A Microbiome Analysis of the Relationship Among Crayfish Ectosymbionts and Their Environment

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A Senior Thesis submitted in partial fulfillment
of the requirements for graduation
in the Honors Program
Liberty University
Fall 2020

Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial
fulfillment of the requirements for graduation from the
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Abstract

The purpose of this project was to determine if there are differences present between the α-diversities of the crayfish microbiome and its surrounding water and sediment. Furthermore, this project sought to discover if these differences hold when microbiomes are evaluated between crayfish of first and second stream orders. Finally, this project sought to determine if the presence of branchiobdellidan ectosymbionts on the crayfish caused further differences in the crayfish microbiome. While the hypothesized patterns between crayfish, ectosymbionts, and stream order were not found to exist, a significantly different microbiome was observed between water, sediment, and crayfish, and the α-diversity of the crayfish microbiome more closely resembled that of the sediment when higher levels of ectosymbionts were present on the crayfish.

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Introduction

Crayfish Role in Freshwater Ecosystems

Through detrital processing and burrowing, crayfish can exert significant changes within many freshwater ecosystems as ecosystem engineers. For example, in multiple studies, crayfish have been found to accelerate the conversion of coarse particulate matter (CPM) into fine particulate matter (FPM) through their burrowing behavior when they consume leafy matter and process it into smaller fragments (Creed & Reed, 2004; Huryn & Wallace, 1987). Additionally, crayfish have been known to significantly alter the composition of benthic invertebrate communities while also serving as a food source for multiple predators including sp. raccoons, otters, and fish (Britton et al., 2017; Charlebois & Lamberti, 1996; Schoonover & Marshall, 1951). Their role as both prey and ecosystem engineer often makes them a keystone species within freshwater ecosystems, a role that lends importance to crayfish research. These types of characteristics lead many researchers to use crayfish as model organisms to gain greater insight into the broader world of biology (Skelton et al., 2016).

Crayfish Ectosymbionts

Crayfish serve as hosts to a variety of invertebrate symbionts, the most prominent of which includes the clitellate worm: Branchiobdellida (Ames et al., 2015; Brown et al., 2002). The Branchiobdellidan worm is an ectosymbiont of the crayfish that lives out its entire reproductive cycle on the crayfish (Ames et al., 2015; Brown et al., 2002). Willard Young (1966) noted that the branchiobdellidan worms in his study placed a majority of their cocoons on the

ventral side of the crayfish, with most of these being on the abdominal sternites, cephalic sternites, or pleopods. Very rarely were cocoons found on the dorsal side of the crayfish or anterior to the mouth (Young, 1966). After laying eggs on the abdomen of the crayfish, it is believed that the branchiobdellidan worms will migrate to an area proximal to the gill chamber of the crayfish where they will consume various epibionts such as diatoms and protozoans off of the crayfish gills (Ames et al., 2015; Brown et al., 2002). While branchiobdellidan worms have been found on some freshwater crabs and isopods, their primary host is the crayfish (Ames et al., 2015). Research has repeatedly shown that branchiobdellidan worms are completely dependent on their crayfish hosts to reproduce (Creed et al., 2015; Young, 1966). Creed et al. (2015) demonstrated that this reproductive cycle required a live crayfish and not merely the habitat that the host provides or its chemical cues. However, branchiobdellidan worms are not dependent on crayfish for survival as many worms have been found to survive for long periods of time without a host (Creed et al., 2015; Penn, 1959; Young, 1966).

The dependence of branchiobdellidan worms on their crayfish hosts has led some to speculate about the type of relationship occurring between the crayfish and its ectosymbiont. Initially, researchers believed that the branchiobdellidan worm was a parasite of the crayfish, using its jaws and mouth to extract blood from its host (Young, 1966). However, this hypothesis was eventually discounted as it was demonstrated that the primary contents in the gut of the branchiobdellidan worm were epibionts from the biofilm on the crayfish gills (Penn, 1959). Furthermore, research has since shown a lack of host tissue in the guts of branchiobdellidan worms and a lack of scarring or damage to the host from their ectosymbionts to support the view that the worms were ingesting flesh from their hosts (Jennings & Gelder, 1979). Willard Young

(1966) concludes that, due to their ability to survive off of the crayfish host for many months, branchiobdellidan worms are commensals or at most facultative parasites of the crayfish host. Since this time, however, empirical research has shown that the relationship between crayfish and branchiobdellidan worm is much more complex than this initial proposition and may even be mutualistic at times (Brown et al. 2002; Skelton et al. 2013). Research has repeatedly shown that branchiobdellidans engage in a cleaning symbiosis by eating biofilm off the gills of the crayfish (Brown et al., 2002; Skelton et al., 2013).

While research has definitively shown the method by which branchiobdellidans interact with crayfish, the exact benefits of this relationship to the crayfish remain somewhat unknown (Brown et al., 2002; Skelton et al., 2013). Research suggests that this relationship can vary between mutualism and parasitism depending on multiple different factors (Brown et al., 2002; Skelton et al., 2013). Lee et al. (2009) suggest that the difference in this relationship is dependent on the amount of biofilm (also known as fouling pressure) on the gills of the crayfish. However, research by Brown et al. (2012) suggests that the shift may actually be caused by differing concentrations of worms. In their experiment, crayfish with intermediate amounts of worms had the highest growth levels, more so than crayfish with zero worms (Brown et al., 2012). However, at high densities of worms, the positive effects dissipated and negative effects on growth were recorded (Brown et al., 2012). The results of these experiments suggest that the relationship between crayfish and branchiobdellidan worms is complex and can shift from mutualism to parasitism depending on a variety of factors.

Microbiome

One of the largest groups of organisms within an ecosystem, particularly a freshwater ecosystem, are bacterial assemblages also known as microbiomes. These collections of bacteria play a huge role in freshwater ecosystems by performing vital tasks necessary to the functioning of the ecosystem. These functions include nutrient cycling, reducing toxic nitrogen, decomposing organic matter, and producing proteins (Cardona et al., 2016; Dudgeon et al., 2006; Lear et al., 2009). Additionally, microbiomes can serve as indicators of the health of a freshwater stream (Dudgeon et al., 2006; Lear et al., 2009; Newton et al., 2011). Interestingly, however, bacterial diversity is an understudied area of research in field-based freshwater ecosystems (Schultz et al., 2013; Zeglin, 2015). This is particularly true of studies regarding diversity in relation to aquatic symbionts. Research in this area has only recently been able to gain traction with the introduction of such methods as whole-genome sequencing, which allows for ease of identification for more than one bacterial species at a time (Schultz et al., 2013; Zeglin, 2015).

Metacommunities and Symbiosis

Symbiosis is broadly defined as “Intimate (and not exclusively positive) interspecific relationship with prolonged physical contact” (Silknetter et al., 2020, pg. 3). While there are many types of symbioses such as mutualism, parasitism and commensalism, the general idea behind all of these interactions is how two kinds of organisms can live life together (Silknetter et al., 2020). These interactions can be looked at on a small scale, such as the interaction between a clownfish and an anemone, or they can be looked at on a large scale, known otherwise as an ecosystem. Many different frameworks have been produced to conceptualize the changing

abundance and interaction among organisms within a freshwater ecosystem. Two of these frameworks are the River Continuum Concept and metacommunities.

River Continuum Concept (RCC)

The first of these frameworks references an early article that visualizes the stream ecosystem as aggregates of organisms that exist in a continuous mosaic of populations (Vannote et al., 1980). The River Continuum Concept (RCC), as it is called, looks at the interplay between the varied physical-geomorphic and biological components along a stream continuum to create a dynamic equilibrium of biological communities that result from their surrounding environment (Vannote et al., 1980). For example, biological communities in wooded slow-moving parts of a stream may contain certain invertebrates specialized for that environment. However, biological communities in a faster moving, more open part of that same stream may contain a completely different set of invertebrates (Vannote et al., 1980). This idea has since been confirmed by various researchers, often characterizing streams as “leaky funnels” (Savio et al., 2015, p. 4994) with bacteria and other types of invertebrates entering one part of the stream while other parts of the stream environment filter these same bacteria and invertebrates out (Savio et al., 2015). These filtering methods give rise to a relatively predictable gradient of bacteria from the headwater to the mouth of a stream or river (Savio et al., 2015).

Metacommunities

The second ecological concept that can be used to model symbiosis in stream habitats would be metacommunities. A metacommunity is a small community that has connections with other communities of species that may or may not interact with the community (Leibold et al., 2004). There are two ways in which metacommunities can be synthesized with this current

project. The first way is by looking at the individual crayfish as a metacommunity (Miller et al., 2018). While formal community theory would simply study the crayfish and its microbiome/symbionts as a unique individual unit, the metacommunity framework looks at the crayfish in light of the interactions it has to give rise to its unique community (Leibold et al., 2004; Miller et al., 2018). The second approach used by the metacommunity concept is broader and would look at the stream as consisting of metacommunities within it, each giving rise to a unique bacterial community (Savio et al., 2015). Crayfish, as ecosystem engineers, may cause the disruption of these predictable communities, thus disturbing the overall gradients that may otherwise exist in the lotic environment (Savio et al., 2015).

Project Focus

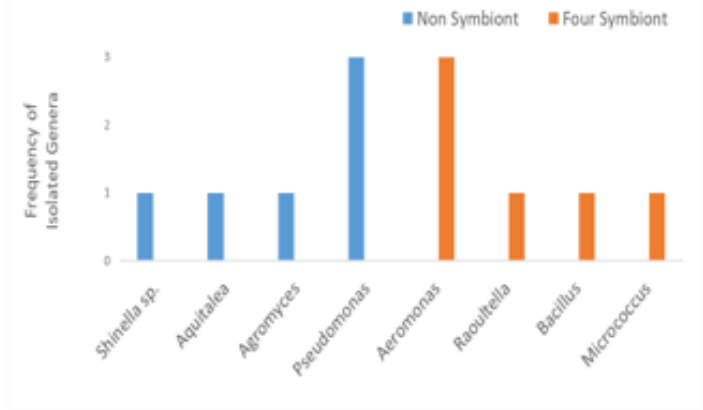
This project seeks to learn more about symbiosis specifically in reference to the relationship between the crayfish and two of its ectosymbionts, the branchiobdellidan worm and the microbiome of the crayfish. The bacterial composition of a stream is typically predictable along the assumptions laid out by the river continuum concept and metacommunity theory (Savio et al., 2015). However, when disturbances such as a dam or other obstruction are added to the environment, the bacterial community is often disrupted (Schultz et al., 2013). This project seeks to determine if crayfish act in some way as a unique microhabitat for both branchiobdellidan worms and microbiomes that is different from the surrounding stream and soil. It has been suspected that crayfish act as a successive filter of bacteria, creating a unique microbiome on their different layers of carapace and gill (Skelton et al., 2016). This research project seeks to build on that understanding to determine if the crayfish microbiome is further shaped by the presence or absence of the ectosymbiotic branchiobdellidan worm.

Previous Research

This lab previously researched the microbiome of the crayfish in relation to branchiobdellidan worms. In this lab-based study, crayfish were split into two categories, an experimental group inoculated with four annelid worms and a control group with no annelid worms. Crayfish were swabbed and the swabs were plated on a TSA gel. 16S gene sequencing was subsequently performed to determine the identity of bacteria from the crayfish in the two groups. Results (Figure 1) showed no overlap between the identities of the four most common bacteria in the experimental and control groups that grew on the plates (Holman et al., 2016).

Figure 1

Identities of the four most common bacterial species



Note. Samples are from the control group (blue) and the experimental group (red). From "Ectosymbiotic relationships between the Appalachian Brook Crayfish (*Cambarus bartonii*) and the Branchiobdellidan *Cambarincola ingens* in relation to dissolved oxygen uptake and gill bacteria," by Holman, T., Davis, J., & Harris, K. (2016) [Poster presentation]. Liberty University Research Week, Lynchburg, VA, United States. Reprinted with Permission.

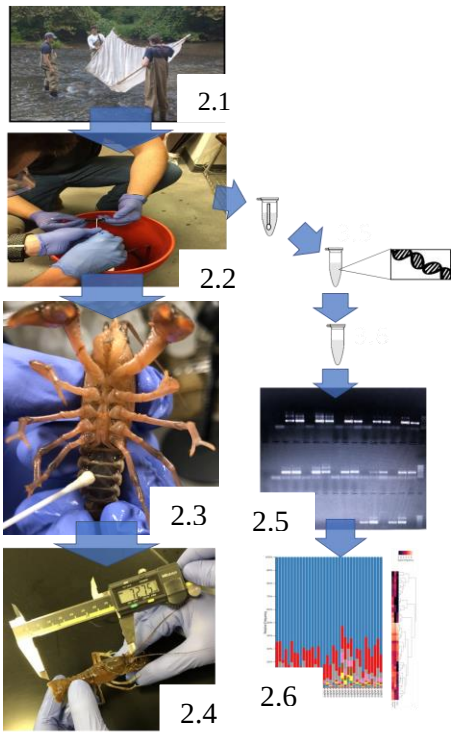
This indicated that the presence of branchiobdellidan worms may be acting to significantly alter the microbiome of the crayfish (Holman et al., 2016). However, this study was limited by both the sample size and the mere fact that the experiment was performed in a lab which may not have accurately reflected the results as they would be found in nature.

Current Study

In this study, bacterial swabs were collected from crayfish at five different collection sites along first and second order streams of the Opossum Creek and its tributaries. In addition to swabbing the crayfish, swabs were collected of the surrounding water and sediment. DNA from the swabs was then extracted and PCR/sequencing was performed on the 16S gene of bacteria in the swabs. From there, sequencing was used along with QIIME2 analysis to compare bacterial species from the different samples (Figure 2). By performing metagenomic sequencing on bacteria collected from the field instead of the lab, results would be more accurate and provide a larger picture of the overall microbiome. As a very small fraction of bacteria from a given sample can even grow on TSA plates, this research sought to expand on the previous study by using metagenomic sequencing to increase the percentage of bacteria that could be identified from a sample instead of merely the bacteria that could grow on a TSA plate (Staley, 1985).

Figure 2

Experimental methods



Note. Crayfish collection (2.1) followed by swabbing of both crayfish and water (2.2-2.3). Extraction of DNA was then performed after taking physical measurements of the crayfish (2.4). PCR success was measured using gel electrophoresis (2.5) and successful samples were compared using QIIME2 (2.7).

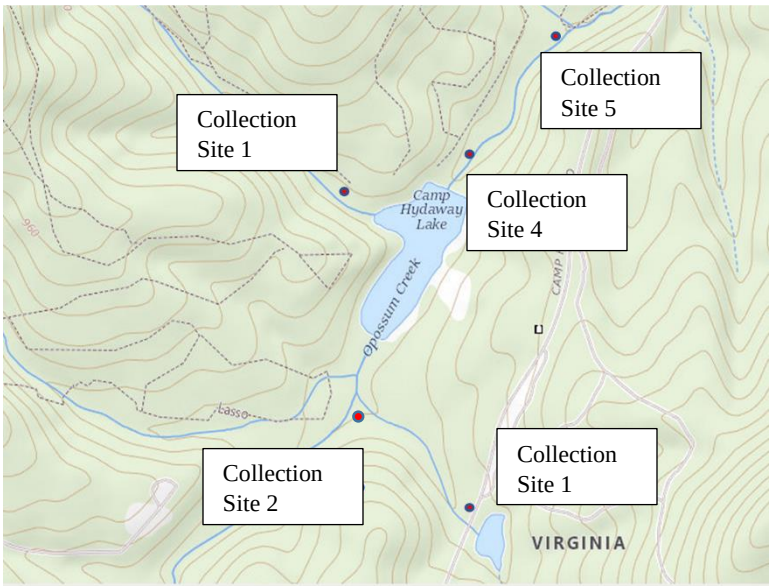
Methods and Materials

Swabbing and Collections

Five different collection sites were used in this experiment along first and second order streams of Opossum Creek and its tributaries (Figure 3). At each collection site upon initial arrival, a 20-meter length was flagged after measurement at 0, 10, and 20-meter points along the length. In addition to this, before the water was disturbed, flow rate, optical dissolved oxygen,

Figure 3

Collection Sites Used in Project



Note: Adapted from *EarthExplorer* by United States Geographical Survey, (n.d.) (https://earthexplorer.usgs.gov/). In the public domain.

pH, water temperature, and air temperature were measured and recorded. Rayon swabs were used to collect samples from the water and substrate at each flag using aseptic technique, and a kick seine was used to collect crayfish with a range of 4-11 crayfish per collection site. With both the crayfish collections and the swab collections, activity began downstream and moved upstream so as to avoid stirring up the substrate prior to collecting from the downstream sites. Crayfish were removed from the kick seine and placed in a Whirl-Pak with stream water. Swab samples and crayfish were then taken to the lab for processing.

Physical measurements of each crayfish were initially taken along with other measurements to determine total length (TL), carapace length (CL), blotted wet mass (BWM), and gender. All measurements and swabs were taken with sterile nitrile gloves which were

changed out between each crayfish. Additionally, crayfish were double rinsed with DI water before swabbing to remove transient bacteria. Rayon swabs were used to collect microbial samples off the dorsal and ventral cephalothorax as well as the dorsal and ventral abdomen of the crayfish. Using a standardized aseptic technique, swabs were used across a 10-millimeter distance and drawn across this length three times. Swabs were rotated as they were drawn across the crayfish. Once collections had been completed, both environmental and crayfish swab samples were stored in a freezer at -20ºC.

Ectosymbiont Quantification

Quantification of branchiobdellidans was performed by submerging each crayfish in a 10% $MgCl_2$ hexahydrate solution to remove the worms (Skelton et al., 2016). Any remaining worms on the crayfish were removed by examining the crayfish under a stereoscope and using fine tipped forceps to remove the worms. The crayfish were then placed back in a Whirl-Pak after inspection, and the hexahydrate solution was examined for any worms that had fallen off. To ensure that all worms from the crayfish had been quantified, the stream water in which the crayfish had been placed was also examined for any remaining worms that had fallen off when being transported. The worms were stored in a 75% ethanol solution and the crayfish, after examination, were transported back to their respective collection sites and released.

Total Bacterial DNA Extraction

Total bacterial DNA was extracted from each collected swab and stored at -20°C using a Qiagen DNeasy Blood and Tissue kit according to protocol.

Polymerase Chain Reaction (PCR) of the 16S Ribosomal Subunit

PCR using a C 1000 Touch Thermal cycler was performed on the 16S small ribosomal

subunit to identify bacterial species within the samples. DNA from the previously performed extraction was used as the initial template for PCR. The following reagents were used for amplification: 13 µL of Ultra Clean PCR grade H_2O, 10 µL of 5 Prime Hot Master Mix, 0.5 µL of forward primer + barcode IL 515F (5µM), a different forward primer with a unique fluorescent barcode assigned to each sample, 0.5 µL of reverse primer 806R (5µM) (AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT), and 1 µL of template DNA. Samples were prepared in duplicate with a negative control. Protocol cycles were as follows:

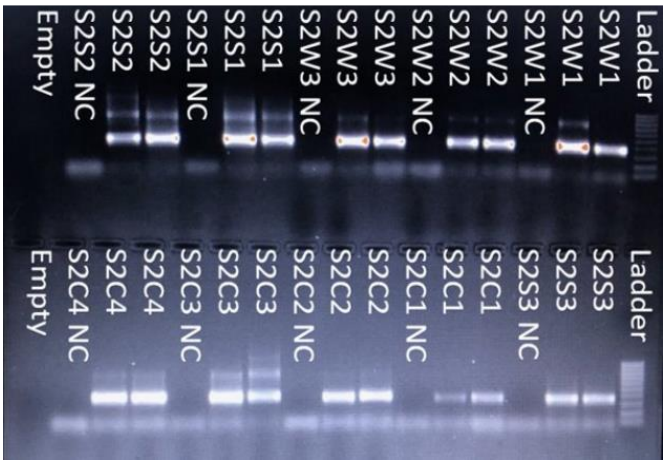
1. 94°C for 3 minutes
2. 94°C for 45 seconds
3. 50°C for 1 minute
4. 72°C for 1.5 minutes
5. Repeat steps 2-4 for 35 cycles
6. 72°C for 10 minutes

Gel Electrophoresis

To ensure that samples had successfully been amplified, gel electrophoresis was used. To make the gel, 50X TAE (242 g Tris base, 57.1 mL Glacial acetic acid, 100 mL of 0.5M EDTA) was diluted and used as gel buffer. A 1.0% agarose gel was made with 12 µL of ethidium bromide, which was added while the gel was stirred. Gel lanes were made using three combs per gel. PCR products were added to wells after the gel had cooled. A low base DNA ladder (100 bp DNA ladder) was added to the first well of each lane as a reference. A ChemiDoc XRS+ gel imager was used to visualize banding. Successful amplification was indicated by a band in the lane for the duplicates and no band in the lanes for the negative controls (Figure 4).

Figure 4

Image of a successful gel



Note. Successful gel images are indicated by a band in each of the duplicate lanes followed by an absent band in the negative control lane indicated by NC.

DNA Quantification and Pooling

To standardize the concentration of DNA from each sample, quantification was performed on each PCR tube using an AccuBlue Broad Range dsDNA quantification Kit. Using the determined values from quantification, the samples were pooled by pulling various volumes of DNA from each tube and pooling into one tube. The volumes varied according to determined concentrations so that 400 ng of DNA was added from each sample. The combined DNA samples were cleaned with Qiagen QIAquick PCR Purification Kit according to protocol.

DNA Sequencing

In total, 132 samples were sequenced, which was performed by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. The sequenced samples were then analyzed using the bioinformatics software QIIME2, and the following analyses were conducted according to QIIME2 protocol (Bolyen et al., 2019).

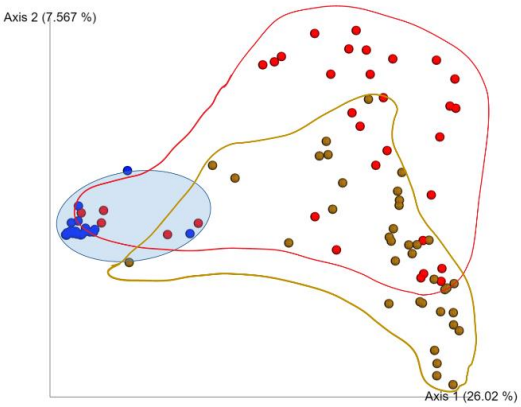
Results

Crayfish Versus Sediment Versus Water

As an initial analysis of the microbiomes, a principal coordinate analysis (PCoA) plot was constructed to compare the relative diversities of bacteria collected from the crayfish shown in red, the sediment near the collection site shown in brown, and the water shown in blue (Figure 5). The plot indicated that the diversities of the crayfish as compared to the surrounding water and sediment were significantly different. This can be seen from the distinct clustering of colors in Figure 5.

Figure 5

PCoA analysis comparing sample types



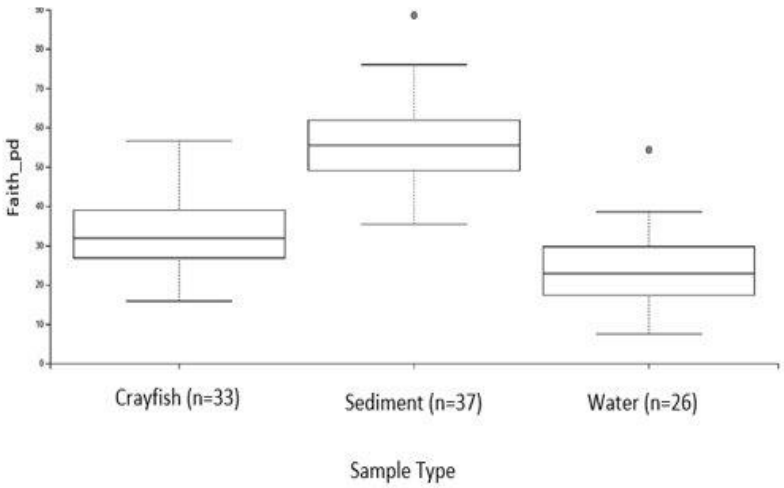
Note. Sediment samples indicated in brown with water samples indicated in blue and crayfish samples indicated in red.

To further confirm whether this separation was statistically significant, a Kruskal-Wallis test comparing the means of each of the three groups' α-diversities was performed. Additionally, a box-and-whisker plot was constructed in Figure 6. Using the Kruskal-Wallis test, *p*-values of less than 0.001 were obtained for comparison of the means of diversity when comparing the

crayfish to the water as well as to the sediment, indicating a statistically significant difference. The values indicated that there was a statistically significant difference in the bacterial composition and diversity between the crayfish and its surrounding sediment and water.

Figure 6

Box-and-Whisker Plot comparing the α-diversities of sample types



Crayfish with Worms Versus Crayfish Without Worms

The second variable studied on the crayfish microbiome was the presence or absence of the ectosymbiotic branchiobdellidan worm. This variable would determine if the presence or absence of branchiobdellidan worms exerted significant changes in the crayfish microbiome. An initial graph was constructed using a PCoA plot to compare crayfish with and without worms (Figure 7). Unlike the plot used to compare crayfish with their surroundings, this PCoA plot did not show distinct clusters between the different variables measured.

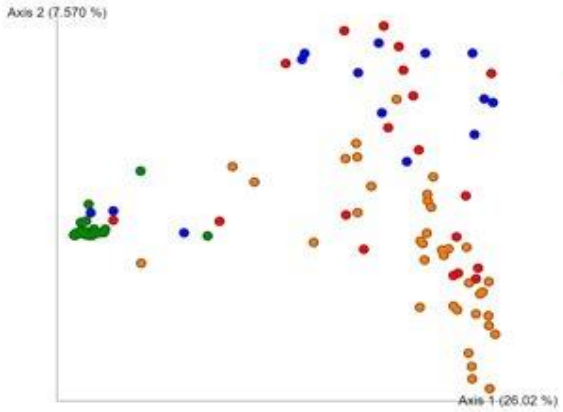
While the PCoA plot indicated that the crayfish with worms were not statistically different from the crayfish without worms, a box-and-whisker plot was still constructed, and a Kruskal-Wallis test was again performed to quantify if differences between the two groups were

statistically significant (Figure 8). A p-value of 0.9419 was obtained, indicating that the two groups were not significantly different in their bacterial diversities in the presence or absence of branchiobdellidan worms.

While a significant difference was not found between the crayfish with worms and crayfish without worms, a trend seemed to appear when viewing the PCoA plot in light of the

Figure 7

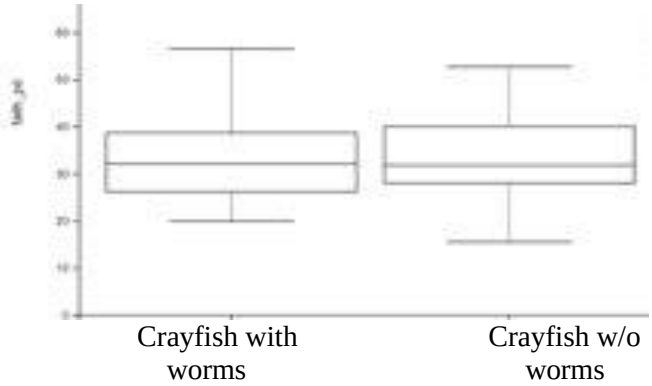
PCoA Plot of Crayfish with and without Worms



Note: Samples from crayfish with worms indicated in red. Samples from crayfish without worms indicated by samples in blue. Samples from sediment are indicated in orange and samples form water are indicated in blue.

Figure 8

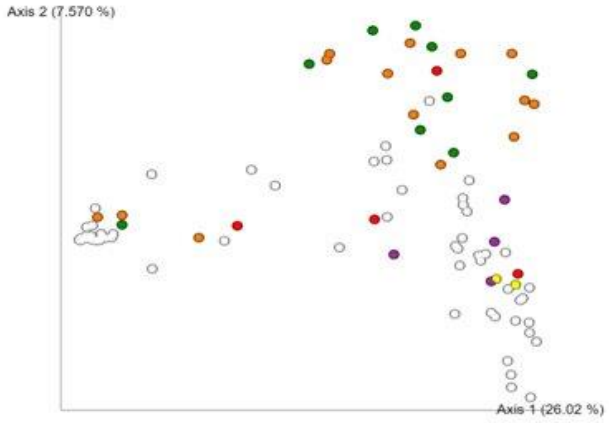
Box-and-Whisker Plot of Crayfish with and without Worms



differing numbers of worms. Crayfish with larger numbers of worms clustered closer to the samples collected from the sediment while crayfish with fewer numbers of worms clustered away from the sediment samples (Figure 9).

Figure 9

PCoA plot of diversity based on the concentrations of worms

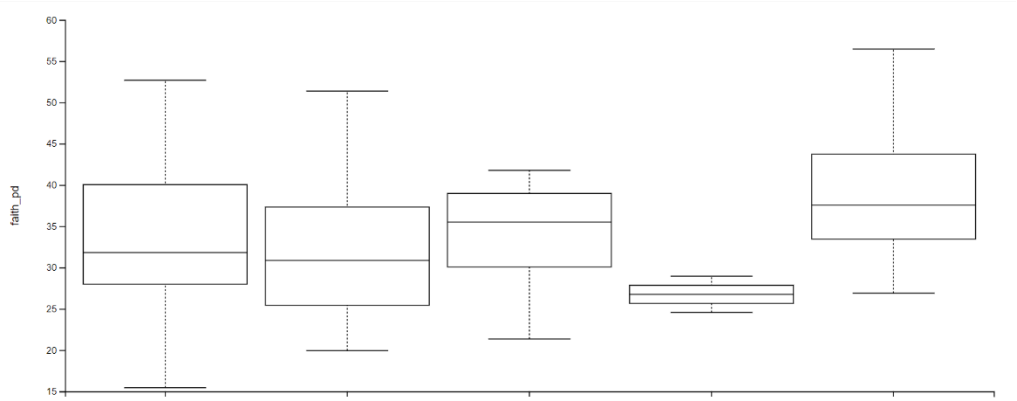


Note: Points in red indicate samples from crayfish with more than 12 worms. Points in orange indicate zero worms. Points in green indicate samples with 1 to 4 worms. Points in purple indicate samples with 5 to 8 worms. Points in yellow indicate samples with 9 to 12 worms.

To confirm whether this trend was statistically significant, a box-and-whisker plot was constructed in which worms were split into five cohorts based on the number of worms present in each sample (Figure 10). A Kruskal-Wallis test was performed in which means of the five cohorts were compared to each other. Ten comparisons were made, none of which indicated statistically significant differences between the cohorts as all ten *p*-values were at least 0.165.

Figure 10

Box-and-Whisker Plot Analyzing Crayfish Samples Based on Cohorts of Worm



Note: Labeled from left to right cohorts are 0 worms, 1-4 worms, 5-8 worms, 9-12 worms, and more than 12 worms.

First Order Versus Second Order

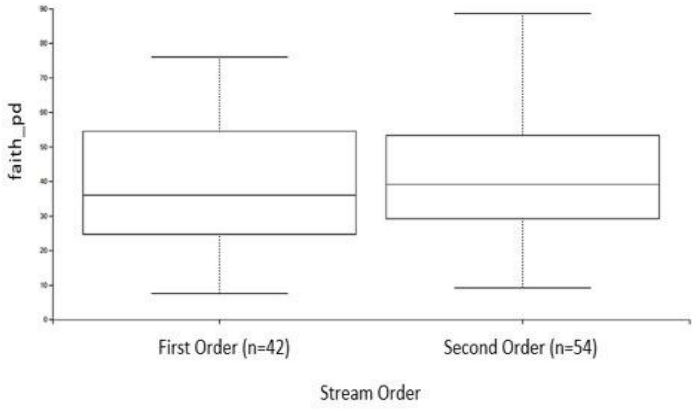
The final comparison used to determine changes in the microbial makeup of the crayfish was the stream orders of the collection sites from which the crayfish were taken. A box-and-whisker plot was used to visualize differences in the diversities of the different samples. A Kruskal-Wallis test was performed to quantify statistical differences between the means of diversity in first and second stream order collection sites (Figure 11). Results from Kruskal-Wallis test indicated that a first and second stream order comparison did not contain a statistically significant difference in bacterial diversity as comparison of the two groups obtained a *p*-value of 0.497.

Bacterial Species

To determine the specific species and their relative abundances, a heat map (Figure 12) was created. Actinobacteria, Bacteroidetes, and Proteobacteria were the most common species,

Figure 11

Box-and-Whisker Plot comparing Stream Orders

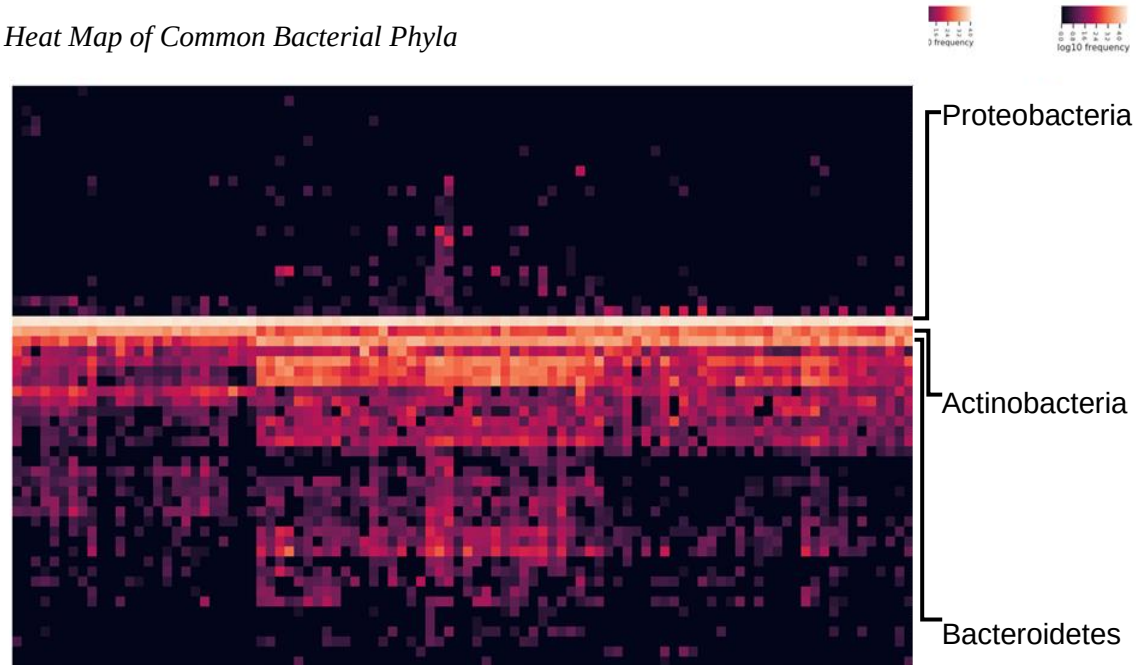


Note: Analysis indicated a *p*-value of 0.497.

being found in 100% of the samples (indicated by the lighter/white bands). These were followed by the phylum Verrumicrobia, which were found in 94% of the samples collected. Proteobacteria was the most common phylum, followed by Actinobacteria and then Bacteroidetes.

Figure 12

Heat Map of Common Bacterial Phyla



Discussion

The purpose of this study was to discover more about the crayfish microbiome, particularly in how it relates to its freshwater surroundings and cohabitating ectosymbionts. While the hypothesized patterns between crayfish and ectosymbionts were not found to exist in these samples, a significantly different microbiome was observed among sample types (water, substrate, and crayfish).

Crayfish Interactions

In this study, the initial hypothesis suggested that branchiobdellidan worms would alter the microbiome of their host crayfish. This was believed to occur because of the interactions that branchiobdellidan worms maintain with their hosts. As noted by multiples researchers, branchiobdellidan worms are known to consume the biofilm of their host organisms (Brown et al., 2002; Jennings & Gelder, 1979; Young, 1966). This ectosymbiont feeding is suspected to act as a disturbance to the microbiome of the crayfish, similar to the way that the presence of urbanization or a natural disaster will alter the microbiome of a stream (Hosen et al., 2017; Reis et al., 2020). For this reason, it was suspected that the presence or absence of branchiobdellidan worms would cause significant changes in the composition of the host microbiome. However, this hypothesis was not supported by the PCoA plot or the Kruskal-Wallis test. These results are the same as found in a 2016 study by Skelton et al. In which they noted that the microbiome of the crayfish carapace was not significantly altered by the presence or absence of worms. Although the biomass of bacteria in this study may have been affected by worm presence, that variable was not measured in this study and does not necessarily influence the diversity and composition of the microbiome on the crayfish carapace (Skelton et al., 2016).

Stream Order and Bacterial Determinations from the Surroundings

The River Continuum Concept conceptualizes a stream as a continuous flow of inputs and outputs from headwaters to river mouths (Vannote et al., 1980). As streams increase from first order, they will initially experience an accompanying increase in species richness that will quickly disappear as the stream orders continue to increase and riparian zone influence over the stream decreases, causing species richness in the stream to decrease with it (Vannote et al., 1980). Instead of allochthonous contributions from the streambeds determining species-richness and type, particular microbiomes within the stream will be regulated by the specific region and conditions surrounding the organisms, an idea termed species-sorting (Henson et al., 2018; Jones & McMahon, 2009). This term originates from metacommunity concept and is specifically concerned with the role that a small environment or "microsite" can play in determining the bacteria that are abundant in that microsite or microsites within it (Henson et al., 2018; Jones & McMahon, 2009). Baas Backing (1934, as cited in Jones & McMahon, 2009) summarizes this idea by noting that "'Everything is everywhere, but the environment selects" (p. 905), highlighting the significance of environment in determining the biological composition of a particular area.

These ideas created two questions for the purposes of this research project. The first was the question of stream order. Multiple research projects have shown differences in microbial diversities of streams with different orders (Kolmakova et al., 2014; Savio et al., 2015). As predicted by the River Continuum Concept, many of these studies have further noted a decrease in bacterial richness of larger stream orders as compared to smaller stream orders (Kolmakova et al., 2014; Savio et al., 2015). This project sought to determine if these trends held true on

crayfish in streams of different orders. Ultimately, a statistically significant difference was not found in this study. While this indicates that the microbiome of a crayfish is not influenced by different stream orders, it is also possible that the stream orders measured in this study were not of a large enough difference to create a statistically significant difference. Whereas many of the streams measured in other studies differed by multiple orders, the streams in this study differed by one order (first versus second order streams). In future research projects, it may be beneficial to study crayfish microbiomes in a wider range of stream orders such as a comparison between first and fifth order streams.

The second question stemming from these concepts was that of species-sorting. To what extent were the diversities of the crayfish bacterial communities a result of the inputs from the surrounding riparian zones and to what extent were they influenced by species sorting and the exact environment surrounding the specific microsites? If environmental filtering played a significant role in determining bacterial composition, it could be suspected that the microbiome of the crayfish would not differ significantly from the surrounding stream and sediment. However, if the crayfish microbiome were being influenced significantly by host filtering, it would be suspected that the crayfish microbiome would differ significantly from its surroundings. The results of this project indicated a statistically significant difference between the microbiome of the crayfish and the microbiome of its surrounding sediment and water. This differed from previous work by Skelton et al. (2016) in which they noted that the crayfish carapaces measured in their study had microbial compositions similar to the surrounding environments. However, overlap in clusters between crayfish and sediment in the PCoA plot

indicated that the environmental filtering may have played a significant, though small, role in determining bacterial composition on crayfish.

Bacterial Species

As noted in the heat map created (Figure 12), the most common phylum of bacteria present across all samples was Proteobacteria, which is similar to previous studies that have found Proteobacteria by far one of the most common phyla on crayfish (Longshaw, 2016). This phylum is characterized as a gram-negative bacterium that contains a very diverse group of microbes that reside anywhere from the human gut to freshwater streams and lakes (Gupta, 2000; Riemann & Winding, 2001). They can be found in large collections of bacteria, often comprising the largest groups in macroaggregates (Weiss et al., 1996). Some species within the Proteobacteria phylum exist on crustaceans as either parasites infecting the reproductive tissues of their hosts or as commensals (Batut et al., 2004; Bouchon et al., 1998). However, in crayfish, this phylum of bacteria has rarely been associated with diseases (Longshaw, 2016). Given that these bacteria are often associated with crustaceans such as crayfish, it is not surprising that Proteobacteria were commonly found in the samples taken in this study. Additionally, multiple studies have shown Proteobacteria to be one of the most common phyla in freshwater biofilms, which is consistent with the results of this study showing Proteobacteria as the most common bacterial phyla in both water and sediment (Battin et al., 2001; Pernthaler et al., 1998; Romani et al., 2016).

The second most common phylum of bacteria found in this study was from phylum Actinobacteria. These gram-positive bacteria are a superficial mix of both fungi and bacteria, with characteristics that allow them to live in a wide variety of both aquatic and terrestrial

environments, playing a large role in much of the decomposition of dead matter that occurs in these different environments (Barka et al., 2016; Maldonado et al., 2005; Ranjani et al., 2016). This phylum of bacteria can often be found in freshwater streams, with some researchers estimating that Actinobacteria may make up more than half of all bacterioplankton in certain freshwater environments (Hahn et al., 2003). For this reason, it is understandable that Actinobacteria were found in a large amount of the samples collected from the streams in this study. However, getting exact data on Actinobacteria can be difficult as this phylum of bacteria is not easily cultured (Hahn et al., 2003). Interestingly, while Actinobacteria have been studied for their possible anti-biofilm properties in some biofilms, they have also been found as the most abundant species in other biofilms (Azman et al., 2019; Bakkiyaraj & Pandian, 2010; Saarela et al., 2004). These anti-biofilm features may also explain why Actinobacteria where abundant on the crayfish samples from this study as researchers have noted that Actinobacteria can act to prevent biofouling, a common problem on crayfish gills (Gopikrishnan et al., 2016).

The final phylum that was found in 100% of samples from this study was the phylum Bacteroidetes. The phylum Bacteroidetes contains roughly 7000 species of bacteria that exist in a myriad of environments from freshwater streams to various different soil types (Thomas et al., 2011). Not only can this phylum be found in freshwater environments, but they are often one of the most abundant with studies finding 40-60% of identified bacteria to be of the Bacteroidetes phylum (Thomas et al., 2011). Given its abundance in freshwater streams, it comes as no wonder that, in this study, Bacteroidetes was one of the most common species in a majority of the water samples. Additionally, Bacteroidetes have been commonly found as a dominant species in both biofilms in marine and freshwater environments as well as on crayfish making it reasonable to

assume that Bacteroidetes would be a common species found within the sediment and crayfish samples from this study (Edwards et al., 2010; Li et al., 2017; Shui et al., 2020).

Future Research

In future studies, it would be beneficial to analyze a wider variety of stream orders than what was studied in this project. Although no significant differences were found between the samples in the first and second order streams, this may simply have occurred because of the similarity between the two streams. However, if this study was exaggerated to a fourth or fifth order stream, it may be found that the bacterial diversities on crayfish differ significantly. In future research, it would be beneficial to compare bacterial diversities within specific sites of the crayfish itself. For example, comparing the gill chamber of the crayfish with its ventral abdomen. Skelton et al. (2016) hypothesizes that the crayfish may be acting as a host filter, creating unique environments purely by the morphology of its body. If this were the case, then it would be suspected that different parts of the crayfish body would yield better or worse environments for particular species and phyla of bacteria as certain areas of the crayfish interact more with the sediment on the bottom of the stream while other parts of the crayfish are restricted to interacting with the water in the stream. Finally, as water quality has often been tied to bacterial vitality, it would be beneficial to study the bacterial diversities on crayfish in relation to the water quality from which the samples were collected.

Conclusion

The initial goal of this project was to study a symbiotic relationship among three types of organisms: the crayfish, the microbiome, and the branchiobdellidan worm in a first and second stream environment. This relationship is understudied as noted by various researchers (Skelton et

al., 2016; Tornwall et al., 2015). Using molecular technique along with next-generation bioinformatics tools, a broader insight into the microbial world was gained. The results of this study showed that the crayfish had a unique microbiome in comparison to its surrounding environment. While this microhabitat differed from the water and sediment samples collected, it did not appear significantly altered when analyzed along a stream-order continuum. This insignificance was also true of samples that were compared with and without ectosymbiotic worms. Ultimately, the definite results of this study indicated that the crayfish provides a unique microhabitat for its associated microbiome. Furthermore, this study indicates that the crayfish's interactions with the stream environment as well as its two ectosymbionts were more complex than what was first hypothesized.

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