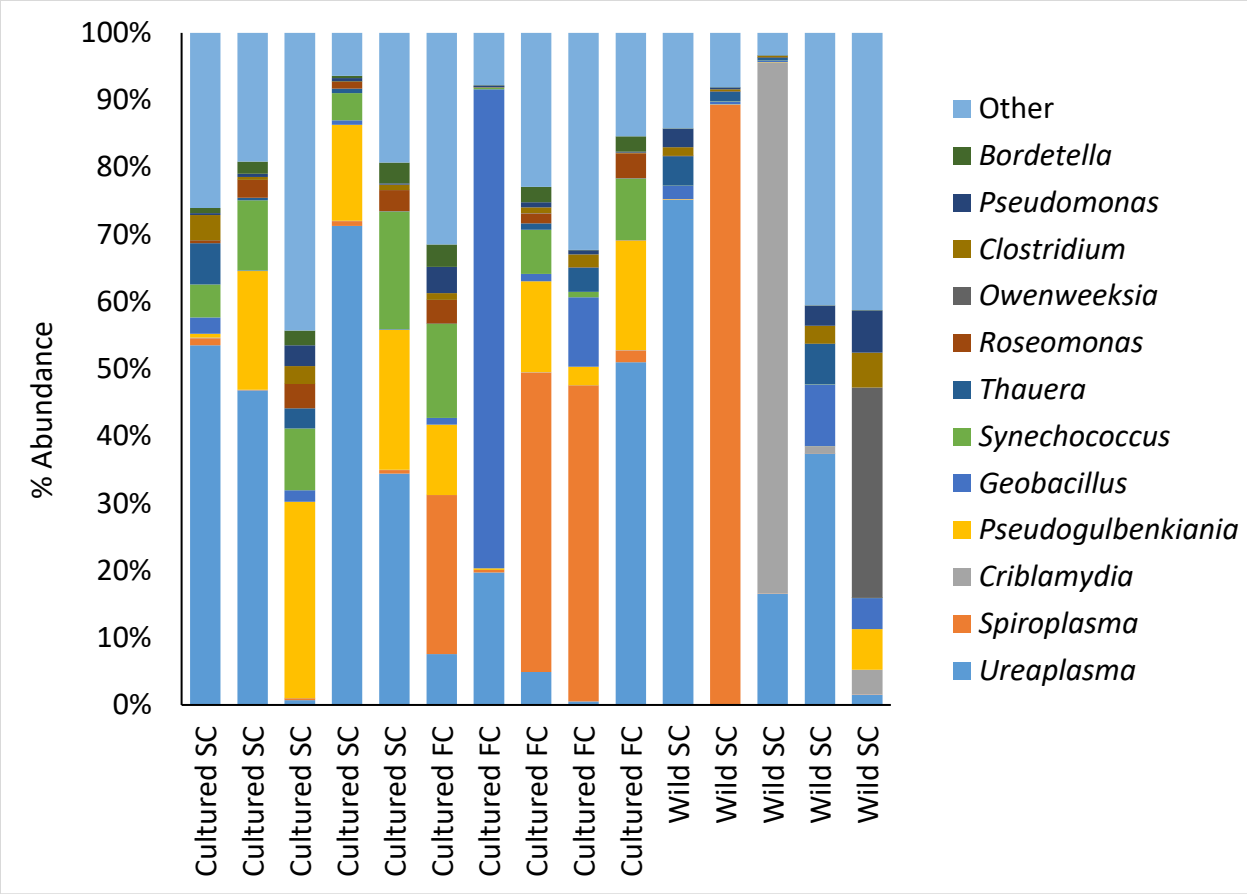

Figure 3-5. 100% abundance bar plot of bacterial genera in individual *Villosa nebulosa*.

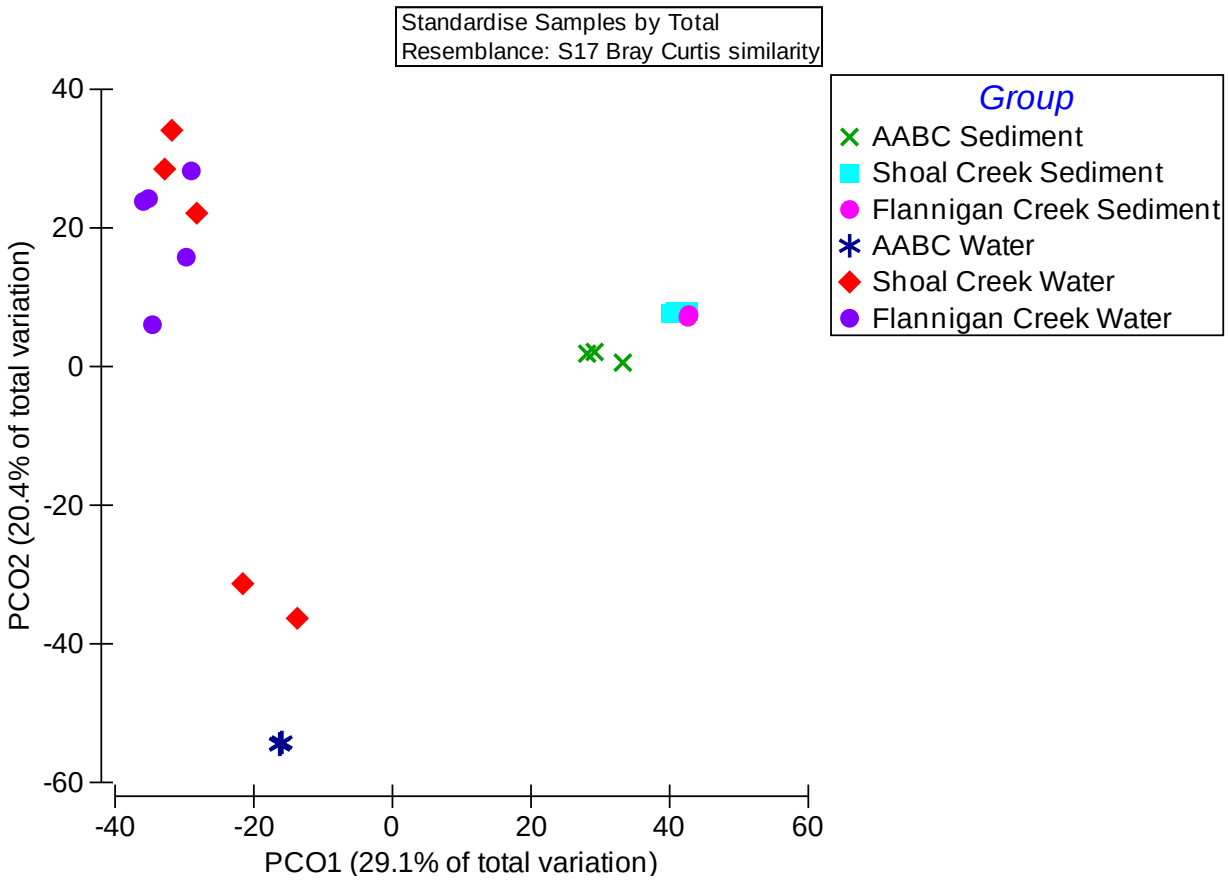

Figure 3-6. PCo A of microbial communities from water and sediment.

Figure 3-7. Extended error bar with the proportion of functional categories at KEGG level 3 between wild and AABC Shoal Creek *Villosa nebulosa*. All sequence reads were used to predict functions against the KEGG database (http://www.genome.jp/kegg/), which is implemented in PICRUSt (http://picrust.github.io/picrust/) bioinformatics software package. Statistical functional categories between groups were analyzed by STAMP.

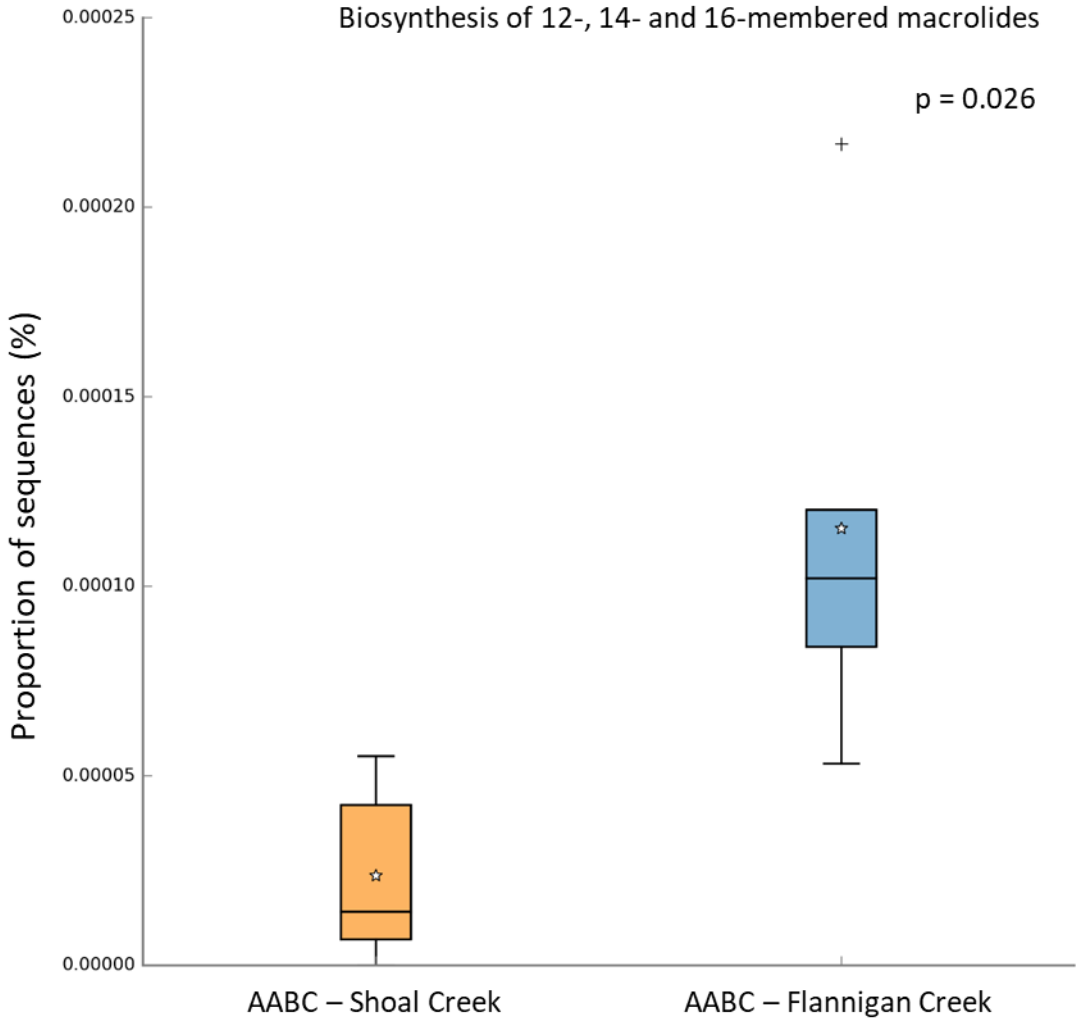

Figure 3-8. Boxplot of the significant difference in proportion of sequences of Biosynthesis of 12-, 14- and 16- membered macrolides between the two cultured populations of *Villosa nebulosa*.

Chapter 4. Characterization of the digestive gland microbiome of the Ohio Pigtoe, *Pleurobema cordatum* (Rafinesque, 1820): evidence on artificial mesocosms inducing dysbiosis

Abstract

In this study, we characterized the microbial community (microbiome) associated with the digestive gland ("gut") of *Pleurobema cordatum* (Rafinesque, 1820) (Ohio Pigtoe) using 16S rRNA gene sequencing. Two populations were compared: Ohio Pigtoe (n = 5) collected from the Tennessee River and Ohio Pigtoe (n = 9) that had been relocated to artificial mesocosms and exposed to 10°C, 20°C, and 30°C thermal regimes for two weeks. We also characterized the microbial communities of their surrounding habitat (water and sediment) in the wild and under laboratory conditions. The gut microbiome of *P. cordatum* analyzed after collection from the Tennessee River was dominated by members of the bacterial phylum Tenericutes (72%). By contrast, the gut microbiome of *P. cordatum* held in artificial mesocosms were dominated by the bacterial phylum Proteobacteria (64%). Interestingly, no difference was observed in the gut microbiome of laboratory-held *P. cordatum* regardless of temperature. The microbial communities of water and sediment from the Tennessee River were diverse and distinct from those found in the mussels. By contrast, the microbial communities of water and sediments in the mesocosms were dominated by Proteobacteria as were the microbial communities in laboratory-held mussels. These results collectively demonstrate that when mussels are moved into artificial rearing environments, their gut microbiome shifted to reflect that of their habitat, i.e., an increase

in Proteobacteria, and the animals lose their ability to maintain their unique microbiome. Moreover, the abundance of Tenericutes (which dominates the gut microbiome in wild *P. cordatum* as well as in other unionids) was reduced from 72% in wild animals to 3% in captive animals. We concluded that the laboratory-held *P. cordatum* became dysbiotic, which may explain the wasting syndrome and associated trickling mortalities that have been observed in captive holding and propagation of this freshwater mussel.

Introduction

The microbiome is a community of symbiotic microbes and their genes that are important for many host physiological processes (Tuddenham and Sears, 2015). In healthy individuals, the gut microbiome is composed of a diversity of microbes that are known to benefit host development and health (Kamada et al., 2013;Stecher and Hardt, 2011). The relationship between the host and its associated symbionts can be referred to as the holobiont, and the genetic makeup of the host and all the symbiotic microbes as the hologenome (Rosenberg et al., 2007). Together they contribute to the hologenome evolutionary concept in which genomic natural selection should consider the host and all of its symbiotic microbes as a single entity (Rosenberg et al., 2007).

It is well known that environmental disturbances are one of many factors that can elicit significant changes in the gut microbiome (Shade and Handelsman, 2012). When a host is exposed to unfavorable environmental conditions (e.g. captivity, toxins, temperature shifts), a shift in bacterial diversity and composition is observed leading to dysbiosis (Egan and Gardiner, 2016;Petersen and Round, 2014). Dysbiosis is characterized by a reduction in diversity, loss of beneficial microbes, and an increase in pathobionts (Petersen and Round, 2014). When the gut microbiome becomes disrupted, some bacterial endosymbionts can act as opportunistic pathogens potentially compromising host health. For example, some studies on marine species have shown temperature (Burge et al., 2014) and pH (Asplund et al., 2014) changes caused a shift from mutualistic to pathogen dominated bacterial communities (Lokmer and Mathias Wegner, 2015;Fan et al., 2013;Hernandez-Agreda et al., 2017). Our previous research has shown that gut microbiomes from cultured Alabama Rainbow (*Villosa nebulosa*) were different from those of wild *V. nebulosa*, suggesting rearing environment strongly modulates the microbiomes

of unionids (Aceves et al., 2018). Unfortunately, there are only a few studies describing the role of bacterial endosymbionts in freshwater mussels and those have relied on culture dependent methods (Carella et al., 2016;Grizzle and Brunner, 2009;Harris, 1993). In this study, we sought to explore how environmental and temperature changes affect the gut microbiome of another freshwater mussel species, the Ohio Pigtoe, *Pleurobema cordatum*.

Historically, the Tennessee River in northern Alabama was a diversity hotspot for unionids (Hughes and Parmalee, 1999), and in fact Muscle Shoals represented the most diverse faunal assemblage on the planet (Garner and McGregor, 2001;Williams et al., 2008) Williams, Bogan, and Garner 2008). Historic Tennessee River Pleurobemini included many species restricted to large rivers in the genera *Elliptio*, *Fusconaia*, *Hemistena*, *Plethobasus*, *Pleurobema* and *Pleuronaia*. The Ohio Pigtoe, *Pleurobema cordatum* was once the most abundant mussel in the Tennessee River (Scruggs, 1960). Its historical range included the upper Mississippi River and St. Lawrence River drainages, covering western New York to Michigan, Wisconsin, Iowa, and Kansas, and in the south, from Arkansas to Alabama. Currently in Alabama the Ohio Pigtoe remains in small numbers within Guntersville Dam and Wilson Dam tailwaters in the Tennessee River, and is considered a species of moderate conservation concern (Williams et al., 2008).

The decline of *P. cordatum* and other rare Pleurobemini historic to Muscle Shoals, represents a national conservation priority. Many Tennessee River endemics are now conservation priority species protected under the Endangered Species Act. Several conservation efforts with these species have been thwarted, because of the difficulty to successfully hold these animals long-term in a captivity (P. Johnson, unpublished data). To address these data gap ongoing research efforts are aimed at characterizing respiration rates and overall metabolic functions using *Pleurobema cordatum* as model for other large river Pleurobemini. The objective

of this study was to 1) describe the gut microbiome of wild and captive Ohio Pigtoe held in different temperatures in an artificial rearing environment and 2) evaluate how experimental conditions affect their digestive gland microbiome.

Materials and Methods

Sample collection. Five *P. cordatum*, water, and sediment samples were collected from the Tennessee River in Guntersville Dam tailwaters, Madison Co., AL in July 2017 (N 34.57661° W -86.56685°) at an approximate depth of 4.5 meters in 29° C water ('wild samples'). In October 2017, additional *P. cordatum* (n = 14) were collected from the identical locality and relocated to artificial mesocosms at the University of Alabama (Tuscaloosa, Alabama). *Pleurobema cordatum* were acclimated at 20° C for one week prior to a two-week exposure period at three different thermal regimes (10°C, 20°C, and 30°C). *Pleurobema cordatum* were kept in river sediments and fed algae cultured from Black Warrior River stock once daily and maintained on a full dark schedule. After the fulfillment of respiration, filtration, and excretion experiments, *P. cordatum*, water, and sediment samples were collected from each experimental tank and transported in coolers at ~10°C to Auburn University (Auburn, Alabama) for microbial community analysis.

DNA extraction, PCR, and sequencing. Approximately 25 mg of digestive gland tissue was aseptically removed from each *P. cordatum* (wild, n=5, 10°C, n=3, 20°C, n=3, and 30°C, n= 3). The DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), following the Gram-positive bacterial DNA protocol, was used to extract total DNA from gut samples. Water samples (100 ml) were centrifuged at 2,000 *g* for 5 min at 4°C, supernatant was discarded, and the pellet was filtered through an autoclaved funnel onto a 0.22 µm polycarbonate membrane. Using sterilized forceps the filter was placed into a 5 ml PowerWater Bead tube and stored at -80°C until DNA

extraction was performed using the MoBio PowerWater DNA isolation kit (Qiagen, Valencia, CA). Sediment samples were stored at -80˚C until DNA extraction was performed using approximately 25 mg of sediment with the MoBio PowerSoil DNA isolation kit (Qiagen). The concentration of extracted DNA was quantified using spectrophotometry (Nanodrop 2000, Thermo Scientific, Rochester, USA) and their purity assessed using the absorbance ratios at 260 nm and 280 nm (OD 260/OD 280).

Samples were deemed free of PCR inhibitors by amplifying a conserved region of the 16S rDNA using universal primers 63V and 1387R (Larsen et al., 2015). PCR conditions were as follows: initial denaturation at 95°C for 5 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and final elongation step at 72°C for 10 min. Quantities were adjusted to 20 ng/µL before sequencing. A total of 40 samples were submitted to MR DNA (Shallowater, TX) for PCR amplification and Next Generation sequencing using the Illumina MiSeq platform. The primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') was used, targeting the V4 variable region of the 16S rRNA gene. All samples were sequenced as pair end reads (2 x 300) following the manufacturer's protocol. Resulting sequences were processed using MR DNA's proprietary pipeline. Briefly, sequencing data were joined, barcodes and primers were removed, followed by the removal of sequences <150 bp and ambiguous base calls. After denoising, chimeras were removed, and operational taxonomic units (OTUs) were generated as proxies for bacteria species, defined by the current prokaryotic species concept (≥ 97% 16S rRNA sequence similarity (Rossello-Mora and Amann, 2001)). Taxonomic classifications were obtained using BLASTn against a curated database derived from Greengenes (DeSantis et al. 2006), Ribosomal

Database Project II (Cole et al. 2014), and National Center for Biotechnology Information (Geer et al. 2009).

Data analysis. After standardization to the lowest number of reads, rarefaction curves, diversity indices (Good's coverage, abundance-based coverage estimation (ACE), Chao1, and Shannon evenness), observed OTUs, and shared OTUs (Venn diagrams) were generated using Mothur v.1.35.1 software (Schloss et al., 2009). A JCLASS tree was also generated in Mothur and viewed in Mega5 (Tamura et al. 2011). A one-way ANOVA was performed on all diversity indexes, followed by a Tukey's post hoc test ($P < 0.05$). PRIMER v6 (Clarke and Warwick, 2001) was used to generate non-metric multidimensional (MDS) plots using the Bray-Curtis similarity index to visualize the differences between samples and analysis of similarity (ANOSIM) to test whether there were significant differences between different groups and OTU relative abundance. Additionally, similarity percentages (SIMPER) analysis was used to define the taxon contributing to the maximum dissimilarity observed between samples. Cut-off for low contributions was set at the default of 90.

To determine predictive metagenomic functions of gut microbiome between wild and captive P. cordatum, we used PICRUSt (Langille et al., 2013). OTUs were classified (MR DNA) with 97% identity to sequences in the Greengenes database (version 13.5) as required by the PICRUSt Galaxy tool (Afgan et al., 2018), http://huttenhower.sph.harvard.edu/galaxy). A biom file was created and imported into Galaxy where the data were normalized by copy number. Metagenomic function in the form of functional orthologs determined by KEGG Orthology was predicted. The resulting table was collapsed to a KEGG pathway hierarchy level of 3. Predicted functions were compared between groups using Statistical Analysis of Taxonomic and Functional Profiles (STAMP) version 2.1.3 (Parks et al., 2014).

Results

Alpha diversity. A total of 3,631,151 sequences corresponding to 15,046 OTUs were obtained. After normalization to the lowest sequencing read (n = 19,539) and an additional abundance cut-off set at < 0.00011%, 13,447 OTUs remained in the study. Rarefaction curves were generated for each sample to graphically display species richness covered in the analysis showing a high coverage of the bacterial diversity (data not shown), which was confirmed with a Good's coverage estimation of $\geq$ 91% in all samples. Gut samples exhibited a significantly lower diversity and evenness than water and sediment samples (Figure 1). Expected richness estimates (ACE and Chao 1) were significantly higher in sediments than in mussels and water samples. Similarly, in artificial mesocosms, richness estimates were significantly higher in sediment than those found in mussels and water samples (Figure 2). Interestingly, the Simpson diversity index was not significantly different between laboratory-held *P. cordatum* and their surrounding environment.

The bacterial community of the digestive gland of wild *P. cordatum* was overwhelmingly dominated by the phylum Tenericutes (72%) (Figure 3A). Mollicutes was the most abundant (72.2%) class from this phylum. There were an additional seven phyla (> 1.0 % abundance level) identified in the community, but all of them were in less than 8% abundance. By contrast, water and sediment samples from the Tennessee River displayed a more even community with Proteobacteria (32%) and Actinobacteria (32%), and Proteobacteria (32%) and Bacteroidetes (18%) being the dominant phyla in water and sediment samples, respectively (Figure 3B & C).

Samples (*P. cordatum*, water, and sediment) from the artificial mesocosms were grouped by temperature treatment (10°, 20°, and 30° C) and compared to each other. Since no significant differences were found in the microbial communities within temperature treatment groups (Table 1), samples were grouped collectively, e.g., gut, water, and sediment for alpha- and beta-diversity analysis. In contrast to wild *P. cordatum*, the most dominant phylum from laboratory-held mussels was

Proteobacteria (63.6%), followed by 6 bacterial phyla at an abundance > 1.0% (Figure 3D). In mesocosm water samples, Proteobacteria (93.7%) was the most dominant phylum, followed by Cyanobacteria (3.2%), Actinobacteria (2.9%), and Other (0.2%) (Figure 3E). Mesocosm sediment samples showed that Proteobacteria (50.1%) was the most dominant phylum, followed by 11 other phyla with an abundance > 1.0% (Figure 3E and 3F).

Core microbiomes. Venn diagrams were generated to compare the number of distinct and shared OTUs between groups. In the Tennessee River system, 426 OTUs were shared between the gut, water, and sediment, representing 6.2% of the total OTUs (Figure 4A). However, for the laboratory system, 337 OTUs were shared between the gut, water, and sediment, representing 3.4% of the total OTUs (Figure 4B). The number of distinct OTUs was higher for wild *P. cordatum* (1,174) than laboratory-held mussels (1,033). On the other hand, sediment samples from the laboratory environment had almost twice the number of distinct OTUs (9,424) than the Tennessee River (5,603). Water samples from the Tennessee River were also found to have a higher number of distinct OTUs (2,105) than in the mesocosms (906).

Beta diversity. Differences in microbial communities (beta diversity) of all gut, water, and sediment samples were visualized by MDS plot (Figure 5), and confirmed using ANOSIM (Tables 1 & 2). Microbial composition was distinct between groups (Table 2), but there was no difference among *P. cordatum* held at different temperatures (Table 1). Overall, MDS plot and ANOSIM results for the laboratory ecosystem showed overlapping and no significant differences in microbial composition between treatment groups (e.g. 10°C water vs. 20°C water, plot not shown) and within groups (e.g. laboratory-held mussels at 10° vs. 10°C water, plot not shown) but as groups, ANOSIM results indicates some overlap and in microbial composition between the Tennessee River water and mesocosm water (Table 2). A stress value below 0.2 indicates that an MDS plot is a good spatial representation of the difference in the data. Figure 6 summarizes the clustering analysis of all samples analyzed.

SIMPER analysis based on OTUs revealed large differences in digestive gland bacterial communities between wild and laboratory-held *P. cordatum* (Table 3). Between the wild mussels, the most abundant OTUs were OTU_2 (25.73%) and OTU_3 (22.29%) (classified as *Ureoplasma*) and among laboratory-held *P. cordatum*, OTU_8 (classified as *Phyllobacterium*) was the most abundant (9.62%). However, OTU_8 was observed to be the most abundant ascribed species found in only one mesocosm-held *P. cordatum* (86.3%), yet OTU_15 (classified as *Pseudomonas*) contributed to a lesser dissimilarity (6.99%) between wild and laboratory *P. cordatum* and was found in all mussels. Since the abundance of OTU_8 dominated the gut microbiome of one mussel, the ascribed species contributed to the highest dissimilarity between the wild and laboratory-held mussels.

Predicted functions using PICRUSt. An error bar chart showed that 162 functional categories based on KEGG (level 3) were significantly different between wild and captive *P. cordatum* (Figure 7). Pathways related to energy metabolism and carbohydrate metabolism were more enriched in wild *P. cordatum*, whereas, pathways such as ABC transporters and transcription factors were among the few that were more enriched in captive *P. cordatum*.

Discussion

Understanding the host-microbe relationship is important for conservation efforts since the lack of critical symbionts in captive animals may impair reproduction, ontogenesis, nutrition, and health (Rodriguez-Ruano et al., 2018;Burgos et al., 2018;Lavoie et al., 2018;Bahrndorff et al., 2016). Currently, our knowledge on which bacteria are components of the ‘normal’ gut microbiome of unionids is very limited (Aceves et al., 2018). Traditional culture-based approaches were used to isolated bacteria from apparently healthy mussels after the die-off events that occurred in the Middle Fork Holston and Clinch rivers, Virginia (Starliper et al.,

2008) and in the Pickwick Reservoir, Tennessee River, Alabama (Starliper et al., 2011). Most of the bacterial groups that Starliper et al. (2008) identified were sampled aseptically from whole tissue homogenates and from both rivers including motile *Aeromonas* spp., *Pseudomonas* spp., and *Serratia* spp., which are well-known opportunistic pathogens in some species of fish. These results were in agreement with previous studies that identified members of the class Gammaproteobacteria as the dominant bacterial group in the gut of aquatic organisms including fish (Al-Harbi and Uddin, 2003;Skrodenyte-Arbaciauskiene et al., 2006;Tanaka et al., 2003). Unfortunately, culture-dependent methods are biased towards those bacteria that can be isolated under laboratory conditions (less than 10%) resulting in a very distorted composition of the 'normal' bacterial community (Amann et al., 1995). The availability of culture-independent methods that cover the entire bacterial diversity present in a given sample has revolutionized our approach to understanding symbiotic relationships between bacteria and their host (Su et al., 2012;Tarnecki et al., 2017).

Herein, we characterized the microbial communities found in wild and laboratory-held Ohio pigtoes and sought to understand the influence of water and sediment on the core microbiome of these mussels. Similar to what we have previously reported in the freshwater mussel *Villosa nebulosa* (Aceves et al., 2018), the gut microbiome of wild *Pleurobema cordatum* was dominated by Tenericutes, specifically the bacterial class Mollicutes. The bacterial communities in the surrounding aquatic environments contained insignificant numbers of members of the phylum Tenericutes (0.4% in sediments and 0.2% in water), which highlights the selective mechanisms that the host uses to enrich their digestive gland for desirable bacteria. Zhang et al. (2016) reported a similar finding in the Chinese mitten crab, where Tenericutes dominated their gut microbiota, but were in low abundances in their surrounding water and gills.

Findings from Zhang et al. (2016) and our study suggest co-evolutionary mechanisms between specific groups of Tenericutes and their aquatic hosts (Hird, 2017). In our study, the bacterial community in water was dominated by Proteobacteria and Actinobacteria, which were found in less than 7% in the digestive gland of wild *P. cordatum*. Clearly, mussels are capable of selectively acquiring and enriching for Tenericutes in their digestive gland. We are uncertain of the role that these bacteria play in mussel health as there are only a few examples on this class being symbionts in aquatic invertebrates (Zhang et al., 2016;Zhang et al., 2014;Chen et al., 2015;Neulinger et al., 2009;Kostanjsek et al., 2007). To determine the predictive functional profiles of the gut microbiome of *P. cordatum*, the present study used PICRUSt (Langille et al., 2013). The most abundant functional pathways in wild *P. cordatum* were those associated to the functions of nutrition metabolism, such as carbohydrate and energy metabolism, which has also been reported in herbivorous fish (Liu et al., 2016;Yang et al., 2019). In captive *P. cordatum*, there was a high abundance of ABC transporters, known to play critical roles in bacteria in oligotrophic environments as these systems function to transport substrates across the cell membrane (Davidson et al., 2008;Horikoshi et al., 2010).

Noteworthy, the majority of the OTUs ascribed to the class Mollicutes were assigned to the genus *Ureaplasma*, a well-studied group that causes urinary tract infections in humans (Combaz-Söhnchen and Kuhn, 2017). *Ureaplasma* are characterized by lacking cell-wall and very small genomes with limited biosynthesis capabilities which explains their parasitic (both intra and extracellular) or saprophytic lifestyles. However, the 16S sequence similarity between the Mollicutes OTUs recovered from *P. cordatum* (and those previously recovered from *Villosa nebulosa*) and the *Ureaplasma* sequences found in GenBank and GreenGenes was lower than 85% (data not shown). This indicates the majority of OTUs recovered from the digestive gland

of unionids have not previously been reported and likely belong to a new genus, possibly a new family, within the class Mollicutes.

Pleurobema cordatum relocated to artificial mesocosms experienced a dramatic shift in their gut microbiome. Tenericutes abundance dropped from 72% to 3% while Proteobacteria increased from 7% to 64%. A surprising result from this study was the lack of temperature effect in the gut microbiome of mussels. During the 21 day exposure we hypothesized mussels held at warmer temperatures (30°C) during this season would experience dysbiosis. However, even at cooler temperatures, mussels became dysbiotic by losing their normal bacterial community and becoming dominated by Proteobacteria, including a significant increase in abundance of opportunistic pathogenic bacteria belonging to the genera *Pseudomonas* and *Corynebacterium.* Proteobacteria is recognized as the microbial signature of gut dysbiosis (Shin et al., 2015). The captive *P. cordatum* exhibit a gut microbial community significantly different to those found in their surrounding water and sediment. However, it is tempting to speculate that the large numbers of Proteobacteria (94%) found in mesocosm water overwhelmed the selective mechanism of the mussels to regulate their gut communities. On the other hand, the observed loss of homeostasis in their gut microbiome could be due to adverse physio-chemical conditions in the artificial systems that incapacitated the host to the point of being unable to maintain their normal microbiome. We lack sufficient data to conclude if the observed changes in the host microbiome were due to the composition of the bacterial community in water and sediments, that was drastically different from the communities found in their native environments, or if the stress of relocation affected the host to the point of losing their balanced gut community.

Under captive conditions, the gut microbiome of animals can change (Bahrndorff et al., 2016), with the host losing the beneficial microbes that it needs to survive. In two species of non-

human primates, Clayton et al. (2016) reported that when the primates were relocated to captivity, their microbiomes may have become dysbiotic, shifting to a human-associated bacterial community. Studies on other animals have reported similar findings that captive microbiomes differ from their wild counterparts and that there is a reduction in bacterial diversity in captive animals (Borbón-García et al., 2017;Kueneman et al., 2016;Loudon et al., 2014). In this study, wild *P. cordatum* and those relocated into artificial mesocosms were collected from the same locality but at different times. Therefore, we were unable to characterize the temporal shift of the captive Ohio Pigtoe. Unfortunately, unionids, in particular this species, are hard to find in large numbers in the wild and few individuals were available for evaluation. Although we recognized seasonal variations could occur in the gut microbiome of wild *P. cordatum*, we think it is unlikely (based on spatiotemporal studies conducted in other aquatic species, see (Larsen et al., 2013;Pierce et al., 2016;Zurel et al., 2011)), that seasonality resulted in such drastic changes in alpha-diversity at the phylum level. Compo et al. (2018) found that the most predominant phylum in the fecal microbiota of commercial mink was Firmicutes, even if comparing between years (2014 vs. 2015), life stage (adult females vs. weaned kits), and season (summer vs. winter).

Our results show that metabolic, dietary, and other physiological studies conducted in artificial mesocosms need to take into account that captive animals are likely to experience dysbiosis in unfavorable conditions and should perhaps aim at identifying the bias introduced in their results by using dysbiotic animals. Future studies are ongoing to further characterize the Mollicutes found in other unionid species and to explore the differences in microbial communities between wild and artificial rearing environments, including the influence of environmental factors on the gut microbiome.

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Table 4-1. One-way ANOSIM results of pairwise comparisons of OTUs between temperature treatment groups.

Pairwise tests	**R-value**	**P-value**
10°C *P. cordatum* vs. 20°C *P. cordatum*	-0.185	0.9
10°C *P. cordatum* vs. 30°C *P. cordatum*	0	0.5
20°C *P. cordatum* vs. 30°C *P. cordatum*	0.185	0.2
10°C *P. cordatum* vs. 10°C water	0.519	0.1
10°C *P. cordatum* vs. 10°C sediment	1	0.1
20°C *P. cordatum* vs. 20°C water	0.667	0.1
20°C *P. cordatum* vs. 20°C sediment	1	0.1
30°C *P. cordatum* vs. 30°C water	0.704	0.1
30°C *P. cordatum* vs. 30°C sediment	1	0.1
10°C water vs. 20°C water	-0.111	0.7
10°C water vs. 30°C water	-0.185	0.9
20°C water vs. 30°C water	-0.111	0.7
10°C sediment vs. 20°C sediment	0.111	0.4
10°C sediment vs. 30°C sediment	0.444	0.2
20°C sediment vs. 30°C sediment	0.259	0.2

Table 4-2. One-way ANOSIM results of pairwise comparisons of OTUs between groups.

Pairwise tests	**R-value**	**P-value**
Wild *P. cordatum* vs. Tennessee River sediment	1	0.018
Wild *P. cordatum* vs. Tennessee River water	0.964	0.008
Tennessee River sediment vs. Tennessee River water	1	0.018
Laboratory-held *P. cordatum* vs. Mesocosm water	0.566	0.0001
Laboratory-held *P. cordatum* vs. Mesocosm sediment	0.981	0.0001
Mesocosm water vs. Mesocosm sediment	0.555	0.0001
Wild *P. cordatum* vs. Laboratory-held *P. cordatum*	0.904	0.0005
Tennessee River sediment vs. Mesocosm sediment	1	0.005
Tennessee River water vs. Mesocosm water	0.559	0.001

Table 4-3. One-way SIMPER analysis of the top-ten genera contributing to the most dissimilarity between wild and laboratory-held *P. cordatum*. Each genus listed is in percent abundance

Genus	**Wild *P.cordatum***	**Laboratory-held *P. cordatum***	**Contribution to dissimilarity (%)**
Ureaplasma	59.36	0.54	30.76
Pseudomonas	1.11	14.25	6.89
Corynebacterium	0.15	10.33	5.37
Phyllobacterium	0.01	9.66	5.05
Mycoplasma	9.66	0.97	4.54
Delftia	0.42	7.15	3.56
Treponema	5.28	0.00	2.76
Staphylococcus	0.03	4.26	2.22
Rothia	0.12	4.14	2.20
Spiroplasma	3.22	1.51	2.01

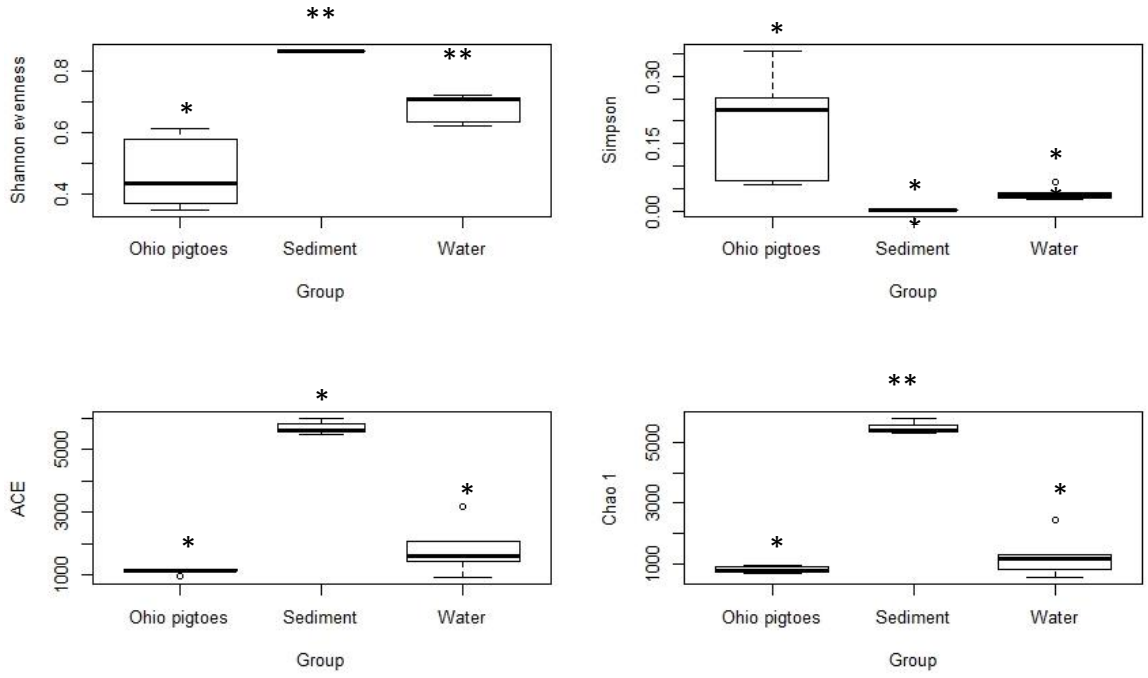

Figure 4-1. Diversity indices as boxplots of the Tennessee River system.

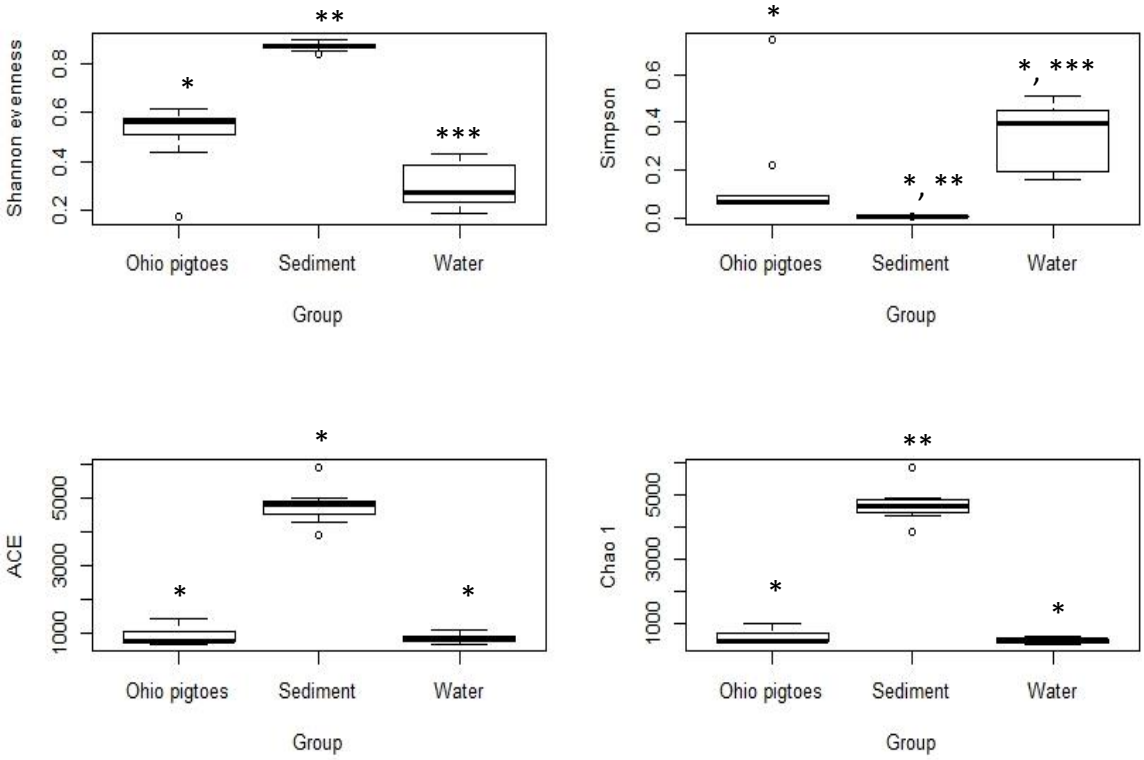

Figure 4-2. Diversity indices as boxplots of the mesocosm system.

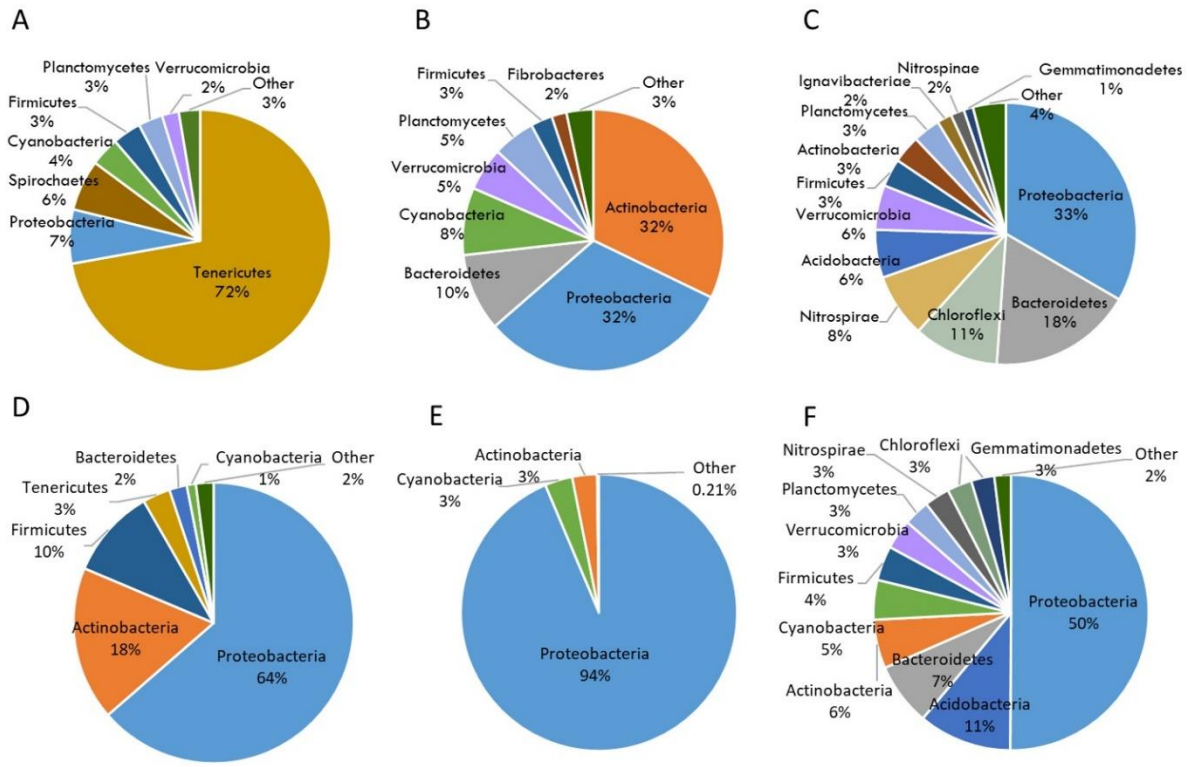

Figure 4-3. Phyla composition of the digestive gland microbiome in (A) Wild *Pleurobema cordatum* (B) Tennessee River water (C) Tennessee River sediment (D) Laboratory-held *Pleurobema cordatum* (E) Mesocosm water and (F) Mesocosm sediment. All phyla < 0.1% abundance are included as other.

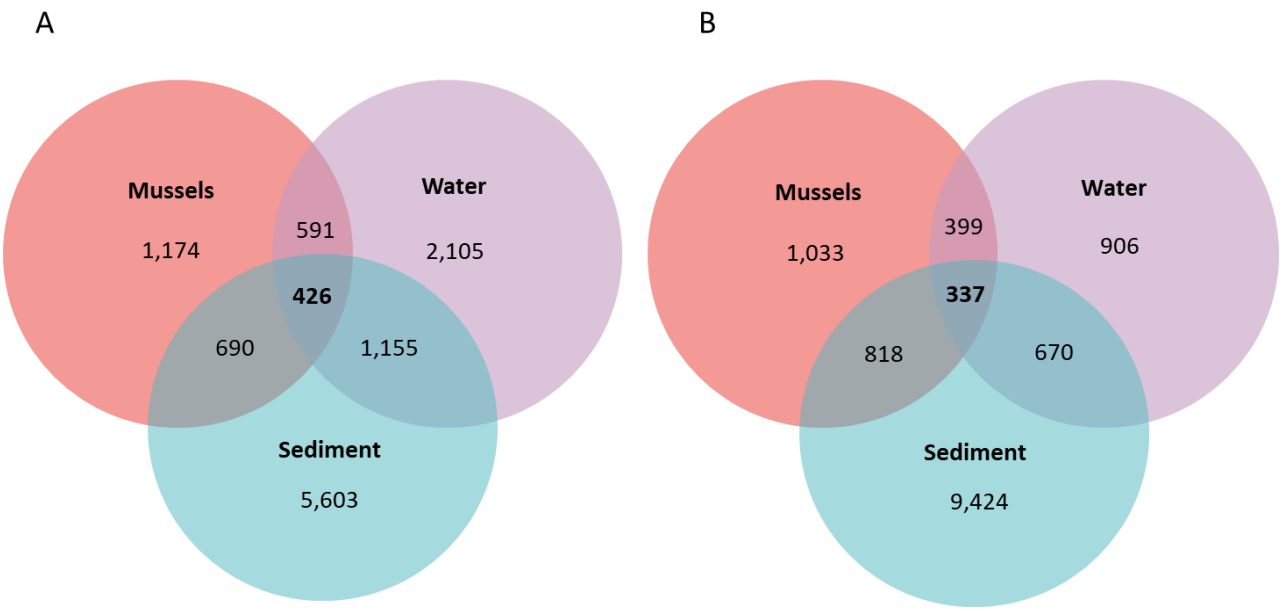

Figure 4-4. Venn diagrams showing unique and shared OTUs between mussels, water, and sediment from (A) Tennessee River and (B) Mesocosms.

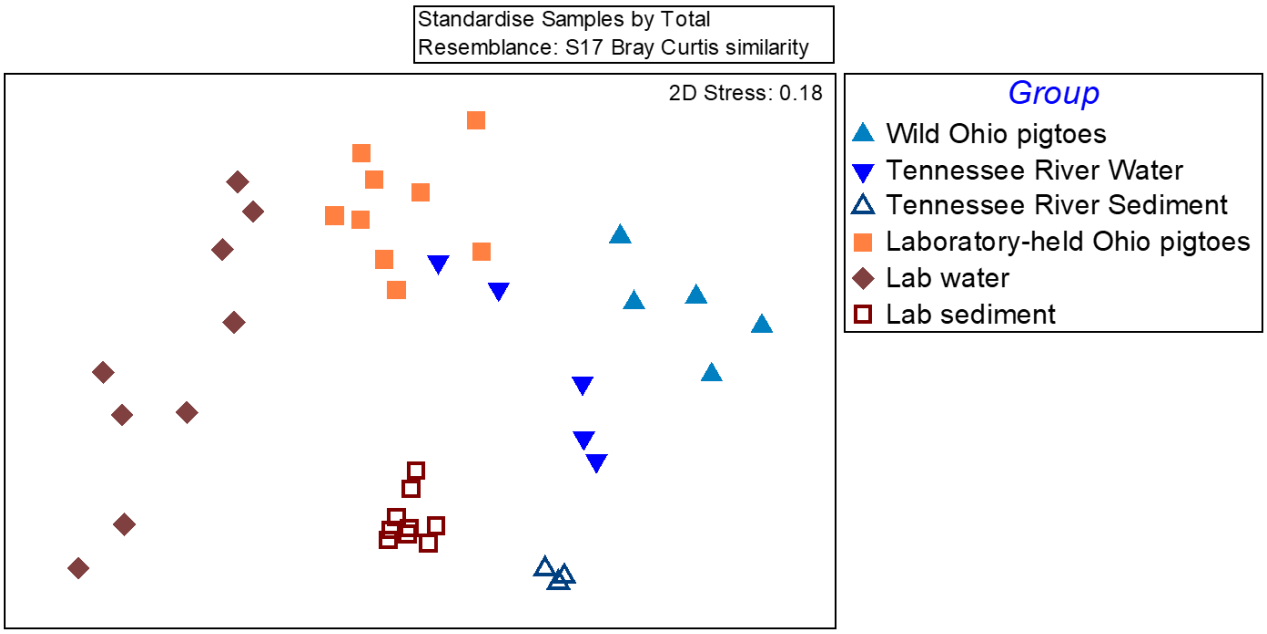

Figure 4-5. Multidimensional scaling plot of all mussel, water, and sediment samples by system, based on OTU abundances.

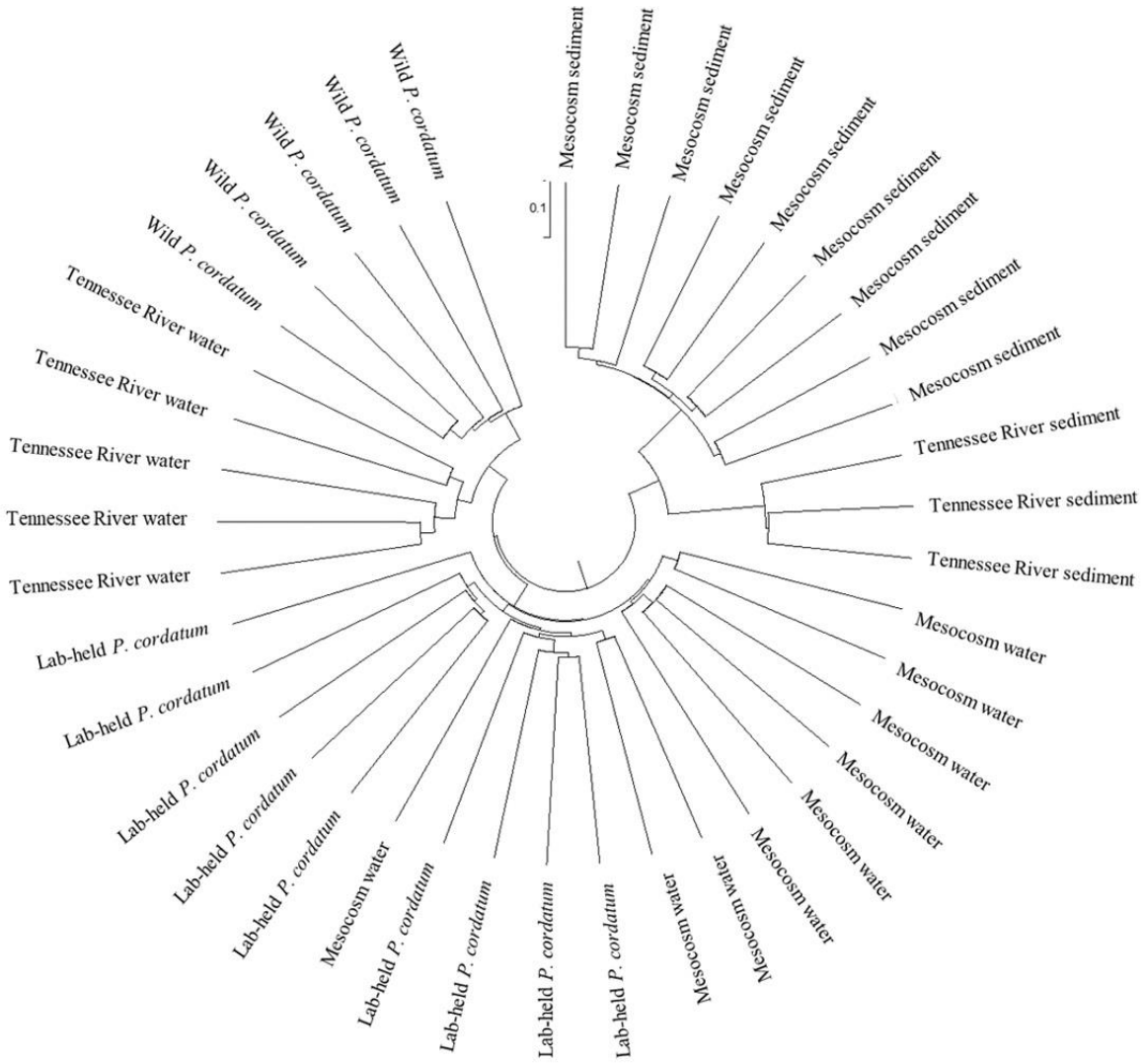

Figure 4-6. A dendrogram illustrating the hierarchical arrangement of the sequence samples showing all replicates per group. The scale bar on the dendrogram presents the percentage of dissimarity between two samples.

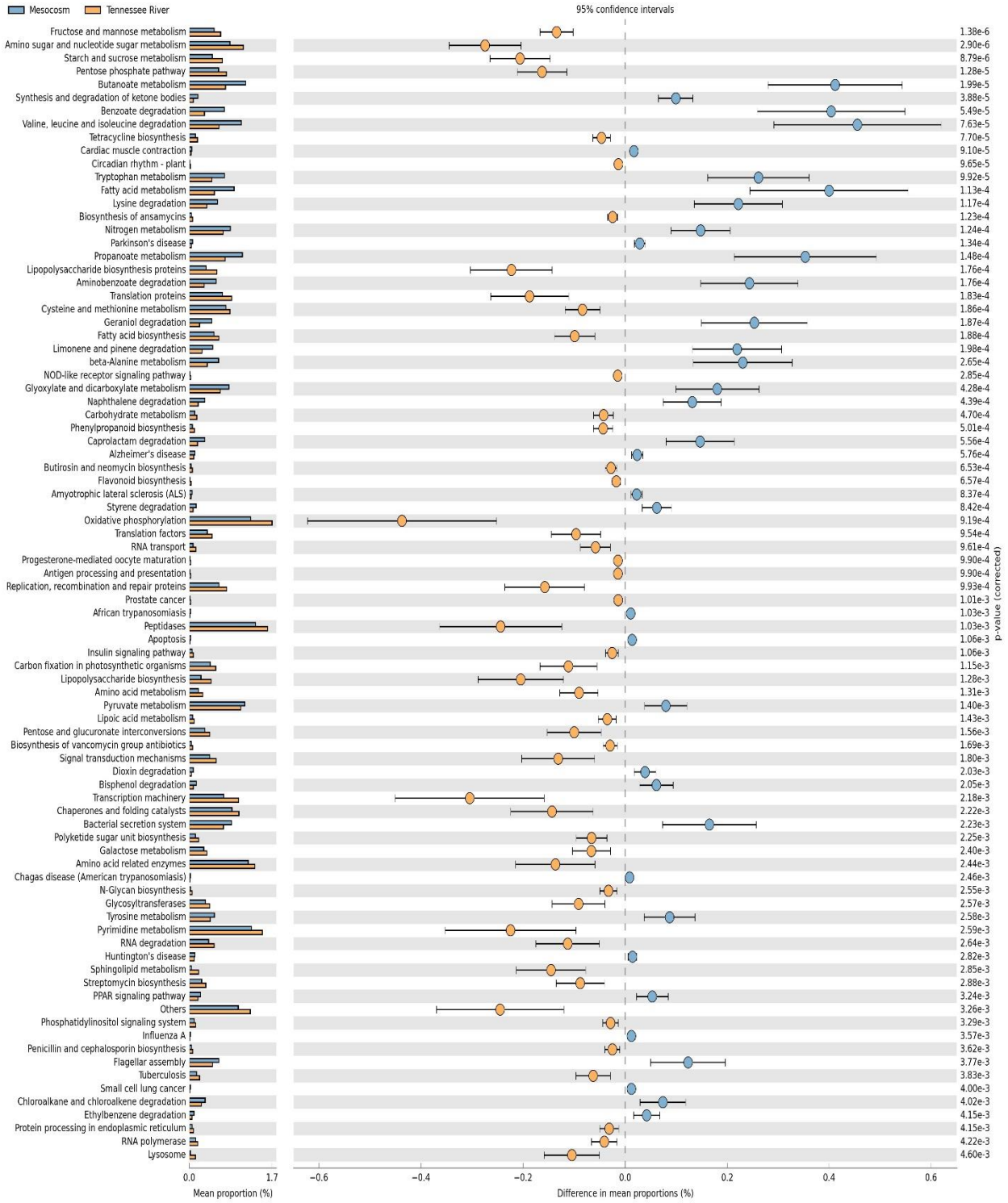

Figure 4-7. Extended error bar with the proportion of functional categories at KEGG level 3 between wild and laboratory-held *Pleurobema cordatum*. All sequence reads were used to predict functions against the KEGG database (http://www.genome.jp/kegg/), which is implemented in PICRUSt (http://picrust.github.io/picrust/) bioinformatics software package. Statistical functional categories between groups were analyzed by STAMP.

Chapter 5. The effects of antibiotics and bacterial challenge on the digestive gland microbiome of *Villosa nebulosa*

Abstract

The gut microbiome is a community of symbiotic microbes that is important for many host physiological processes. When the homeostatic equilibrium between the host and its' gut microbial symbionts is disrupted, the gut can become dysbiotic, characterized by an unbalanced gut microbiome, typically associated with an observed decrease in overall bacterial diversity and an increase in pathogens. The objective of this study was to determine if the use of antibiotics induced dysbiosis of the digestive gland ('gut') of freshwater mussels and as result they became more susceptible to bacterial infections. Our previous research found that the bacterial phylum Tenericutes was the most predominant phylum in the gut microbiome of *Villosa nebulosa* (Alabama Rainbow). We chose tetracycline as a selective antibiotic to eliminate (or reduce) the Mollicutes (Tenericutes) present in the digestive gland of *V. nebulosa*, and we used ampicillin as a broad-spectrum antibiotic that is not selective for Tenericutes. Mussels were treated with antibiotics for 10 consecutive days and had a withdrawal period of 5 days (no antibiotic treatment) before the first sampling point (t = 15 days). The withdrawal period extended for another four days. At that point, each treatment was divided into two sub-treatments (challenge and non-challenge). Mussels were challenged by immersion with a cocktail of two strains of *A. hydrophila*, a proven pathogen for fish, and sampled 24 hours post-challenge (t = 20 days). The gut microbiome was characterized using 16S rRNA gene sequencing. Our results showed that tetracycline-treated mussels had a significantly lower abundance of Tenericutes (3.6%), than

ampicillin-treated (37.5%) and control (33.9%) mussels. After challenge, with no mortalities, there was an observed increase of bacterial diversity in tetracycline-challenged mussels.

Introduction

Bivalves are important biological indicators of ecosystem health. In particular, freshwater mussels (Bivalvia: Unionidae) appear to be appropriate sentinel organisms to assess environmental perturbations because of their global distribution, sessile adulthood, and their ability to filter large volumes of water (Haag and Williams, 2014;Vaughn, 2018). As suspension feeders, unionids rely on bacteria as food source (Nichols and Garling, 2000) but at the same time establish symbiotic relationships with specific prokaryotic groups (Aceves et al., 2018).

In recent years, research into human health and disease has suggested that many chronic diseases, such as, diabetes, bowel, and obesity disorders, are driven by a disturbance (or shift) in the normal microbiome, known as dysbiosis (Sekirov et al., 2010;Selber-Hnatiw et al., 2017;Petersen and Round, 2014). One of the many roles of the microbiome is to provide protection against pathogens, a phenomenon known as colonization resistance (Stecher and Hardt, 2011), which can be severely compromised by antibiotic treatment. Disturbances due to antibiotics often eliminate or destroy portions of the microbial community, providing an opportunity for remaining community members or new colonists (i.e. pathogens) to establish (Wegner et al., 2013). The effect of antibiotics has served as a paradigm for disruptions in human – (Becattini et al., 2016), mice – (Buffie et al., 2012) and aquaculture system – (Zeng et al., 2017) associated microbial communities.

According to the U.S. Food and Drug Administration, the therapeutic use of antibiotics refers to the treatment of clinically sick animals, as well as, the use of antibiotics to prevent and control disease. Therefore, the appropriate concentration of the drug, for an adequate duration in a particular area must be achieved so that the targeted pathogen is eliminated (Willing et al., 2011). When members of the natural microbiome are exposed to antibiotics that affect their

growth without killing them, there is selection for resistance (Aminov, 2010). Antibiotics alter the composition of the gut microbiome and most studies are consistent in showing that various taxa recover to different extents, with the abundance of most taxa returning to prior levels within a few days or weeks (Dethlefsen et al., 2008).

Previous research on characterizing the gut microbiome of *Villosa nebulosa* has found that Mollicutes (phylum Tenericutes) is the most abundant bacterial class in freshwater mussels. Mollicutes are a class of wall-less bacteria with genera that are well-known intracellular pathogens of plants and animals. The susceptibility of several human origin *Mycoplasma* spp. to antibiotics that inhibit protein synthesis has long be recognized (Taylor-Robinson and Bebear, 1997), although some mycoplasmas are known to carry the tetracycline resistance gene tet (M) (Chopra and Roberts, 2001;He et al., 2016). Tetrayclines are a broad-spectrum antibiotic that act as a bacteriostatic agent, binding to the 30S ribosomal subunit and inhibiting protein synthesis by preventing the binding of aminoacyl-tRNA to the ribosomal acceptor (A) site (Brysker 2005). Mycoplasmas have a natural resistance to the drugs of β-lactams due to their lack of cell wall (Foschi et al., 2018). Ampicillin (class β-lactams) is a broad-spectrum antibiotic that acts as a bactericidal agent, inhibiting bacterial cell wall synthesis by inactivating transpeptidases on the inner surface of the bacterial cell wall membrane (Drawz and Bonommo, 2010).

To improve our understanding of the risks caused by antibiotics to non-targeted aquatic species, I evaluated the effects of two broad-spectrum antibiotics on the gut microbiome of *Villosa nebulosa*. In addition to inducing gut dysbiosis in *V. nebulosa,* I challenged mussels with two strains of a well-known opportunistic pathogen, *Aeromonas hydrophila*. *Aeromonas hydrophila* is ubiquitous in warm-water systems and is known to cause diseases in amphibians, reptiles, birds, and mammals (including humans) (Janda and Abbott, 2010). A virulent strain of

A. hydrophila (vAh) emerged in West Alabama in 2009 causing a devastating outbreak of motile *Aeromonas* septicemia (MAS) in U.S. farmed-raised catfish (Hossain et al., 2014). I hypothesized that mussels with dysbiotic gut microbiomes (i.e. tetracycline-treated) will be more susceptible to septicemia.

Materials and Methods

Sample collection. Juvenile *V. nebulosa* (offspring of Shoal Creek populations) were held in suspended upwelling systems deployed in an AABC rearing pond for ~12 months before collection (320 individuals) and transported in coolers, with aerators, to Auburn University (Auburn, Alabama). Water changes were performed during transport and upon arrival to the E.W. Shellfish Fisheries Center to reduce ammonia levels. *Villosa nebulosa* were fed daily with commercially available *Nancholoropsis* species and shellfish diet (Reed Mariculture), with a 20% water change and a photoperiod of 12:12 during the study.

Experimental design. The experimental design consisted of three treatments: control (no antibiotics), ampicillin, and tetracycline. Eight tanks were used per treatment (=24 tanks total), with 12 individual *V. nebulosa* per tank. Mussels were held in fish net breeder pins in 37.9-liter glass aquaria (water level ~35 liters), with continuous aerated, dechlorinated city water. Mussels were allowed to acclimate to their tank conditions for 4 days before antibiotic trials began. For 10 consecutive days, *V. nebulosa* were exposed to 100 µg/L of ampicillin and 50 µg/L of tetracycline, followed by a withdrawal period of 5 days (no antibiotic treatment) before the first sampling point (t = 15 days). Temperature and water quality checks were monitored twice weekly, with parameters maintained between 20.9 to 23.1°C, ammonia concentration 0.5 ppm, nitrate 0-20 ppm, nitrite 0-3 ppm, 75 ppm hardness, and 7.2-7.8 pH for all tanks over the course of the study period.

After the first sampling point, the withdrawal period extended for another four days before the bacterial challenge. The virulent strain of *A. hydrophila* (vAh), ML119, and an *A. hydrophila* isolate from a Dwarf Wedgemussel (*Alasmidonta heterodon*) were grown as follows. Briefly, from -80°C glycerol stocks, the isolates were passed onto tryptic soy agar plates and in tryptic soy broth with constant shaking and incubated at room temperature for ~18 hours. The purity and isolation of *A. hydrophila* was confirmed using API 20E test strips (bioMérieux). To increase the virulence of *A. hydrophila*, I passed ML119 through channel catfish by intraperitoneal injection. Briefly, healthy catfish fingerlings were inoculated with the virulence *A. hydrophila* isolate (~10^6 cfu/fish). After 24 h, the pathogen was reisolated on blood agar from ascitic fluid. After the 5 day withdrawal period, each treatment was divided into two sub-treatments: ampicillin-control, ampicillin-challenged, control-control, control-challenged, tetracycline-control, and tetracycline-challenged. *Villosa neulosa* were challenged by immersion with the two strains of *A. hydrophila* with the final cell density of ~ 1 x 10^9 CFUs/mL, in which 100 mL of each bacterial culture was added to a 1-gallon challenge bucket/tank. *Villosa nebulosa* were kept immersed in the aerated challenge suspension for 1 hour, placed back into their respective tanks and sampled 24 hours later (t = 20 days).

DNA extraction, PCR, and sequencing. Approximately 25 mg of digestive gland tissue was removed aseptically from each *Villosa nebulosa* (samples were not pooled together). The DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), following the Gram-positive bacterial DNA protocol, was used to extract total DNA from gut samples. The concentration of extracted DNA was quantified using spectrophotometry (Nanodrop 2000, Thermo Scientific, Rochester, USA) and their purity assessed using the absorbance ratios at 260 nm and 280 nm (OD 260/OD 280). Samples were deemed free of PCR inhibitors by amplifying a conserved region of the 16S

rDNA using universal primers 63V and 1387R (Larsen et al., 2015). Quantities were adjusted to 20 ng/µL before sequencing.

Samples were submitted to MR DNA (Shallowater, TX) for PCR amplification, DNA clean-up, and Next Generation sequencing using the Illumina MiSeq platform. The primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') was used, targeting the V4 variable region of the 16S rRNA gene. The HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used and PCR conditions were performed as follows: initial denaturation at 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, and final elongation step at 72°C for 5 min. After PCR, products were checked in a 2% agarose gel for successful amplification and relative band intensity. Multiple samples were pooled together based on their molecular weight and DNA concentrations and purified using calibrated Ampure XP beads. All samples were sequenced as pair end reads (2 x 300) following the manufacturer's protocol. Resulting sequences were processed using MR DNA's proprietary pipeline. Briefly, sequencing data were joined, barcodes and primers were removed, followed by the removal of sequences <150 bp and ambiguous base calls. After denoising, chimeras were removed, and operational taxonomic units (OTUs) were generated as proxies for bacteria species, defined by the current prokaryotic species concept (≥ 97% 16S rRNA sequence similarity (Rossello-Mora and Amann, 2001)). Taxonomic classifications were obtained using BLASTn against a curated database derived from Ribosomal Database Project II (Cole et al. 2014), and National Center for Biotechnology Information (Geer et al. 2009).

Data analysis. After standardization to the lowest number of reads, rarefaction curves, diversity indices (Good's coverage, abundance-based coverage estimation (ACE), Chao1, Shannon evenness and Simpson's Index), observed OTUs, and shared OTUs (Venn diagrams)

were calculated using Mothur v.1.35.1 software (Schloss et al., 2009). A one-way ANOVA was performed on all diversity indexes, followed by a Tukey's post hoc test ($P < 0.05$). Metastat analysis (White et al. 2009) was used to identify which phyla were significantly different between treatment groups. PRIMER/PERMANOVA + (Clarke and Warwick, 2001) was used to generate non-metric multidimensional (MDS) plots, using the Bray-Curtis similarity index, and principal coordinate analysis (PCOA) to visualize the differences between samples. Analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were used to test whether there were significant differences between different groups. Similarity percentages (SIMPER) analysis was used to define the taxon contributing to the maximum dissimilarity to observe differences between groups. Cut-off for low contributions was set at the default of 90.

Results

Alpha diversity. Data were analyzed by day and by treatment. At day 15, a total of 3,264,433 bacterial sequences were recovered, representing 15,282 OTUs, after an additional abundance cut-off of < 0.00012%. After standardization to the lowest number of sequences (n = 42,994) 14,956 OTUs remained in the analysis. Sequence coverage was > 95% for day 15 antibiotic samples. There were no significant differences of diversity indices between groups. Ampicillin-treated mussels exhibited the highest bacterial species richness; however, individuals from the control varied in their species richness and dominance compared to the other groups (Figure 1).

At day 20, 2,409,180 bacterial sequences representing 15,742 OTUs were recovered from control-challenged *V. nebulosa*, after an additional abundance cut-off of <0.000124%. After standardization (n = 29,732 bacterial sequences) 15,056 OTUs remained in the study. Sequence

coverage was > 95% for day 20 control-challenged mussels. Total expected richness as calculated by ACE was statistically significant between ampicillin-control and tetracycline-control *V. nebulosa* (Figure 2). All groups displayed a more individual variability in their bacterial species richness than at day 15.

At day 20, 2,112,519 bacterial sequences were recovered from antibiotic-challenged *V. nebulosa*, representing 15,369 OTUs, after an additional abundance cut-off of <0.000141%. After standardization to the lowest number of sequences (n = 35,035), 15,020 OTUs remained in the study. Sequence coverage was >97% for day 20 antibiotic-challenged samples. Interestingly, tetracycline-challenged mussels exhibited the highest bacterial species richness than any other challenge group; however, species dominance was relatively similar among all groups (Figure 3).

At the phylum level, there were significant differences between groups at day 15 (Figure 4). Metastats analysis revealed a statistically significant higher abundance of Tenericutes in control mussels (34%) and ampicillin-treated mussels (37%) compared to tetracycline-treated mussels (3%) (similar pattern was also noted at day 20 between control groups). The predominant phyla of tetracycline-treated mussels was Firmicutes (60%), followed by Proteobacteria (30%). From the Firmicutes phylum, the most abundance class was Bacilli, with abundances being higher in control (39.7%) and tetracycline-treated *V. nebulosa* (59.5%) compared to ampicillin-treated *V. nebulosa* (20.6%) (Figure 7). Interestingly, less common phyla varied between treatment groups, with some unique members in each microbial community. For instance, ampicillin-treated samples had higher abundances of Actinobacteria (4%), Bacteroidetes (3%), and Planctomycetes (2%), while the control and tetracycline-treated groups had a lower abundance of these representatives (<1%). Overall, at day 20, all control groups began to display similar phyla composition, with Proteobacteria (>56.8%) being the most

prominent phylum, and phyla, such as, Firmicutes and Tenericutes varying in abundance between groups (Figure 5). At day 20, after challenge, all groups showed similar phyla composition and abundance compared to control-non-challenged mussels (Figure 6). Overall, Proteobacteria was the predominant phylum, followed by Tenericutes and Firmicutes.

Core microbiomes. Venn diagrams were generated to compare the number of distinct and shared OTUs between groups (data not shown). The number of shared OTUs between groups decreased between the two sampling points. A total of 5650 OTUs were shared between day 15 groups. Ampicillin-treated *V. nebulosa* had a higher number of unique OTUs (1705) in their gut microbiome than control (1468) and tetracycline-treated (760) mussels. The ampicillin-treated group and control group contributed to the highest number of shared OTUs (45.3%) from the overall core microbiome. At day 20, control-non-challenged groups of *V. nebulosa* shared 5170 OTUs, with control-control mussels having the highest number of unique OTUs (1947), followed by tetracycline-control (1721) and ampicillin-control (1556) groups. Again, the ampicillin-treated group and control group shared the highest number of shared OTUs (37.9%) from the overall core microbiome. At day 20, challenged groups of *V. nebulosa* shared 4575 OTUs, with tetracycline-challenged having the highest number of unique OTUs.

Beta diversity. PCO analysis and MDS were generated to visualize the dissimilarities and positions of samples from the different groups in ordination plots. At day 15, there were significant differences between all groups at the OTU level (Figure 8). PERMANOVA results revealed that the control group and tetracycline group were the most significant with a larger t statistic and lowest p-value (Table 1-1). At day 20, control groups (non-challenged) showed nearly indistinguishable microbial communities at the OTU level (Figure 9), with a very low R statistic between all comparisons, and only ampicillin-control vs. tetracycline-control groups

having a significant p-value (Table 1-2). PCO analysis and PERMANOVA showed that the microbial community structure at day 20 challenged groups were all statistically significant between each other (Figure 9), with ampicillin-challenge and tetracycline-challenge groups contributing to the highest among those differences (Table 1-3).

SIMPER analysis revealed that at day 15, control *V. nebulosa* had a higher abundance of *Ureaplasma* (Tenericutes) compared to ampicillin-treated mussels and this genus contributed to the highest dissimilarity between the two groups. In comparison, tetracycline-treated *V. nebulosa* had a higher abundance of *Geobacillius* (Firmicutes) compared to ampicillin-treated and control mussels and contributed to the highest dissimilarity when compared to those groups.

Discussion

Antibiotics have ecological effects on the diversity and composition of the gut microbiome, subsequently impacting the interactions between the host and its' microbial community and how the host regulates basic physiological processes (Tuddenham and Sears, 2015). This new environmental state means that the microbial community observed after antibiotic treatment is very different from the original community, often associated with a reduction in overall diversity and increase in pathogens (Stecher and Hardt, 2011). The effects of antibiotics on the human gut microbiome include increased susceptibility to intestinal infections, basic immune homeostasis, and deregulation of host metabolism (Francino, 2016). For example, the depletion of the gut microbiome by using antibiotics and subsequent pathogen overgrowth has caused major diseases such as *Clostridium difficile* colitis in humans and mice models (Leffler and Lamont, 2015;Buffie et al., 2012).

A few reviews have summarized the literature on microbial communities associated with bivalves and other aquatic invertebrates (Carella et al., 2016;Grizzle and Brunner, 2009;Harris,

1993). Recently, Pierce and Ward (2018) summarized culture-independent studies on the oyster microbiome, with topics covering their microbial community composition, spatial and temporal trends, tissue-specific microbiomes, and shared OTUs (core microbiomes). The authors also briefly described their unpublished study on the use of antibiotics to understand the reduction in bacterial diversity in the gut and how it influences *Vibrio* accumulation in eastern oysters (*Crassostrea virginica*). Rungrassamee et al. (2016) conducted disease challenges using *Vibrio harveyi* on two species of shrimp, which resulted in the alteration of their normal intestinal microbiota. However, no studies have focused on characterizing the gut microbiome associated with freshwater mussels after antibiotic treatment and their increased susceptibility to disease. Since these animals are important biological indicators of water quality and water clarity in freshwater ecosystems, it is critical to understand the effects of antibiotics on their gut microbiome and how it impacts the ecosystem services they provide.

In this study, as predicted, I found that ampicillin and control groups had a significantly higher abundance of Tenericutes in their gut microbiome than the tetracycline group did. Unfortunately, I was unable to induce mortalities in challenge mussels and therefore the deleterious effect (if any) of a dysbiotic gut microbiome could not be tested. On the contrary, our results show that the challenge may have caused rebiosis, that is, establishing the microbial community back to its' healthy state (Petersen and Round, 2014). After challenge with *A. hydrophila*, mussels treated with tetracycline experienced a significant abundance of Tenericutes in comparison with non-challenge mussels. Additional experiments will need to be conducted before the use of *A. hydrophila* can be recommended for restoration of a healthy gut microbiome in mussels but this was certainly an interesting result. In mice, a group of researchers found that three different strains of *Lactobacillus* were able to individually restore ampicillin-induced

dysbiosis in their microbiome (Shi et al., 2018). A study on the dysbiosis of black molly (*Poecilia sphenops*) microbiomes after streptomycin treatment found that two candidate probiotic bacterial species *Phaeobacter inhibin* and *Bacillus pumilus* were able to prevent antibiotic-induced mortalities without significantly changing the microbial community structure or diversity (Schmidt et al., 2017). Zeng et al. (2017) found that antibiotics such as oxytetracycline had a significant effect on microbial population genetic structures in catfish pond water and sediments. Overall, our study found the general trends of dysbiosis to be similar to what has been observed in mice (Shi et al., 2018), swine (Looft et al., 2012), rats (Manichanh et al., 2010) and humans (Dethlefsen et al., 2008), where antibiotic treatment causes a decrease in microbial diversity and a shift in community composition.

Bacterial species evenness was higher in ampicillin-treated mussels than control and tetracycline-treated groups at day 15 and day 20 – control groups. However, at day 20 – challenged, tetracycline-treated mussels exhibited a higher species evenness. Ampicillin-challenged and control-challenged groups had similar Shannon evenness and Simpson indices. The shift in observed phyla between the three groups at day 15 was evident, with control and ampicillin groups having three predominant phyla (Tenericutes, Firmicutes and Proteobacteria) and tetracycline-treated mussels having a significantly higher abundance of Firmicutes (class Bacilli). The *Geobacillus* genus that belongs to this bacterial class was the most abundant in gut samples from tetracycline-treated mussels. *Geobacillus* genera include thermophilic gram-positive spore-forming bacteria that are utilized in the biotechnology industry as sources of thermostable enzymes, substrate fermentation, and biofuel production (Studholme, 2014). It has also been proposed that *Geobacillus* plays a minor role in the global carbon cycle as

decomposers of plant biomass and that their spores are distributed by atmospheric transport (Zeigler, 2014).

At the beta-diversity level, the ordination plots show that treatment significantly contributed to the differences in microbiome structure. The more significant changes occurred at day 15 and day 20 challenged. The only significant difference (p-value <0.05) in microbial assemblages at day 20 control groups was between ampicillin-control and tetracycline-control, although the R-value shows that communities were overlapping. This lack of significance between control groups at day 20 was also observed at the alpha-diversity, with the most predominant phyla in all groups being Proteobacteria (>57% in all three groups). Although mussels became dysbiotic by being held in recirculating aquaria (shift to Proteobacteria), it is evident that water quality parameters did not cause a tank effect in the observed results from this study.

In conclusion, tetracycline treatment induced dysbiosis in the gut microbiome of *V. nebulosa*, characterized by a reduction in Tenericutes and an increase in Firmicutes. Interestingly, the bacterial challenge with *A. hydrophila* caused tetracycline-induced microbiomes to shift back to their normal state, accompanied by a high species richness that was predominated by members of Proteobacteria, Tenericutes and Firmicutes. Antibiotic treatment can be important chemotherapeutics against harmful pathogens, but they can also disrupt the microbial community and promote bacterial colonization by opportunistic pathogens. Herein, this study provides the first characterization of the gut microbiome of the freshwater mussel *V. nebulosa* after treatment of two-broad spectrum antibiotics.

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Table 5-1. PERMANOVA analysis at day 15.

PERMANOVA	t statistic	p-value
Ampicillin, Control	1.453	0.03
Ampicillin, Tetracycline	1.985	0.0011
Control, Tetracycline	2.590	0.0001

Table 5-2. ANOSIM analysis at day 20 control groups.

One-way ANOSIM	R statistic	P-value
Ampicillin, Control, Control-Control	0.026	0.273
Ampicillin-Control, Tetracycline-Control	0.291	0.002
Control-Control, Tetracycline-Control	0.085	0.093

Table 5-3. PERMANOVA analysis at day 20 challenge groups.

PERMANOVA	t statistic	P-value
Ampicillin-Challenge, Control-Challenge	1.477	0.026
Ampicillin-Challenge, Tetracycline-Challenge	1.764	0.002
Control-Challenge, Tetracycline-Challenge	1.500	0.012

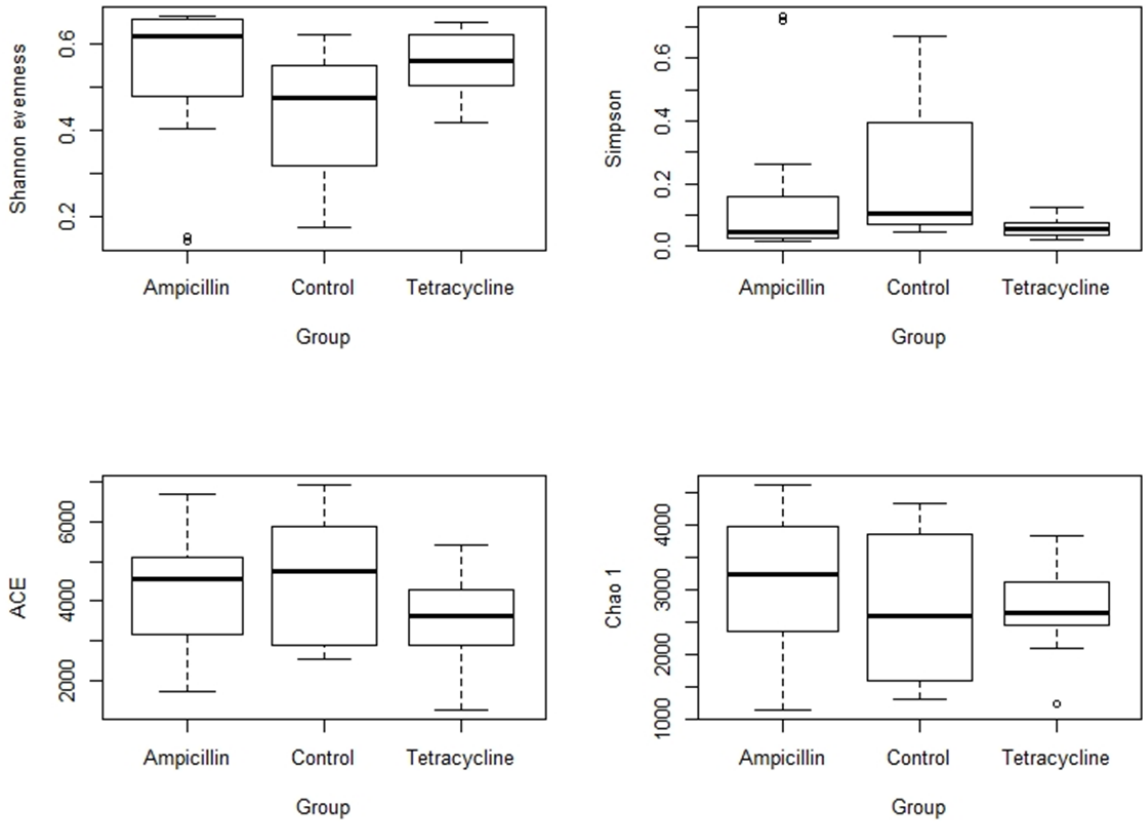

Figure 5-1. Diversity indices as boxplots of day 15 groups.

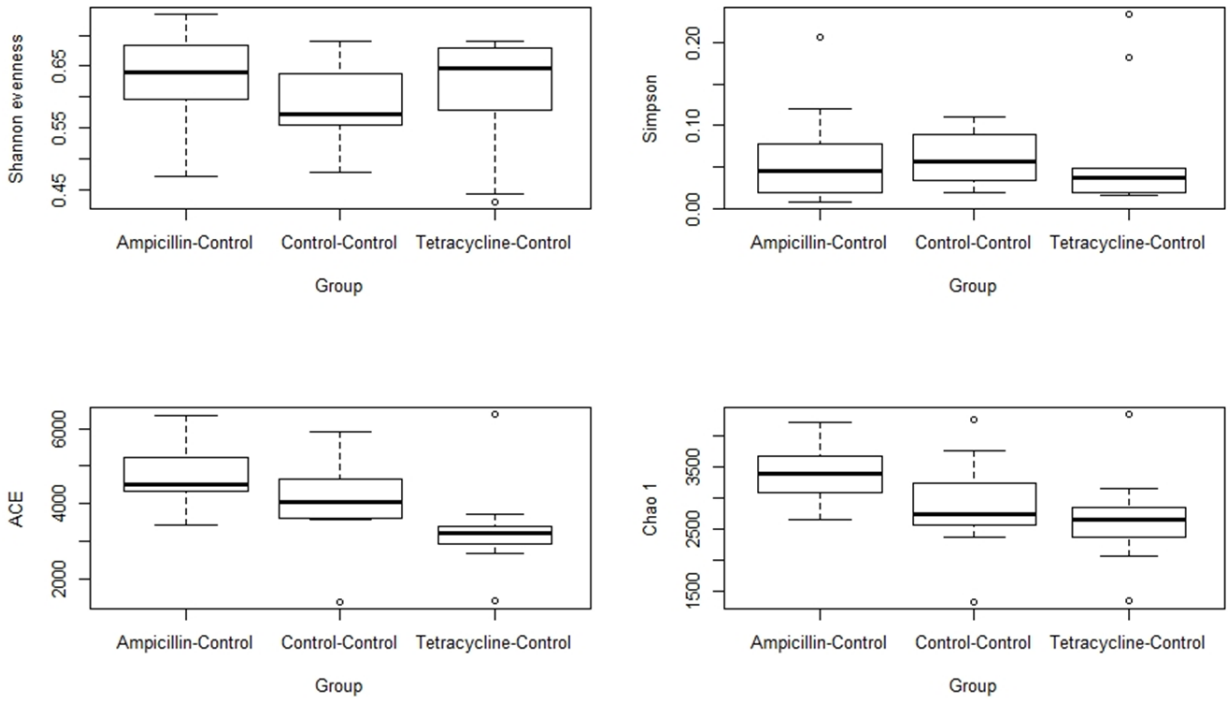

Figure 5-2. Diversity indices as boxplots of day 20 non-challenged (‘control’) groups.

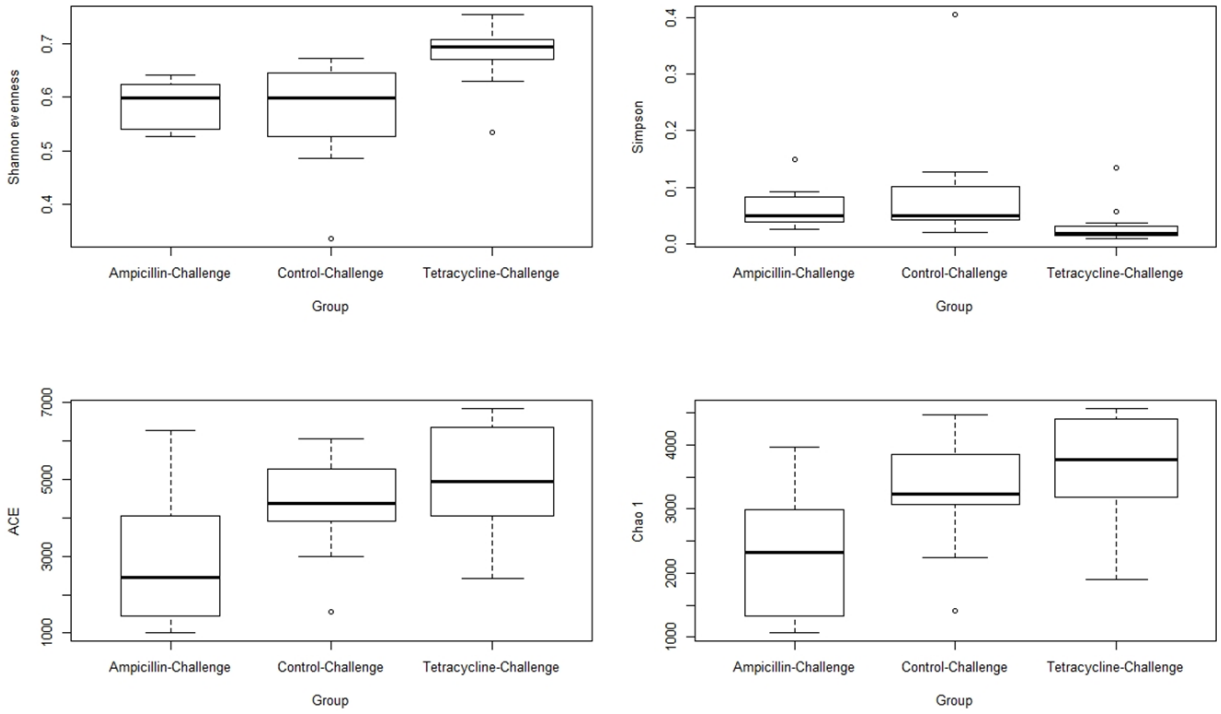

Figure 5-3. Diversity indices as boxplots of day 20 challenge groups.

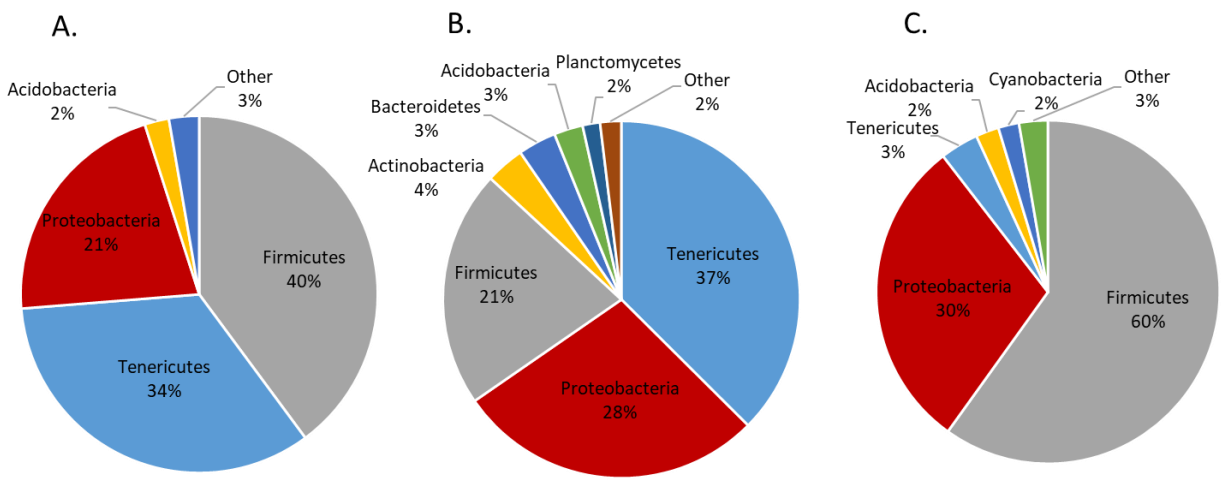

Figure 5-4. Phyla composition of the digestive gland microbiome of *Villosa nebulosa* at day 15 (A) Control (B) Ampicillin-treated and (C) Tetracycline-treated.

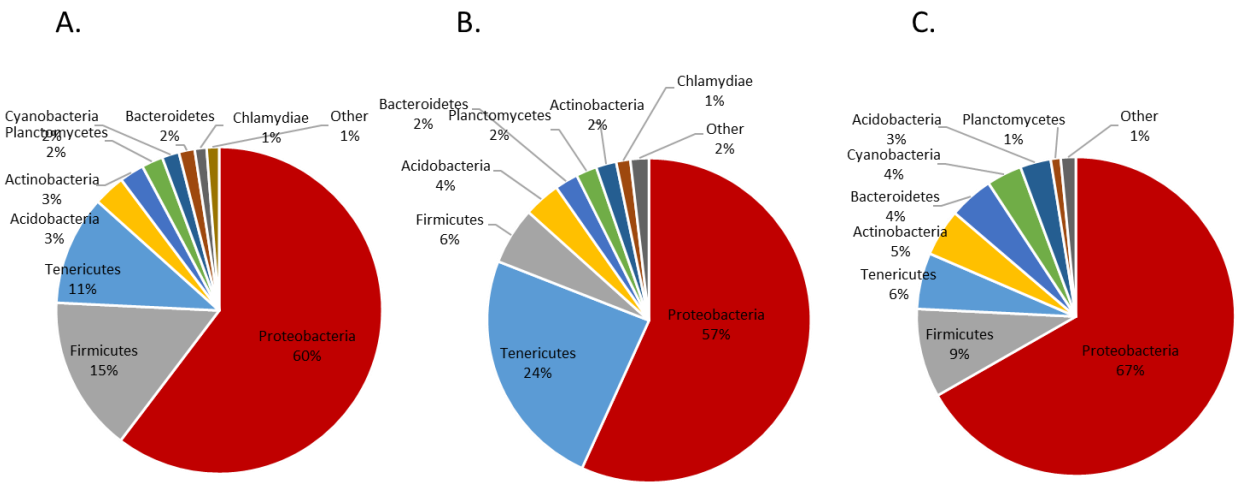

Figure 5-5. Phyla composition of the digestive gland microbiome of *Villosa nebulosa* at day 20 control groups (A) Control-Control (B) Ampicillin-Control and (C) Tetracycline-Control.

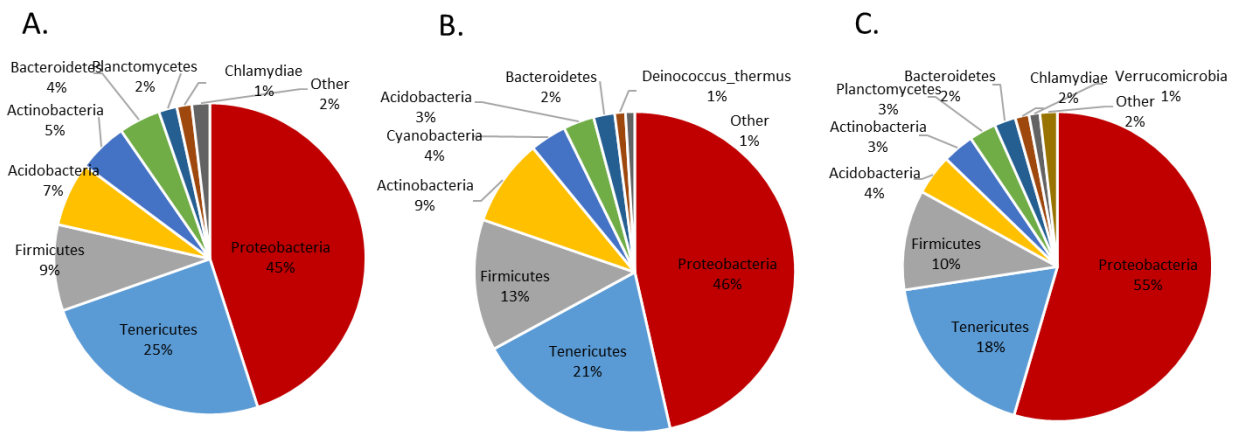

Figure 5-6. Phyla composition of the digestive gland microbiome of *Villosa nebulosa* at day 20 challenge groups (A) Control-Challenge (B) Ampicillin-Challenge and (C) Tetracycline-Challenge.

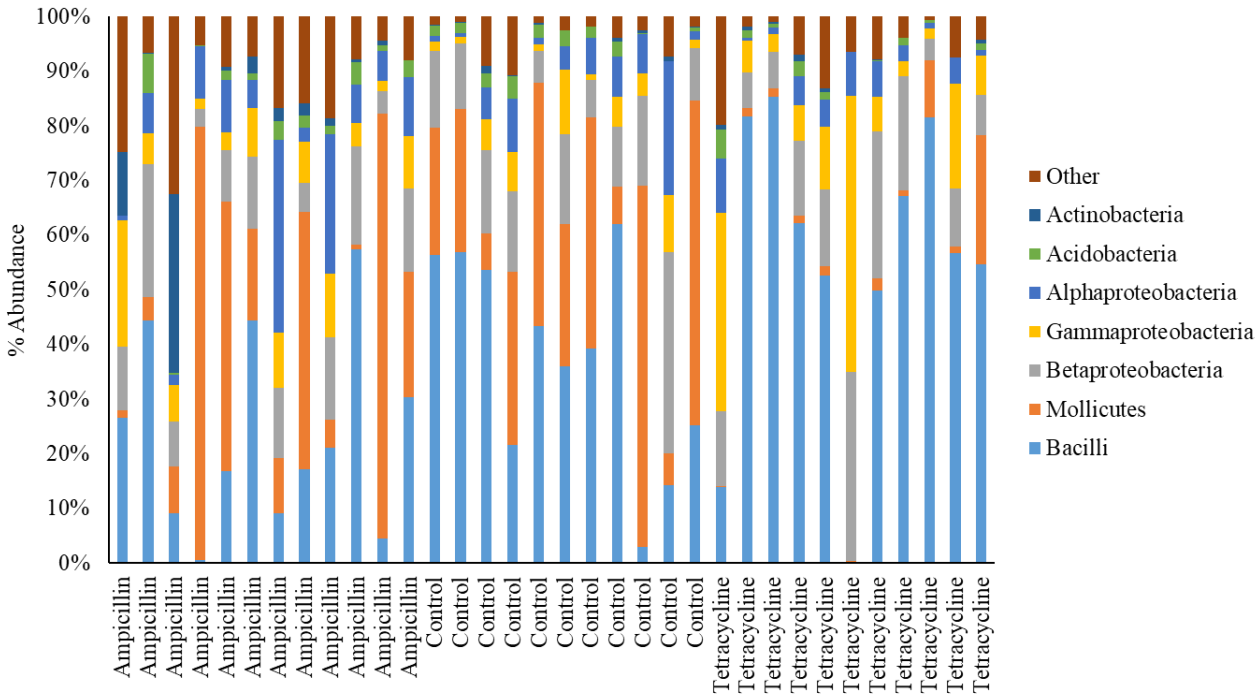

Figure 5-7. Bacterial class composition of the digestive gland microbiome of *Villosa nebulosa* at day 15.

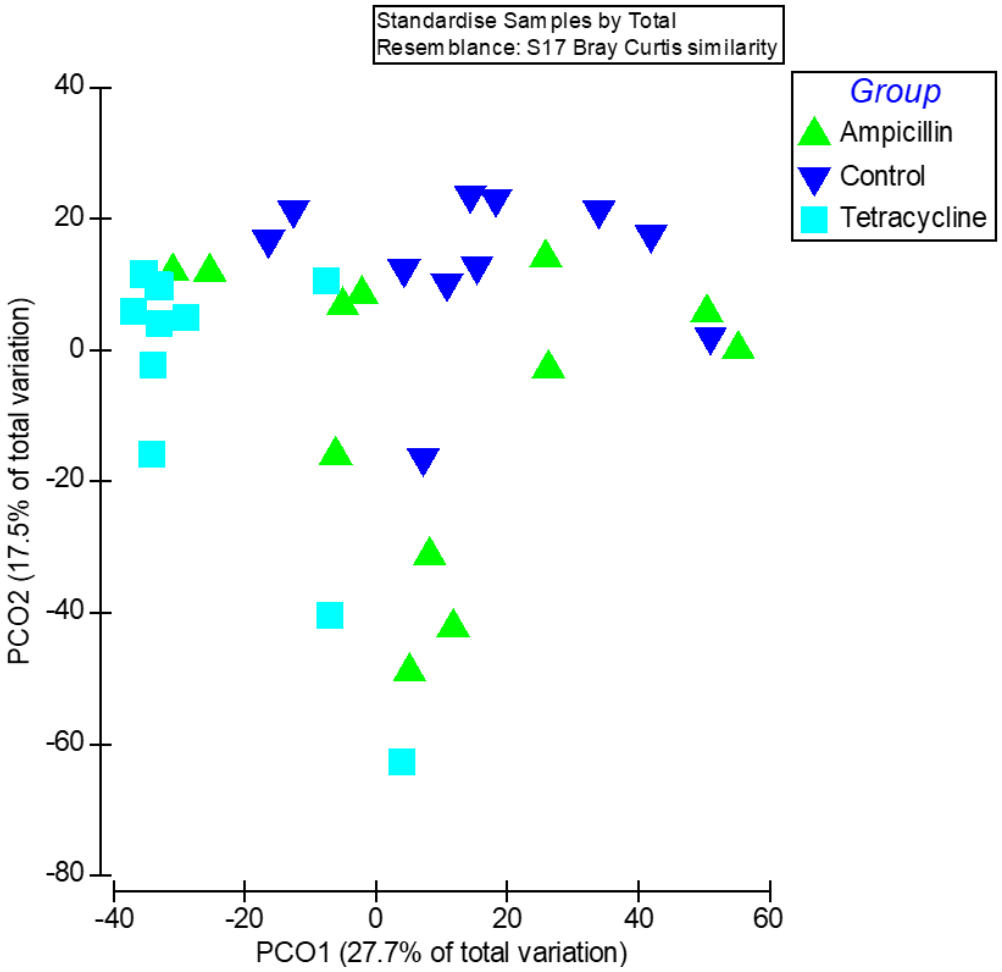

Figure 5-8. PCo plot of digestive gland samples at day 15.

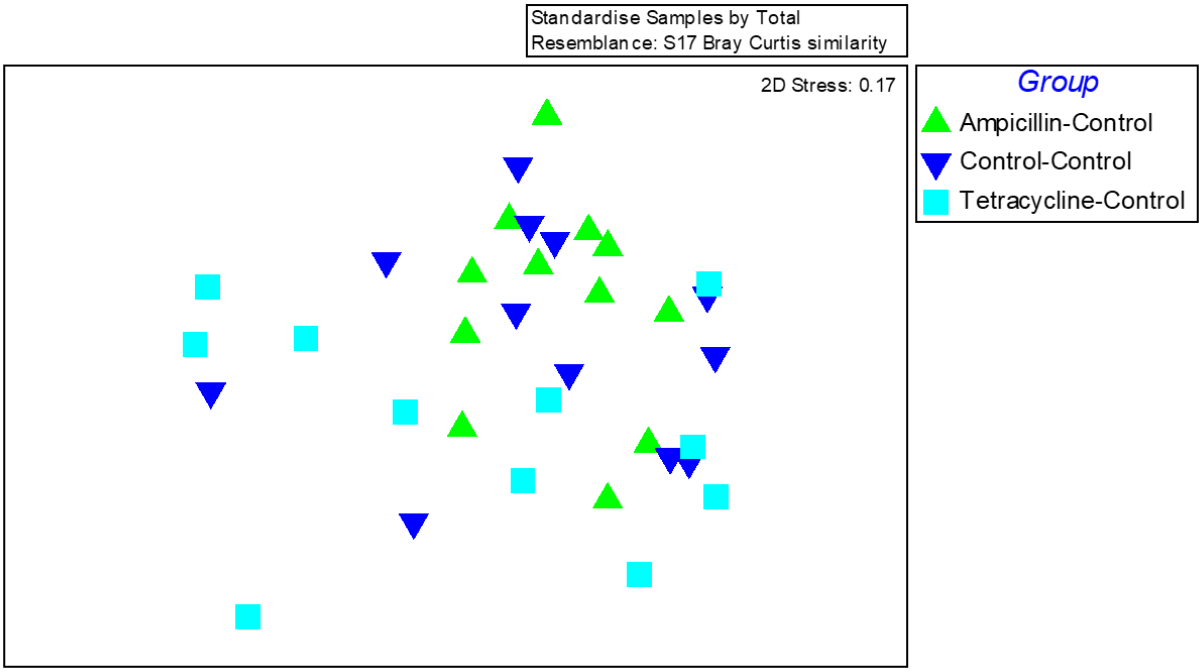

Figure 5-9. Multidimensional scaling plot of digestive gland samples according to groups treatments at day 20 – non-challenged groups.

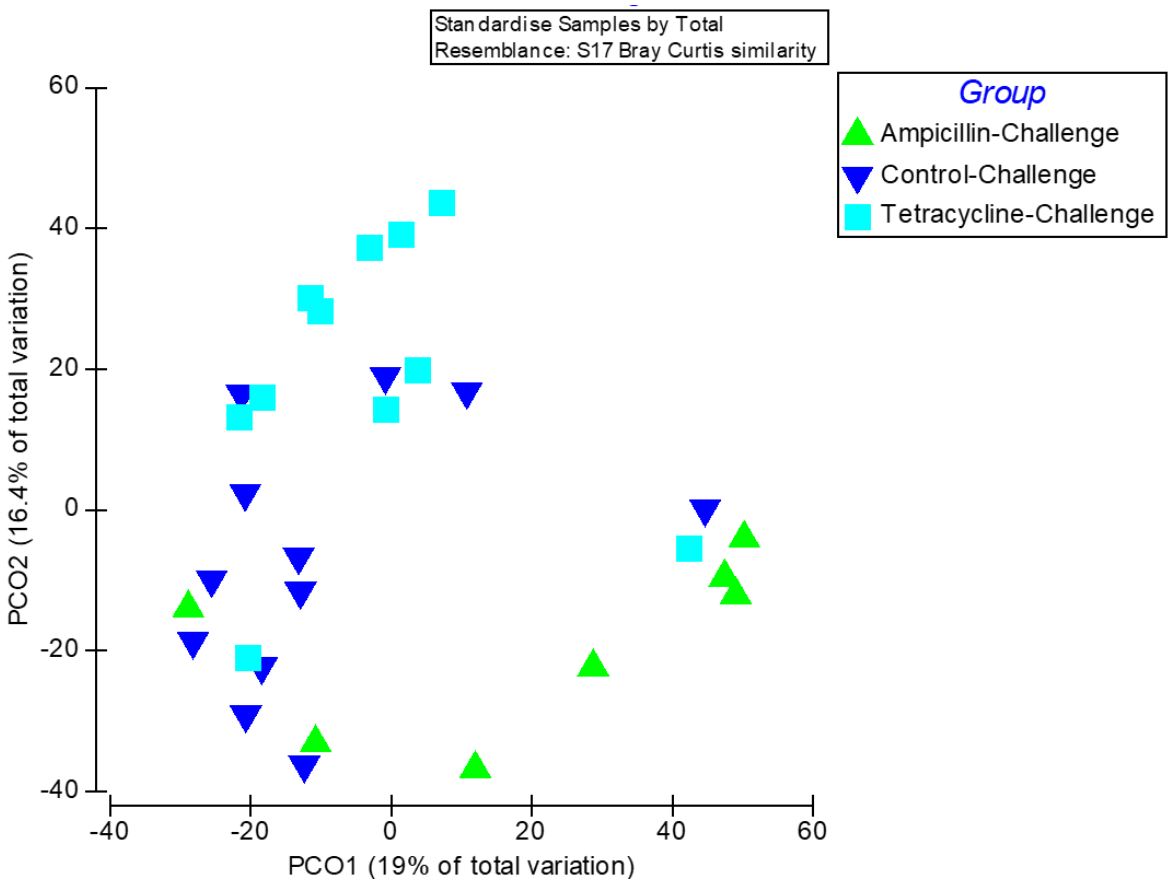

Figure 5-10. PCo plot of digestive gland samples at day 20 – challenge groups.