Microbial Assemblages in Relation to Host and Environmental Surroundings

Mark Fischer

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Acceptance of Senior Honors Thesis

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Honors Program of Liberty University.

_____
Kyle Harris, Ed.D.
Thesis Chair

_____
Matthew Becker, Ph.D.
Committee Member

_____
Cynthia Goodrich, Ed.D.
Honors Assistant Director

_____
Date

Abstract

The goal of this study was to determine if the microbiome between two crayfish microhabitats were different from one other, in addition to the surrounding water and sediment. This project was a field-based study in which all environmental samples and crayfish were collected from a central Virginian freshwater stream. Molecular techniques were employed to sequence bacterial DNA from the various sample types and Qiime 2 was used to analyze statistical differences. The results from this study aligned well with the initial hypothesis in that the alpha-diversities between all of the sample types were statistically different ($P<0.05$) except for the gill chamber and surrounding water microbial assemblages. In addition, the Beta-diversity was statistically different when the bacterial composition of one sample type was compared to the composition of the other samples ($P<0.05$). This data aids in our understanding of the significance of microbes within a freshwater ecosystem and how they are distributed among freshwater organisms, such as the crayfish.

Microbial Assemblages in Relation to Host and Environmental Surroundings

In the last several years, significant focus has been given to the area of microbiome research. These communities of bacteria inhabit almost every microhabitat on the planet, from human digestive systems to freshwater bodies. This heavy focus is, in part, due the ever-expanding realization of the importance of microbiomes to the health of the organisms in which they dwell (Allison et al., 2008; Shui et al., 2020). A plethora of research has been conducted on the effects of microbiota within a marine context, however, the relationship between freshwater organisms and their microbiome is understudied within freshwater ecosystems (Shultz et al., 2013).

Role of Crayfish within Freshwater Environments

Crayfish are crustaceans that are known to inhabit many freshwater ecosystems including streams, rivers, lakes, and ponds (Skelton et al., 2013). Crayfish are widely distributed around the world in freshwaters and are considered to be a keystone consumer and ecosystem engineer (Brown & Lawson., 2010; Creed & Reed., 2004). Crayfish can significantly alter community structure and ecosystem processing by creating, modifying, or maintaining habitats by controlling the rate in which resources are made available to other species. The primary mode used by crayfish involves their influence of detrital processing rates, leading to the bioturbation of fine and coarse sediment (Creed & Brown., 2004). Crayfish are also involved in a number of symbiotic relationships with ectosymbiotic organisms and microorganisms within freshwater ecosystems. Due to the large role crayfish play within a freshwater context, they are commonly used as a model organism in organismal biology (Helms et al., 2013; Skelton et al., 2017).

Symbiosis

Symbiotic relationships are prevalent throughout all species and consist of intimate interspecific interactions with prolonged physical contact (Moran., 2006; Silknetter et al., 2019). They have been shown to affect populations, communities, and ecosystems by creating novel flows of material and energy throughout food webs (Tipton et al., 2019). Host specificity can have considerable variation among symbionts in which some symbionts display an extremely high degree of plasticity in host selection, while others show perfect fidelity to a host species (Sepahvand et al., 2020). The mechanisms underlying these interactions are largely unknown and understudied.

Symbiotic interactions are described as a dynamic continuum which can vary from parasitism to mutualism depending on biotic and abiotic factors of the environment in which they coexist (Skeleton et al., 2014; Thomas et al., 2016). Mutualism has been defined to be an interaction between two organisms in which both species involved receive a measurable net benefit (Silknetter et al., 2019). Commensalism lies at the middle of the dynamic spectrum and occurs when one organism benefits from the symbiotic relationship, while the other organism has no net cost or benefit. Lastly, parasitism occurs when one organism benefits at the expense of the other organism's health. This study focuses on two symbiotic relationships in which crayfish partake in with ectosymbiotic organisms (Silknetter et al., 2019).

Crayfish Ectosymbionts

The first symbiotic relationship that is important within the context of this research project exists between crayfish and branchiobdellidan worms (BWs). BWs have been found to reside on multiple body parts of *Cambarus spp.* including the carapace, ventral abdomen, and

gill chamber (Hoverson, 2020; Skelton et al., 2013). The relationship between BWs and crayfish is considered to be obligate for BWs, because successful reproduction has only been recorded on a live crustacean host, commonly, on the ventral abdomen region (Ames et al., 2015; Creed et al., 2015). The ventral abdomen has also been noted to be a common site for BW eggs to be found, due to the protection that is afforded by the crayfish host (Yazicioglu et al., 2016). It is for this reason that the microbiome of the ventral abdomen was investigated in the current project.

The second site that BWs have been known to inhabit is the gill chamber of crayfish. BWs located in the gill chamber have been found to partake in a cleaning symbiotic relationship in which they remove fouling agents from the gills (Brown et al., 2002; Skelton et al., 2014). Most of what is known about cleaning symbiotic relationships comes from the study of marine ecosystems while freshwater cleaning symbioses are understudied. This relationship between the crayfish and BWs varies along a dynamic continuum with parasitism at one end of the spectrum and mutualism towards the other (Skelton et al., 2017; Thomas et al., 2016). The outcome of the symbiotic relationship is altered in contingency with varying host-symbiont factors. The relationship between crayfish and BWs has been shown to lean more to the mutualistic end of the spectrum when a moderate number of BWs have been found in the gill chamber (Brown et al., 2012; Lee et al., 2009). The BWs are then able to graze on the biofilms and particulate matter on the gill filaments without harm to the crayfish. However, if the load of BWs increases, the bacteria and particulate matter are no longer able to support the worms (Farrell et al., 2014; Thomas et al., 2016). At this point, BWs have been noted to target the gill filaments themselves, causing gill scaring, shifting the symbiotic relationship closer to parasitic due to the harm inflicted on the crayfish by BWs (Lee et al., 2009; Skelton et al., 2014).

The second major symbiotic relationship that is fundamental to this project exists between crayfish and their microbiome. Bacteria, fungi, and other microscopic organisms reside over the length of the crayfish. Bacteria have been documented to be the primary source of food for BWs, which could potentially influence the BW load experienced by crayfish (Brown et al., 2002; Skelton et al., 2017).

Microsites

Microsites or microhabitats consist of a particular domain on or inside the host's body that provides residence for symbionts (Dittmer et al., 2014; Sepahvand et al., 2020). Microhabitats or specific tissues may vary with respect to the resources they offer, or the risk of mortality within each domain (Johnson et al., 2012). This can affect the distribution of symbionts throughout a host organism. A study was conducted with ghost shrimp to determine whether their ectosymbiotic organism (copepods) were selective in terms of inhabiting certain microsites on the ghost shrimp. The study concluded that one species of copepods was primarily found on the carapace and gill chamber of ghost shrimp, while the second species had a stronger preference for the chelae and anterior walking legs or ventral abdominal region (Sepahvand et al., 2020). This phenomenon has also been observed between crayfish and BWs in which they have been found to inhabit the gill chamber, carapace, and ventral abdomen at higher frequencies in comparison to other microhabitats on crayfish. For this reason, the gill chamber and ventral abdomen have been selected to the focus of this study to examine how the microbiome of each microsite is similar or different due to environmental factors in addition to symbiotic relations.

Microbiome

The field of microbial research has increased within past years, as its importance in determining the health of various ecosystems and organisms has been realized (Shui et al., 2020; Skelton et al., 2017). Microbial biodiversity is a key component to the sustained health of host organisms in addition to the larger community (Dudgeon et al., 2006). With bacterial assemblages being the largest group of organisms within a freshwater ecosystem, it is important to understand what roles they play within an ecosystem, but also in terms of singular organisms (Tipton et al., 2019). Bacteria have been shown to have important functions within freshwater environments such as decomposing organic matter, nutrient recycling, reducing toxic nitrogen, and competitively excluding potentially pathogenic microbes (Cardona et al., 2016; Skelton et al., 2017). Despite the growing knowledge of the importance of bacterial functions within freshwater environments, it is severely understudied (Dudgeon et al., 2006). This project aims to shed light onto the selective nature of bacteria on a host organism, and how their composition compares to the surrounding environment.

River Continuum Concept

One important concept that was created to better understand the distribution of macroinvertebrates within freshwater streams is the river continuum concept (RCC). The RCC was first hypothesized in 1980 to describe how rivers are longitudinally linked systems in which biotic assemblages are orderly, due to the connection of downstream ecosystems with those upstream (Vannote et al., 1980; Sedell et al., 1989). This concept explains the mechanism of biodiversity within a river system and can be used to make assertions about the distribution of bacteria and other microbes along a river in a predictable manner (Tornwall et al., 2015). The

RCC also highlights the complexity of the relationship between crayfish and the microbial assemblages that exits within the surrounding freshwater environment. While this project is only assessing crayfish within a second order stream, the RCC model was still be applied in the formation of the hypothesis. Due to the constant flow of bacteria both in the water in addition to the surrounding sediment, we predicted that the gill chamber would have a similar microbiome in comparison to the surrounding water samples that were taken. It would also be reasonable to state that the ventral abdomen of the crayfish would have a similar microbiome with the surrounding sediment due to their benthic nature and ability to burrow under rocks and other sediment.

Project Focus

The primary focus of this research project was to assess the relationship between two microsites on freshwater crayfish located in a central Virginia stream and to compare their bacterial composition to the surrounding environment. This was accomplished by extracting and sequencing bacterial DNA that was collected from swabs of crayfish in addition to the surrounding water and sediment. Qiime 2 will then be used to analyze the data and assess the relationships between the microbiomes of both microsite and environmental samples. The goal of this project is to increase our understanding of microbe distribution within a freshwater environment in relation to crayfish. This knowledge could then be used in the future to determine the overall health of freshwater ecosystems based off of the bacteria present or absent on key species. This project also aims to shed light on the complex symbiotic relationships that exist between crayfish and their microbiome and ectosymbionts.

Previous Research

This lab previously researched the microbiome of crayfish specifically in relation to their carapace, and how the microbiome is affected by the presence or absence of BWs (Holman et al., 2016; Hoverson., 2020). A study was conducted in vitro in which the experimental group had four BWs placed on the carapace, while the control group had no BWs present. DNA sequencing was then performed on the bacteria in each group. The findings of this experiment demonstrated that there was no overlap between the bacterial species found on the experimental group compared to the control group with no BWs (Figure 1; Holman et al., 2016).

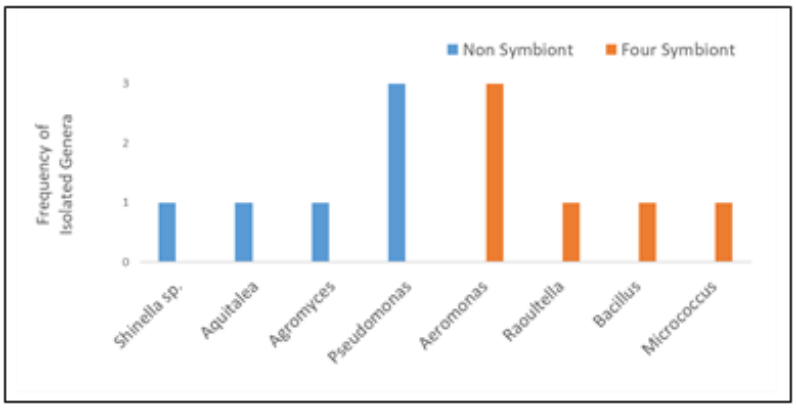

Figure 1. Most common bacterial species on crayfish carapace. Blue bars represent bacterial species from the control group while orange bars represent bacterial species from experimental group. From "Ectosymbiotic relationships between the Appalachian Brook Crayfish (*Cambarus spp.*) and the Branchiobdellidan *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 study seemed to suggest that BW presence significantly altered the microbiome of the carapace of crayfish. This phenomenon was further researched by a field-based study that examined the microbiome of crayfish that were collected from a central Virginia stream (Hoverson., 2020). The carapace was swabbed, and bacterial DNA was sequenced to determine whether the previous discovery was representative in nature. However, the diversity of bacterial species between the two groups was not statistically significant in this study ($P>0.05$). The major finding from this project demonstrated that there was a statistically significant difference between the microbiome of the crayfish carapace, water, and sediment ($P<0.05$). These relationships seem to support the hypothesis that there is a host filtering mechanism that occurs on crayfish to alter their microbiome, separate from the effect of BW density cleaning symbiotic relations (Figure 2; Skelton et al., 2017).

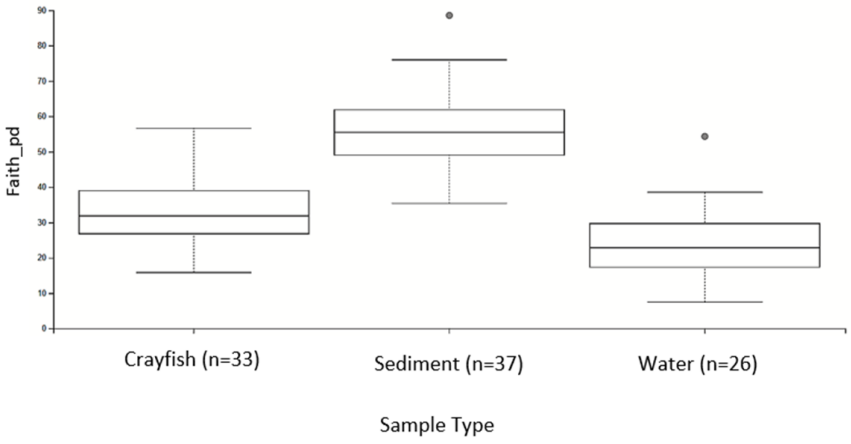

Figure 2. Box-and-whisker plot comparing the α-diversity between sample types.

Current Research Project

This study seeks to further our understanding of the crayfish microbiome within a freshwater ecosystem by specifically targeting two microsites located on crayfish. The foundation of this project was built off findings from previous studies that examined branchiobdellidan worm distribution patterns along *Cambarus spp*. of crayfish in addition to preliminary microbial studies of the carapace (Brown et al., 2012; Hoverson., 2020). For these reasons, the gill chamber and ventral abdominal region were selected as the focal points for our microbial study. First, crayfish were collected from Opossum creek which is located in central Virginia (Figure 3). Bacterial swabs were taken on-site of the surrounding water and sediment. The crayfish were then brought back to the lab and were swabbed on the same day using aseptic technique at the two microsites. DNA extractions, PCR, and sequencing were performed on the bacteria DNA to isolate the 16s gene which is commonly used to identify the species. QIIME 2 was then used to analyze the data and perform statistical tests to determine the significance of relationships between the various sample types. The use of these methods allows for a better understanding of the diversity of the crayfish microbiome within their natural environment. This increases the applicability of the data within freshwater ecosystems and helps us better understand the various symbiotic relationships that can alter freshwater organisms.

Materials and Methods

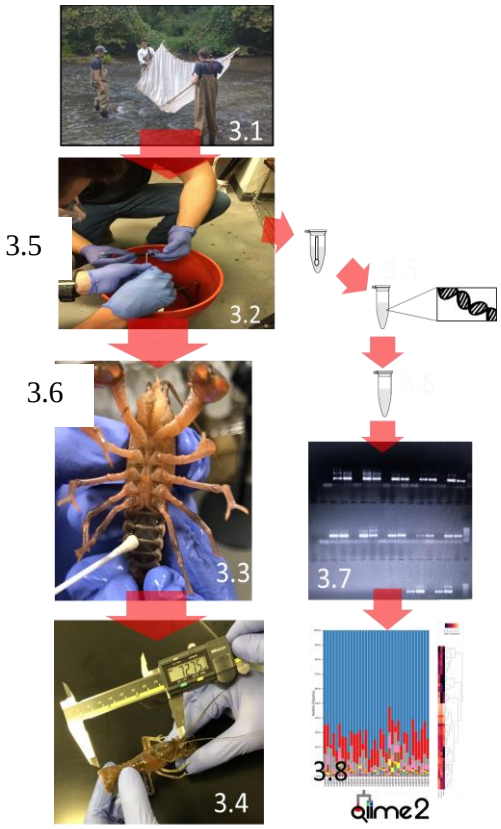

Figure 3. Experimental Methods. (3.1) Crayfish collection using kick-seine technique. (3.2-3.3) Crayfish were taken back to lab and were swabbed in duplicate using aseptic technique. (3.4) Physical characteristics of crayfish were recorded such as blotted wet mass (BWM), weight (g), and sex. (3.5) DNA was extracted from swabs. (3.6) PCR was performed to amplify bacterial 16s gene. (3.7) Gel electrophoresis was used to verify that samples were successfully amplified, and that no contamination was present. (3.8) Qiime 2 was used to analyze statistical relationship between microbiome of various sample types.

Crayfish Collection

Crayfish were collected from Opossum Creek, located in Lynchburg, Virginia. Upon arrival to the collection site, environmental swabs were taken of the water and sediment using aseptic technique. Crayfish collections started upstream and moved down the river until enough

crayfish were collected. A kick seine was used to capture the crayfish, which were then transferred into separate Whirl-Pac's to avoid cross contamination during travel to the lab.

Microbial Swabs

Environmental swaps of the surrounding water and sediment were taken at the collection site before the stream was disturbed by collection of crayfish. Crayfish swabs were done at the laboratory in which each crayfish was washed in diH_2O twice before swabbing was performed. The person handling the crayfish changed gloves between each organism to avoid cross contamination of samples. Two microsites were swabbed using a single rayon swab at each site. First, the ventral abdomen was swabbed from the last pair of walking legs to the telson for a five second interval. Swabs were taken in duplicates and transferred to sterile 1.5 mL Eppendorf tubes immediately after swabs were taken. Next, the posterior gill filament was removed and swabbed in duplicate. A total of 60 swabs were collected, 10 swabs from both the water and sediment samples, and 10 swabs from each microsite plus a duplicate. Once all microbial swabs had been collected, they were stored at -20°C. Physical dimensions were then taken of each crayfish including total length, blotted wet mass, and sex.

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)

PCR was performed using a C 1000 Touch Thermal cycler on the samples after DNA had been successfully extracted. The bacterial 16S gene was targeted for amplification due to its unique "fingerprint" that varies between species of bacteria. PCR was performed using a C 1000

Touch Thermal Cycler. Each DNA extraction obtained in the previous step was used as template DNA for gene amplification. To amplify, the following reagents were used: 13 µL of Ultra Clean PCR grade H_2O, 10 µL of 5 Prime Hot Master Mix, 0.5 µL of reverse primer 806 R (5 µM) (AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT) + barcode IL 515F (5µM), a different forward primer with a unique fluorescent barcode assigned to each sample, 0.5 µL of reverse primer 806R (5 µM), and 1 µL of template DNA. Each sample was prepared in duplicate alongside a negative control. Protocol cycles were as followed:

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 verify that the bacterial DNA had been successfully amplified and no contamination occurred from the process, gel electrophoresis was implored. Gels were made using a standard procedure which consisted of using 50X TAE (242 g Tris base, 57.1 mL Glacial acetic acid, 100 mL of 0.5M EDTA) which was diluted and used as gel buffer. A 1.0% agarose gel was made with 12 µL of ethidium bromide, which was added after the gel had cooled to room temperature and was being stirred. After the gel was poured into the mold, 3 combs were placed to create wells. A low base DNA ladder (100 bp DNA ladder) was added to the last well of each lane as a reference. Each gel was run for 20 minutes at 200 V or until the bands travelled to the end of the

gel. A ChemiDoc XRS+ gel imager was used to visualize banding. Successful amplification was indicated by matching bands in the two lanes for the duplicates and no band in the lanes for the negative controls (Figure 4).

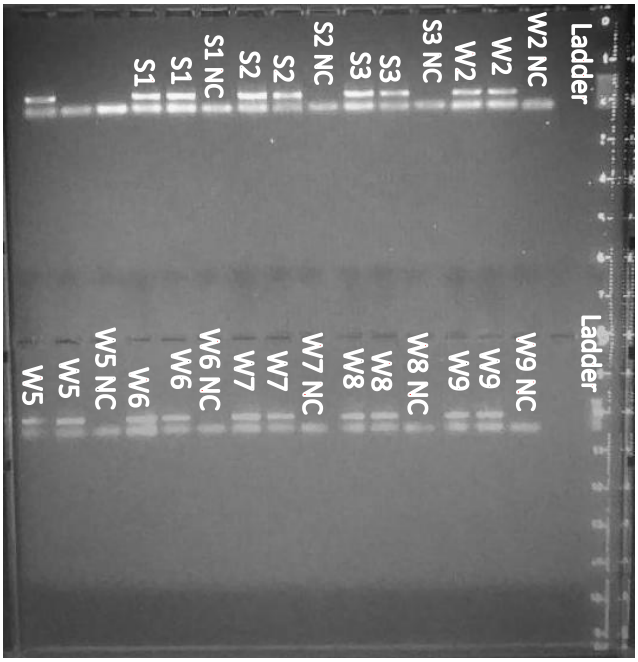

Figure 4. Image of successful gel. Successful gel images are indicated by a band in each of the duplicate lanes followed by an absent band in the negative control lane indicated by NC.

DNA Quantification and Pooling

To standardize the concentration of DNA from each sample, quantification was performed on each PCR tube using a Thermo Scientific NanoDrop 2000 Spectrophotometer. Using the determined concentrations 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

Bacterial DNA samples were then sequenced by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. In total, 40 samples were sequenced, and included in this study. The samples were then analyzed using the bioinformatics software QIIME 2, to determine statistical significance and to create tables to visualize the relationships. The following analyses were conducted using QIIME 2 protocol (Bolyen et al., 2019).

Data Analysis

A Kruskal-Wallis test was used on the data set to compare the α-diversities between the four different sample types. The α-diversities in this project compares the species richness in each sample type and were computed using QIIME II. The Kruskal-Wallis test determined whether the medians of each samples α-diversity was statistically different ($P<0.05$).

The second statistical analyses test used was a permutational multivariate anova (PERMANOVA) test which compared the Beta-diversity between each sample. This measured the specific bacterial composition within each sample type to compare the centroids of each bacterial cluster. The data was then displayed using a Bray-Curtis Principal Coordinate Analysis (PCoA) plot and significance plot.

Results

Alpha-Diversity of Microbial Assemblages

To determine the statistical significance between the α-diversities of various sample types, a Kruskal-Wallis test was used to obtain p-values. The data was then put into the form of a

box-and-whisker plot to visual the differences or similarities between the species richness of various sample types (Figure 5). The initial hypothesis of this experiment predicted that the microbiomes between the gill chamber and water samples would be similar. This was confirmed by the Kruskal-Wallis test, with a P-value of 0.514 was obtained demonstrating that the two sample types were not statistically different.

A P-value of less than 0.05 was calculated when the microbiome of the ventral abdomen was compared to the sediment samples. This demonstrated that the two sample types were significantly different in relation to their α-diversities, meaning that the microbial assemblage richness of the ventral abdomen varied significantly compared to the bacterial richness of the sediment samples. This finding opposed the initial hypothesis which predicted that the two samples would have similar microbiomes due to the large degree of interaction between the two. (Figure 5)

Overall, the microbiome of the crayfish differed from that of the surrounding environment. The only comparison that was not statistically significant was the microbiomes of the gill chamber and water. However, the gill chamber bacterial species richness was statistically different than the sediment, and the ventral abdomen was statistically different from both the water and sediment (Figure 5).

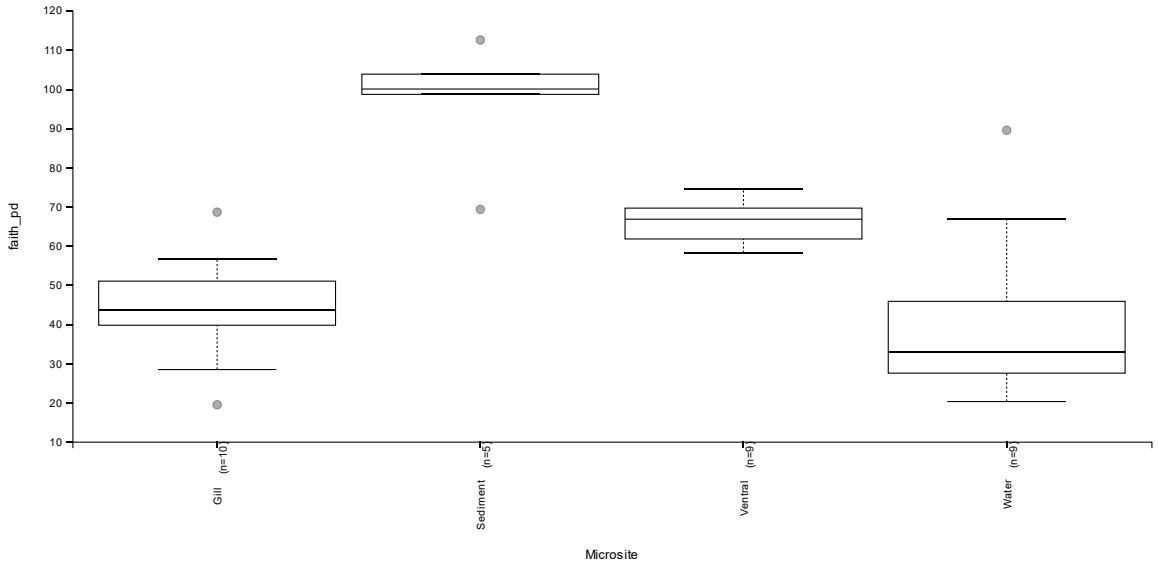

Figure 5. Box-and-Whisker Plot comparing α-diversity between sample types.

The two microsites on the crayfish were also compared to determine the relationship between the microbiomes of the gill chamber and ventral abdominal region. Using the Kruskal-Wallis test, a P-value of 0.001 was obtained demonstrating that the two microsites were statistically different.

Beta-Diversity of Bacterial Composition

The Beta-diversity between each sample type was analyzed using a PERMANOVA test to compare the bacterial composition between each sample's microbiome. A P-value of less than 0.05 was obtained when each individual sample was compared to the other three samples (Figure 6). These results were also expressed using a Bray-Curtis PCoA plot to visualize the difference between bacterial composition of each sample (Figure 7). A Jaccard test was also performed to compare against the PERMANOVA, and similar results were obtained showing that each sample had a statistically different bacterial make-up.

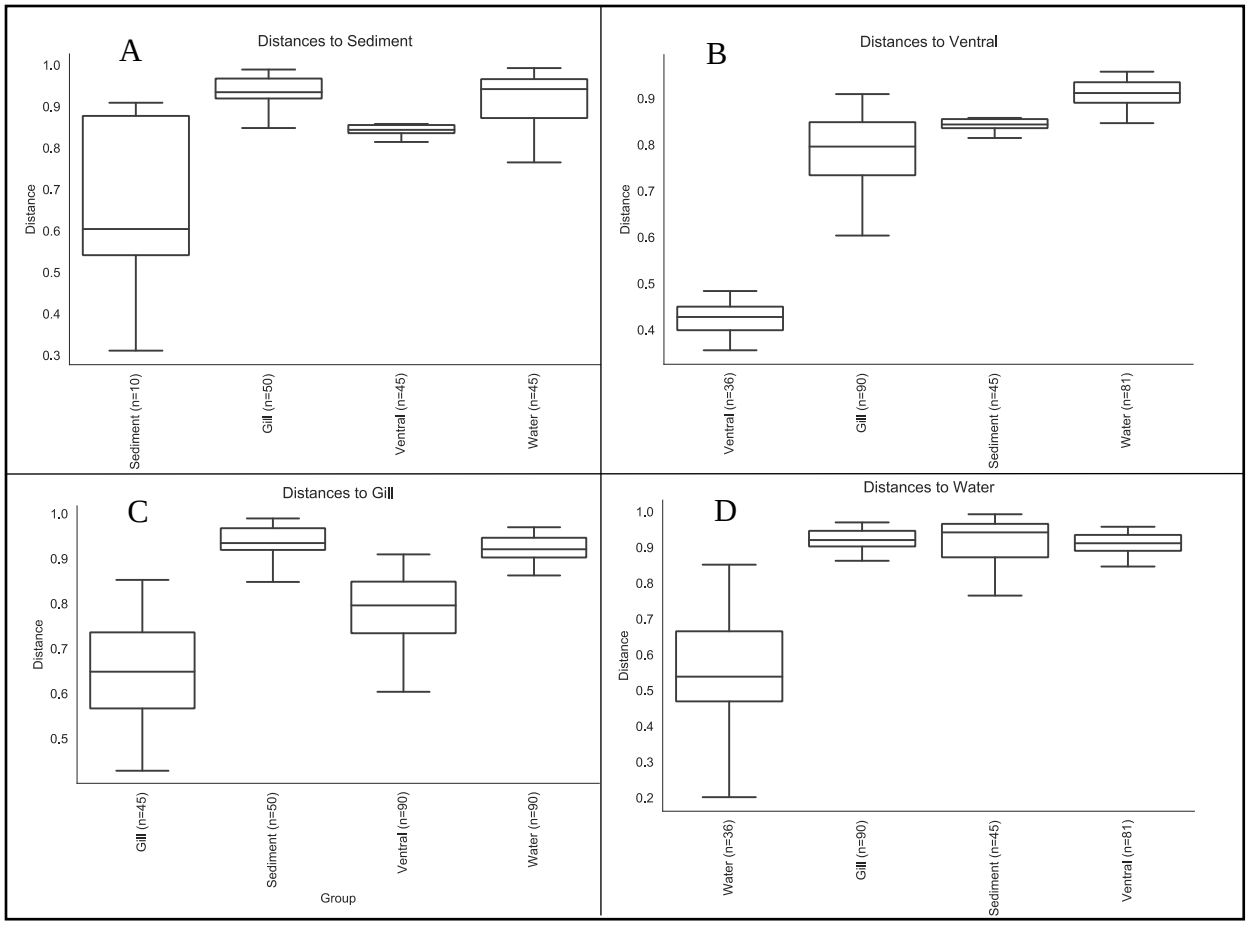

Figure 6. Bray-Curtis pairwise permutational multivariate anova (PERMANOVA) significance plots. Compares distance between microbial compositions of each sample type. All four comparison have statistically different bacterial compositions in comparison to other three sample types ($P<0.05$). (A) Compares sediment bacterial composition to other three sample types. (B) Compares ventral abdomen microsite of crayfish to other three sample types. (C) Shows bacterial composition of crayfish gill chamber in comparison to other sample types. (D) Compares water microbial assemblage composition to other sample types.

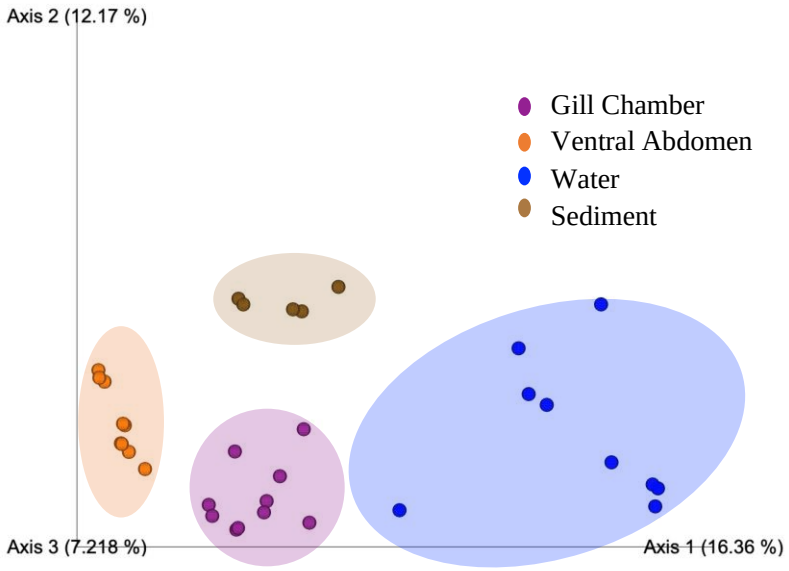

Figure 7. PCoA plot comparing microbiomes of sample types.

Bacterial Species

The microbiomes of both crayfish microsites and environmental samples were dominated by 3-4 major phyla of bacteria (Figure 8). The most common bacteria present throughout all of the samples types was Proteobacteria which accounted for 54.8% of all bacteria sequenced from our samples. Proteobacteria was also the only phylum that was present in 100% of the samples that were sequenced. The second most abundant bacterial phylum from all four samples types was Actinobacteria which comprised of 13.4% of the sequenced samples. Phylum Bacteroidetes accounted for 12.6% of the bacterial samples and was the third most abundant phylum. The sediment and ventral abdomen samples had a disproportionally high percentage of Planctomycetes bacteria and made up 6.9% of these two sample types.

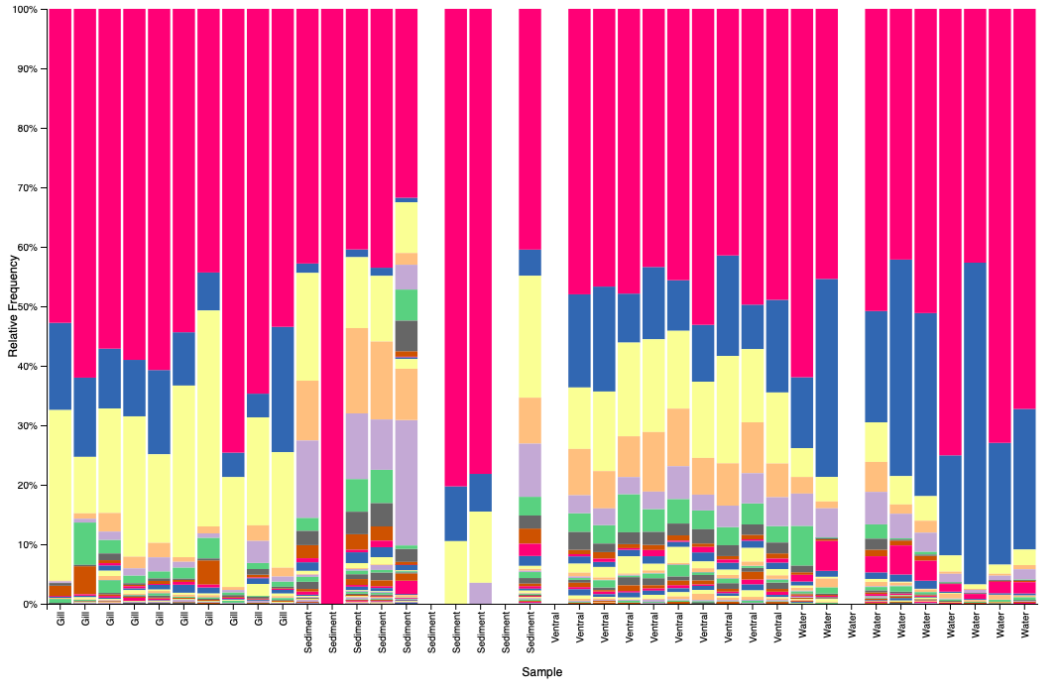

Figure 8. Taxonomic bar plots of bacterial species in each sample type. Red bars represent Proteobacteria phyla of bacteria. Blue bars depict Actinobacteria phylum. Yellow bars represent Bacteroidetes phyla. Orange bars show Planctomycetes phyla of bacteria.

Discussion

The purpose of this study was to explore the microbiome of two crayfish microsites in order to draw inferences between their diversity in light of the surrounding freshwater environment. Many of the relationships between the α-diversities of various sample types were found to be significantly different which supported our initial hypothesis.

Microsite Specificity

In this study, the initial hypothesis suggested that the microbiomes between the gill chamber and ventral abdomen would be different due to a number of host and environmental factors. This assumption was drawn due to a previous study that originally compared the microbial assemblage of the gill chamber to the carapace of crayfish (Skelton et al., 2017). The

study observed that the gill chamber had a limited microbiome in terms of bacterial diversity and that the bacterial richness of significantly different to the surrounding environment. This was attributed to a host immune mechanism that restricted successful colonization of most environmental taxa on the gill filaments (Skelton et al., 2017). Another aspect of this study examined the cleaning symbiosis that takes place in the gill chamber of crayfish in which BWs graze on associated biofilms. While there was no evidence to support the role of BWs in limiting the density of bacteria on gill filaments, they indicated that there could be a possible connection. In contrast to the gill chamber, the microbiome of the carapace of crayfish exhibited a positive correlation with the richness of the surrounding environment. This demonstrated that the richness of the carapace is determined by the richness of the surrounding environment (Skelton et al., 2017).

To build off of these previous findings, we examined two microhabitats in which BWs are commonly found on *Cambarus spp.* of crayfish. The results obtained by this research project had many similarities to the previous studies in regard to the relationship between the two microsites. The gill chamber and ventral abdomen had significantly different microbiomes ($p<0.05$) suggesting that a host filtering mechanism occurs along gill filaments to alter and influence the successful colonization of bacteria. The delicate nature of gill filaments, and the important role that they play in respiration are possible reasons to why the microbial assemblages in the gill chamber are tightly regulated. However, the exact mechanism of this phenomenon is still unknown and could be used as a focal point of future research projects.

Environmental Influences

This project also explored how the α-diversity of crayfish microhabitats compared to the α-diversity of environmental samples. It was hypothesized that the microbiome of the gill chamber microbiome would closely resemble the species richness in the surrounding water, while the ventral abdomen microbiome would be similar to the sediment samples. After the data had been analyzed, the α-diversities between the gill chamber and water samples had a P-value of greater than 0.05 indicating that the two sample types did not have statistically different microbiomes. This finding aligned well with our initial hypothesis but varied from the data of previous research. The previous project that examined the microbiome of the gill chamber in relation to the environment determined that the environmental richness and gill richness were not correlated (Skelton et al., 2017). While this finding did support the difference in diversity between the gill chamber and sediment samples ($P<0.05$); the water and gill chamber had overlapping microbiomes in this study. This could be due to the large extent of interaction between the gill filaments and surrounding water, as water is continually flowing over the gill filaments as oxygen is extracted during respiration. Despite the varying results between our two studies, it does seem as if a host filtering mechanism occurs within the gill chamber to prevent the colonization of potentially pathogenic bacteria on gill filaments. However, these filtering mechanisms do not seem to completely prevent the formation of biofilms containing bacteria that are suspended in the water.

A second statistical test was also used to compare the Beta-diversities between each of the sample types and measured the bacterial composition. The results from the PERMANOVA test showed that the bacterial composition of each sample type was statistically different

($P<0.05$) from each of the other three sample types. This demonstrated that not only the bacterial richness (number of bacteria) differed between sample types, but also the composition of bacteria.

The ventral abdomen was the other microhabitat examined in this study due to the high number of BW cocoons that have previously been found in this area (Hoverson., 2020). When the α-diversity of the ventral abdomen was compared to both the surrounding water and sediment samples, a P-value of less than 0.05 was calculated by the Kruskal-Wallis test. This indicated that the bacterial richness of the ventral abdomen was significantly different compared to both environmental samples. This discovery allowed us to reject the initial hypothesis which we stated that the ventral abdomen would have a similar microbiome to the surrounding sediment. This hypothesis was formed due to the fact that crayfish are benthic organisms that spend most of their lifetime in contact with riverbeds or burrowed between rocks and other loose sediment (Brown & Lawson., 2010). In addition, a different study examined the carapace microbiome of crayfish and discovered that the species richness of the carapace was directly proportional to the surrounding environment (Skelton et al., 2017). Despite these earlier findings, the results obtained from this experiment suggest that a host or environmental filtering mechanism occurs to significantly alter the microbiome of the ventral abdomen in comparison to the surrounding water and sediment. This could be due to the important role performed by the ventral abdominal region of female crayfish in housing and protecting crayfish eggs which are bound to the swimmerets (Yasicioglu et al., 2016). As noted earlier, the ventral abdomen is also where BWs lay their cocoons due to the protection that is afforded by the tail, in addition to the constant flow of water over this body region.

However, these results are from a preliminary study, due to the limited number of samples that were collected. In addition, not all samples were able to be successfully sequenced due to having insufficient amounts of DNA present in the sample. With this being said, future projects could replicate this study with an increased sample size to increase the statistical significance of this projects results.

Bacterial Species

As noted by Figure 8, the most common and abundant phylum of bacteria present across all four sample types was Proteobacteria. This finding aligns well with previous studies that examined the microbiome of crayfish and concluded that Proteobacteria is by far one of the most prevalent species on crayfish (Longshaw, 2016). Proteobacteria are consist of gram-negative bacterium that inhabit a wide variety of environments from freshwater habitats to the human digestive system (Gupta, 2000). Proteobacteria have been considered to be a potentially pathogenic bacteria that often leads to a chronic infection of the host organism. The aim of the invading microorganism is not to kill the host cells, but rather to sustain them, thereby sustaining its own growth and development (Batut et al., 2004). Despite the potentially harmful nature of Proteobacteria, it has been noted that this phylum of bacteria rarely causes a pathogenic state in crayfish (Longshaw, 2016). In addition, a number of other studies have previously discovered that Proteobacteria are one of the most common phyla in freshwater biofilms, which explains why it is the most abundant phyla in both the environmental samples and crayfish samples (Battin et al., 2001; Romani et al., 2016).

The second most abundant phyla of bacterium present in 97% of the samples from this study was Actinobacteria. Actinobacteria are a gram-positive bacterium that live in a wide range

of environments including aquatic and terrestrial habitats. They play a fundamental role in the decomposition of organic matter and replenishing the supply of nutrients in soil and sediment (Ranjani et al., 2016). Actinobacteria are commonly found in freshwater streams and have been estimated to make up more than half of all bacterioplankton within these types of environments (Hahn et al., 2003). Due to their important role in nutrient recycling and composition of a freshwater stream's normal microbiota, it is understandable that a high percentage of Actinobacteria were found on both the environmental and crayfish samples of this study. The highest percent composition of Actinobacteria from this study was found in the water samples (Figure 8) and made up 27.3% of the bacteria. This finding aligns well with previous studies and highlights the importance of Actinobacteria within a freshwater context.

The third must abundant phyla of bacteria found in 97% of the samples was Bacteroidetes which are commonly found in both aquatic and terrestrial environments. It has been previously noted that up to 40-60% of bacteria in freshwater ecosystems can be identified from the Bacteroidetes phyla (Thomas et al., 2011). These findings would explain the high prevalence of Bacteroidetes in this study. Interestingly, the percent composition of Bacteroidetes was 10% higher on the two crayfish microsites in comparison to the surrounding environment samples (water and sediment). This seems to indicate that the crayfish provides a safe environment that allows Bacteroidetes to thrive through the formation of biofilms in the gill chamber and ventral abdomen (Edwards et al., 2010; Shui et al., 2020).

The fourth most abundant phyla that was primarily located on the ventral abdomen and gill chamber of the crayfish was Planctomycetes. Planctomycetes are considered to be an unusual phylum of budding bacteria that are experiencing increased relevance in microbial ecology

research (Fuerst, 1995; Wu et al., 2012). One unique aspect of Planctomycetes is that they are one of the only phyla of bacteria that do not synthesize the otherwise universal bacterial cell wall peptidoglycan. They also experience internal compartmentalization which is usually a unique feature to eukaryotic cells (Fuerst, 1995). The main function of Planctomycetes within freshwater and marine environments involves their role in anaerobic oxidation of ammonium (anammox) (Wu et al., 2012). While this study did observe that the abundance of Planctomycetes bacteria on the two crayfish microsites, the importance of this relationship is still unknown. The discovery of this phylum of bacteria within freshwater ecosystems is still a relatively new finding, and further research will need to be conducted on what type of symbiotic relationship occurs between Planctomycetes and the host organism.

Future Research

Our current research project observed a number of novel discoveries involving the crayfish microbiome in relation to specific microhabitats and the surrounding environment within a freshwater environment. However, these are preliminary findings that are based off a limited sample size. This project could be furthered through the collection and analyzation of a larger sample pool of both crayfish and environmental sample types. This would allow for a more holistic understanding of the complex interactions between crayfish microsites and the surrounding water and sediment.

This study determined that both the gill chamber and ventral abdomen had significantly different bacterial assemblages in comparison to the surrounding environment with the exception of the gill chamber and water. A future biochemical study could be conducted to examine the specific host or environmental filtering mechanisms that occurs to inhibit the successful

colonization of environmental microbes onto the gill chamber and ventral abdomen. This would give greater insight into our understanding of microbiomes within a freshwater context.

Conclusion

The purpose of the study was to gain a better understanding of symbiotic interactions between crayfish and their microbial assemblages within a freshwater environment. These relationships are understudied as noted by previous researchers, despite their importance in the overall health and well-being of the ecosystems in which they exist (Skelton et al., 2016). Through the implementation of molecular techniques and bioinformatic tools, bacterial DNA was successfully sequenced and analyzed to uncover the unique relationship between the various sample types microbiomes. The results of this study demonstrated that all sample types besides that crayfish gill chamber and surrounding water had statistically significant microbiomes ($p<0.05$). This indicates that crayfish exhibit some level of host filtering to selectively inhibit or promote the colonization of certain types of bacteria within various microsites. However, due to the high degree of interaction between the gill chamber and constant flow over water over the filaments, these two samples had similar microbiomes. In addition, the two microsites on crayfish had unique microhabitats which could be influenced by the presence or absence of BWs or could be due to another filtering mechanism. Furthermore, this study indicates that a complex interaction occurs between crayfish microhabitats and microbes in their surrounding environment.

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