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The role of the skin microbiome in amphibian pathogen susceptibility in Turnbull National Wildlife Refuge

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THE ROLE OF THE SKIN MICROBIOME IN AMPHIBIAN PATHOGEN SUSCEPTIBILITY IN TURNBULL NATIONAL WILDLIFE REFUGE

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Krista S. Dodd

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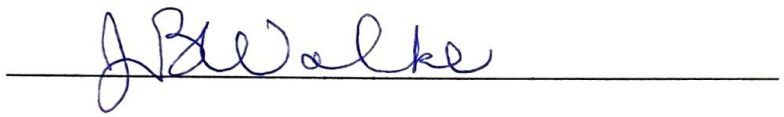

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THE ROLE OF THE SKIN MICROBIOME IN AMPHIBIAN PATHOGEN SUSCEPTIBILITY IN TURNBULL NATIONAL WILDLIFE REFUGE

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The fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), has led to the decline and extinction of many amphibian populations, but some bacteria in the skin microbiome can inhibit its growth. In Turnbull National Wildlife Refuge (TNWR) in eastern Washington, *Bd* is highly prevalent, but the role of the skin microbiome in *Bd* infection dynamics have not been examined in this region. We hypothesized that frogs with lower *Bd* infection intensities would have higher skin bacterial diversity and more abundant anti-*Bd* bacteria, indicative of a more protective function. Our study combined culture-independent and culture-dependent methods to assess the relationship between *Bd* and the microbiome of the Columbia Spotted Frog (*Rana luteiventris*, N=46) and the Pacific Chorus Frog (*Pseudacris regilla*, N=72) in TNWR. We characterized skin bacterial diversity with 16S rRNA gene amplicon sequencing on Illumina MiSeq, and quantified *Bd* infection intensity with qPCR. *P. regilla* had significantly higher Bd infection intensities (14,480 zoospore equivalents, number of zoospores per swab) and prevalence (91.43%) compared to *R. luteiventris* (intensity: 1,647.36 zoospore equivalents, prevalence: 67.74%). To evaluate whether these infection differences correlate with the skin microbiome, a culture-dependent method was used to determine which bacterial isolates produce anti-*Bd* metabolites in *in vitro* co-culture assays, followed by a comparison of culture and culture-independent DNA sequences to determine relative

abundance of anti-*Bd* bacteria on wild frogs. PCF and CSF had significantly different skin microbiomes (p=0.001, pseudo-F=19.07, PERMANOVA), and the two species varied in the interaction between the microbiome and *Bd. Bd* infection intensity was significantly correlated with the skin microbiome in *P. regilla* (Mantel test, r=0.43, p=0.02), which had higher *Bd* levels, but this pattern was not observed in the less infected *R. luv* (Mantel test, r=-0.0086, p=0.93). Lastly, for *P. regilla* only, skin microbiomes varied across wetland sites (PERMANOVA, p=0.004), which could explain the variation in *Bd* infection intensities observed across sites in this species (p=0.038, Kruskal-Wallis). These results are a basis of understanding for the *Bd*-microbiome relationship and for frog conservation in this area.

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INTRODUCTION

Host, Pathogen, Microbiome Symbiotic Relationship

Symbiotic relationships with microbes are key to many ecological processes. In human health, the microbiome (a community of microorganisms found in/on the body of a multicellular organism) is known to help fight against disease including obesity, inflammatory bowel disease, arthritis, autism, and the list is ever increasing (Gilbert et al. 2018). The gut microbiota coevolution was once thought of as a two-way street that benefited the host and microbes, but as it is becoming increasingly apparent, these relationships are also important for defense against pathogens (Shapira 2016). Microbes are now being understood as a symbiotic tool that can help contribute to the harsh drive of natural selection (Bauer et al. 2018).

In humans, the host and microbiome work together to fight disease. The same is true for amphibians, their skin microbiome, and the fungal pathogen *Batrachochytrium dendrobatidis (Bd). Bd* is a single celled motile fungal pathogen found on the skin of many amphibians and it causes chytridiomycosis (Longcore et al. 1999). Chytridiomycosis is an emerging infectious disease that has caused the decline of over 500 amphibian species and the extinction of a presumed 90 amphibian species (Skerratt et al. 2007, Scheele et al. 2019a). It begins its life cycle as an aquatic motile zoospore that initiates colonization of the host's skin cells. Once the zoospore is embedded in the host skin cell, it develops into a thallus (Berger et al. 2005). The thallus matures into a zoosporangium where new zoospores are made. The replication inside the zoosporangium is exponential and it releases new zoospores that enter the water to reinfect the host or infect another nearby amphibian (Berger et al. 2005).

Chytridiomycosis causes hyperkeratosis (skin thickening) of the host, inhibiting gases for cutaneous respiration, absorption of electrolytes and water across the skin, which disrupts action potentials leading to cardiac arrest in highly infected individuals (Voyles et al. 2007, 2009, Rosenblum et al. 2010, Brem et al. 2013, Salla et al. 2015). Since *Bd* is a motile single celled organism, it can be spread by moving zoospores, direct contact between amphibians, or even between host life stages ("Chytrid Fungus | Center for Invasive Species Research" n.d.).

Though amphibian skin can be invaded by *Bd*, the skin is also home to many bacterial species which live in a symbiotic relationship with the host (Rebollar and Harris 2019). The amphibian skin microbiome can contain bacteria with anti-*Bd* properties (Rebollar et al. 2020). Frog populations that have a high proportion of individuals with anti-*Bd* bacteria are more likely to survive *Bd* infection; in addition, removal of the helpful bacteria results in an increase of *Bd* load (Lam et al. 2010, Holden et al. 2015). Bacterial species richness in the microbiome may also help the host fight *Bd* infection, but research for this diversity-function relationship has only been conducted *in vitro* (Piovia-Scott et al. 2017), limiting the understanding of the host's ability to fight infection *in vivo*. *Bd* has been shown to significantly alter the skin microbiome (Jani and Briggs 2014a). Additionally, as infection intensity increases, the relative abundance of bacterial taxa change (Jani and Briggs 2018a). In contrast, the bacterial community can have antifungal properties that may limit the *Bd* growth rate (Walke et al. 2015c). In some instances, it has been found that there is no relationship between *Bd* and the amphibian skin microbiome(Jani and Briggs 2018b).

It is becoming increasingly apparent that the host and microbe symbiosis may be affecting the success of a pathogen that is severely impacting global populations of the amphibian host (Fisher et al. 2009). Unlike the earlier mentioned system, it is not clear yet whether there is an evolutionarily stable system (the microbiome and *Bd* live in balance with one another on the host skin) in the amphibian-Bd-skin microbiome system. It is important to study *Bd* in relation to the amphibian microbiome because some microbes have been shown to inhibit the growth of *Bd* below lethal dose levels increasing, the survivability of the host (Harris et al. 2009, Jani and Briggs 2014b).

Culture-dependent vs Culture-independent

Culture-dependent methods allow bacteria to be grown and physically tested *in vitro.* A frog is swabbed in the environment, and the bacteria from that swab are plated onto a petri dish that contains a growth medium. In most systems, it is estimated that 0.001% to 15% of members of microbiomes are culturable (Walke et al. 2015b). Though roughly 10% of bacteria in the amphibian skin microbiome are culturable (Walke et al. 2015b), culture-dependent methods are still an important tool for understanding the *in vitro* relationship between *Bd* and the microbiome, because it allows for direct testing of frog skin bacterial isolate metabolites against *Bd* in a co-culture assay (Bell et al. 2013, Walke et al. 2015c). The goal of using culture-dependent methods in *Bd* studies is to identify anti-*Bd* bacteria and their metabolites, then potentially use those to create a probiotic for threatened amphibian species. Bioaugmentation of beneficial bacteria may help prevent infection or reduce infection intensity of *Bd* and thus contribute towards the conservation of amphibians (Jankovic et al. 2010, Verschuere et al. 2000, Kueneman et al. 2019).

Culture-independent methods also use a skin swab collected from the environment, but instead of growing the bacteria from that swab in culture, the bacterial DNA from that swab is extracted, amplified, and sequenced to reveal all the bacteria that the swab contains, not just the culturable portion. Culture-independent methods allow for an evaluation of the skin bacterial community, that shows the differences in host species skin microbiomes (McKenzie et al. 2012, Becker et al. 2015b). This step is important for assessing the microbiome as it exists in the environment. Culture-independent assessments reveal the entire microbiome makeup and the bacterial species relative abundance. While this approach allows for the discovery of new bacterial species, the assessment of the cutaneous microbial community with their relative abundances, it does not allow for the assessment of the functional role of the microbiome in regards to *Bd* infection.

There are many ways culture-dependent methods have been utilized to reveal microbial functions. Culture samples in the fermentation processes can help improve renewable energy production (Hallenbeck and Ghosh 2009). Culture-dependent methods can also help with bioremediation by growing and testing microbes that can consume and break down environmental pollutants (Bachmann et al. 2014, Tyagi et al. 2011). Historically, microbial cultures have had a positive effect on discovering ways to protect against biowarfare. For example, *Bacillus anthracis,* a bacterium that causes anthrax (a deadly disease to livestock and occasionally to humans) has been used in biowarfare attacks (Goel 2015). The culturing of this bacterium allowed for antibiotics to be tested *in vitro* and has led to medication that mitigates the deadly effects of this bacterium

(Bachmann et al. 2014, Barrick et al. 2009, Jessup et al. 2004, Tyagi et al. 2011, Lenski 2003, Krause et al. 2014).

The culture-dependent analysis is an important tool for understanding the *in vitro* relationship between *Bd* and the microbiome because it allows for direct testing of the frog skin bacterial isolate metabolites against *Bd* in a co-culture assay. Increasing bacterial species richness can influence the ecosystem function of the microbiome due to aspects like productivity and ecosystem services, but can also impact things like disease resistance, biogeochemical cycling, nutrient/vitamin production and so on (Walke et. al. 2015d). As bacterial species richness increases, microbes begin to compete for resources which may lead to a productive/efficient species being present in the community (Balvanera et al. 2006, Pasari et al. 2013). Having low species richness could lead to the decrease of the services that an ecosystem provides. In the amphibian host-microbiome system, the function of the microbiome could be protection against *Bd.* A diverse microbiome may outcompete *Bd* for nutrients and it could contain bacterial species that produce anti-*Bd* metabolites (Bever et al. 2010, Robinson et al. 2010). The culture-independent analysis is important so that the diversity-function relationship between the microbiome and *Bd* infection can be evaluated *in vivo*. Assessing these two methods together allows for a connection between the diversity-function relationship in the field and the identification of bacterial species with anti-*Bd* properties. The bacterial DNA sequences from both the culture-dependent and culture-independent methods can be aligned so if a bacterium is identified as anti-*Bd in vitro*, then its species relative abundance *in vivo* can be assessed which would give more insight on the complex relationship between *Bd* and the host microbiome (Walke et al. 2015a). By linking both

culture-dependent and culture-independent methods, this project fills a gap in literature because most research done on this topic is focused on one method or the other. If only culture-dependent methods are used, there is only a small fraction of the microbial community assessed. The complete diversity and relative abundance of the microbial community is unknown, which is an important factor in the *Bd*-microbiome relationship (Walke et al. 2017). If culture-independent methods were the only methods used, there would be no functional component of the microbial assessment.

Factors That Affect the Amphibian-*Bd*-Microbiome Relationship

Environmental factors pay a key role in the interaction of *Bd* with the host and host microbiome including time (López et al. 2017), season (Pullen et al. 2010), temperature (Voyles et al. 2012), community dynamics (Jani and Briggs 2014c), amphibian life stages (Marantelli et al. 2004), and geographical location (Molur et al. 2015). Though all those factors can impact amphibian disease susceptibility to *Bd,* this study focuses on time and geographical location. The culture-dependent and culture-independent methods can be interpreted within a broader range of environmental factors, and I focus on geographical location. Temporal variation can affect rainfall, soil pH, and environmental microbial diversity, which can have direct impacts on the survival of amphibians (Estrada et al. 2019, Harrison et al. 2019). Additionally, due to the faster generational time of microbes, the microbiome may be adapting over time to help protect the host species against *Bd* infection so that they can continue to live on the skin of the host species (Zilber-Rosenberg and Rosenberg 2008, Henry et al. 2021). When *Bd* infection intensity is assessed during the same season, year, and amphibian life stage, the infection intensity can still differ throughout geographical location (Molur et al. 2015).

Geographical Information System and *Bd* Infection

Geographical Information System (GIS) software programs allow the organization, analysis, and display of geographically referenced information. They are an increasingly important tool in wildlife biology and have been used to track disease outbreaks in various species (Belsare et al. 2020). Layers consist of geographic information and are added to the basemap. In this project, we created a GIS database of Columbia Spotted Frog (*Rana luteiventris*, CSF, N=46) and the Pacific Chorus Frog (*Pseudacris regilla*, PCF, N=72) samples collected from 17 different wetland sites in Turnbull National Wildlife Refuge (TNWR) over a 3 year period. The original database consisted of frog *Bd* infection intensity, environmental variables, dates collected, and location collected. The spatially referenced database allowed me to link data collected for each frog to specific locations on the ground and visualize patterns of infection intensity. GIS allowed me to take a very cluttered paper map and turn it into an interactive version that was more interpretable and easily understood by viewing different layers separately instead of all at once. Building a GIS dataset allows for analysis of environmental variables and aids in future studies. Additional analysis was performed in RStudio to complement maps created. Also, by using GIS, I was able to create accurate and readable interactive site maps customized to the needs of field researchers collecting data in TNWR.

The study location is TNWR in Cheney, WA. This wildlife refuge was a great place to study amphibians and *Bd* due to it having over 130 wetlands, marshes, and lakes within 3,000 acres, which makes it a really good habitat for a few frog species of the Inland Northwest (“Turnbull National Wildlife Refuge | About Us | U.S. Fish & Wildlife

Service" n.d.). TNWR is also characterized by underground interconnecting streams between the wetlands. The amphibians studied were two frog species, the CSF and the PCF. The CSF can travel up to 3.1 miles away from water (Patla, DA et. Al. 2005) and the PCF can travel ½ mile away from water (Carrasco, J. 2017) (though there is less research on the PCF's range).

One of the research questions being investigated in this study is appropriate for GIS based methodologies. Namely, if *Bd* spreads by direct contact, moving zoospores, or amphibian life stages, and both frog species can move throughout TNWR, then why is infection intensity data different at different wetland sties? By mapping the *Bd* infection intensity data over multiple years (separated by frog species), patterns of infection intensity and location could be assessed. Environmental variables, such as flow patterns of streams and wetland location, could be added to site data collected in the field and could lead to an understanding of differences seen in infection intensity differences between wetland sites (research discussed below). GIS could be used as a predictive tool to track *Bd* spread over time and predict the spread of infection based on that. Additionally, if future research wanted to target wetland locations with high infection intensities, GIS could make it easier and faster for researchers to locate those areas.

Previous Research in Walke Lab

Globally, there is variation in *Bd's* pathogenicity and host susceptibility, and variation in bacterial communities may contribute to the observed variation in pathogen susceptibility (Rebollar et al. 2016, Campos 2020). For example, frog species that were not susceptible to *Bd* had similar skin microbiomes to each other and were enriched in *Pseudomonas* and *Acinetobacter*, suggesting a relationship between *Bd* and the

microbiome (Rebollar et al. 2016) Previous research conducted by Walke and Campos (Campos 2020) in Cheney, WA at TNWR showed that the CSF and PCF had significantly different *Bd* infection intensities (*Bd* load per individual) and prevalence (number of individuals in a population with infection at any level), with PCF having higher infection levels (Campos 2020). Given that the host microbiome and *Bd* interact, it is likely that *Bd* is impacting the microbiome of the host and/or the host microbiome is influencing *Bd* growth on the skin; however, it is unknown whether this relationship exists in these frog species in TNWR (Harris et al. 2009). To fully understand the tripartite system, culture-independent, culture-dependent, temporal, and geographic analyses need to be used together, which is the approach of my thesis.

PURPOSE

The purpose of this project is to assess the diversity-function relationship in the amphibian cutaneous microbiome, to assess bacterial diversity, composition and *Bd* inhibitory capabilities in relation to *Bd* infection levels, and to build a map to visualize *Bd* infection data in TNWR.

OBJECTIVES

The objectives of this study are to 1) evaluate the relationship between *Bd* infection intensity and microbiome composition and diversity, 2) determine whether this relationship varies between host species that vary in infection level, 3) identify bacteria with anti-*Bd* properties and determine their relative abundance on frog skin in the field and 4) to create a more interpretable, aesthetically pleasing map that allows visualization of TNWR and sample points with *Bd* infection data to follow up on patterns seen that

location affects *Bd* infection intensity and explore more environmental variables that may lead to those differences.

HYPOTHESES

I hypothesize that 1) frogs with high *Bd* infection intensity will have different bacterial communities than frogs that are uninfected or have low infection intensity, 2) frogs with more diverse bacterial communities will have lower *Bd* infection intensity, 3) anti-*Bd* bacteria will be in higher relative abundance on frogs with low/no infection intensity compared to highly infected frogs, 4) the two frog species will differ in microbiomes regarding composition, diversity, and species relative abundances, and 5) geographical location will impact *Bd* infection intensity of both frog species. Specifically, I expected to see higher bacterial diversity in the CSF, since this species has lower *Bd* levels. More diverse microbial communities may use more and variable resources thus limiting them for *Bd*, and diverse communities may be more likely to contain bacteria that produce anti-*Bd* metabolites.

METHODS

Overview – Three Part Project

My project consists of three parts. The first part used data from previous research about *Bd* infection in TNWR along with culture-independent methods to assess the *Bd* microbiome relationship in this geographical location. The culture-independent methods were also used to assess the diversity of the microbiome on the frog skin as well as the relative abundance of the bacterial species that make up the microbiome. The second part was a culture-dependent method that grew bacteria from the frog skin swabs, isolated them based on their colony morphology, and tested the isolate's metabolites against *Bd in*

vitro. These methods were used to identify bacteria that produce anti-*Bd* metabolites. The bacterial DNA sequences from both methods were aligned, and the relative abundance of anti-*Bd* bacteria were determined. Those results were then compared to the *Bd* infection data to assess the anti-*Bd* bacteria's relative abundance compared to *Bd* infection intensity. The third part of my project used ArcGIS Pro to map out the sample locations, assess their average infection intensity, and assess possible environmental factors contributing to *Bd* infection intensity.

Culture-Independent Methods

Sample Collection

I conducted a culture-independent method using frog skin swab samples previously collected from TNWR in 2019 (Campos 2020). In this method, the skin of amphibians was swabbed, and the entire sample was sent in for sequencing (Schleifer, K. 2004). The required permits were obtained to sample these amphibians in TNWR: Institutional Animal Care and Use Committee (IACUC) permit (Permit # WALKE 2018-04-02 and 2021-05-01), a Turnbull National Wildlife Refuge Research and Monitoring Activity Special Use Permit (Permit # TBL-21-002r, TBL-20-002r, and TBL-19-005r) for approval of handling live amphibian specimens in refuge wetlands and a Washington State Scientific Collection Permit (Permit # BROWN 18-223 and BROWN 21-272). In May and June of 2019, 31 Columbia Spotted Frogs (*Rana luteiventris*) (CSF) and the 70 Pacific Chorus Frogs (*Pseudacris regilla*) (PCF) were sampled from 15 wetland sites at TNWR in Cheney, WA. The frogs were caught individually by hand or using dip nets. Each frog was handled with new nitrile gloves and were placed into a sterile Whirl-Pack bag prior to swabbing. Each frog caught was rinsed with 50 mL of sterile, deionized

water to remove any transient microbes and environmental debris (Lauer et al. 2007). Two people were required to swab each frog. One surveyor handled the amphibian while the other one swabbed it. The frog was swabbed 10 times (up and down) on the dorsal side, each hind leg 5 times in one direction, and each hind foot 5 times in one direction. The swabs were placed into 1.5 mL microcentrifuge tubes, immediately placed on ice, then stored at -80 degrees C until further processing (Campos 2020). The sample swabs in sterile 1.5 mL microcentrifuge tubes were stored in the lab at -80°C until DNA extraction (described below) (Campos 2020).

Batrachochytrium dendrobatidis (Bd) quantification

The same DNA sample from each frog was used for both *Bd* (Campos 2020) and microbiome analyses (present study). DNA was extracted from swab samples using the Qiagen Dneasy Blood & Tissue Kit with lysozyme pre-treatment and an aliquot of extracted DNA was sent to the Piovia-Scott Lab at Washington State University-Vancouver to perform a Taqman quantitative PCR (qPCR) assay for *Bd* quantification in 2019 and the USGS National Wildlife Health Center for the qPCR analysis of samples collected in 2020 and 2021 (Boyle et al. 2004, Campos 2020). Results indicated that *Bd* was highly prevalent in TNWR (84.16% tested positive for *Bd*) and that there were significant differences in *Bd* infection intensity between the two frog species, with the PCF having higher levels of *Bd* compared to the CSF (Campos 2020). Because *Bd* is prevalent at TNWR, and there was a significant difference of *Bd* infection intensity between the two frog species, a thorough analysis of the microbial communities on these same frog swabs was done and compared to *Bd* data to test for a relationship.

Molecular characterization of skin bacterial communities

To characterize the skin bacterial communities from these samples, a culture-independent method was used by extracting, amplifying, and sequencing bacterial DNA directly from the swab instead of culturing on agar plates, which only captures 1-10% of the bacterial community (Walke et al. 2015c). The V4-V5 regions of the 16S rRNA gene of all bacteria in the samples were amplified and barcode-tagged using PCR and primers 515F+barcode and 926R (Parada, Needham, & Fuhrman, 2016; Quince, Lanzen, Davenport, & Turnbaugh, 2011) following the 16S Illumina MiSeq amplicon protocol from the Earth Microbiome Project (Caporaso et al., 2012, 2011). Total volumes of reagents were: 48 µL Qiagen UltraClean PCR grade H2O, 40 µL QuantaBio 5 Prime Hot Master Mix, 2 µL Illumina Forward primer + barcode 515F, 2 µL Illumina Reverse primer 926R, and 6 µL of DNA from the sample. Reactions were run in a thermocycler set for 25 µL samples and for the following conditions: 1) 94°C for 3 min, 2) 94°C for 45 sec to denature the DNA, 3) 50°C for 1 min to anneal the DNA, 4) 72°C for 1.5 min to elongate the DNA, 5) 72°C for 10 min, and 6) held at 4°C. Steps 2 to 4 were repeated for 35 cycles. Each sample had a negative control, and samples were redone until they were clean. PCR amplicons were confirmed with 1% agarose gel electrophoresis, pooled at equimolar concentrations (determined using Qubit 4.0 Fluorometer), cleaned using the Qiagen PCR Purification kit, and sequenced using a 250bp single-end approach on the Illumina MiSeq instrument at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute at Harvard University.

Sequence processing

The sequences were processed using the freely available bioinformatics program, QIIME2 (Quantitative Insights Into Microbial Ecology 2) (Bolyen et al. 2019, Quast et al. 2013a) After installing QIIME2 onto a desktop, a share folder had to be created to share files between the computer and QIIME2. Once that was completed, a manifest file was created dictating each sample's ID, their file path that QIIME2 used, and a column indicating if the direction was in a single read direction. The q2-demux plugin was used to quality score the single-read, demultiplexed sequence data. The deblur q2-quality-filter was used to filter the sequence quality. The quality sequences were visualized using QIIME2 View, and sequences were not trimmed to maintain the maximum sequence quality. The bacterial species composition of each sample was identified, and taxonomy assigned by comparing sequences to the SILVA database (Quast et al. 2013b) with the classify-sklearn naïve Bayes taxonomy classifier. The identified sequences were clustered based on sequence similarity using the Deblur method (Amir et al. 2017) into bacterial features, or Amplicon Sequence Variant (ASVs), which was used to calculate measures of alpha diversity (ASV richness and Faith's Phylogenetic Diversity). The sequences and the Deblur feature table were filtered from any Chloroplasts, Mitochondria, Unassigned, or known lab contaminant sequences, (*Pseudoalteromonas, Vibrio, Halomonas, Idiomarina, Marinobacter, Marinomonas, Salinisphaera, and Salinarimonas).* After this process was completed, the feature count went from 3,906 to 3,544. To standardize sampling effort, the bacterial community sequencing data was rarefied (i.e., to make sure that all samples have the same number of sequences) to 3,000 sequences per sample. This number was determined by using the alpha rarefaction plots (Figure 1, Figure 2, Figure 3)

to visualize the number of sequences per sample required to represent the diversity of bacteria on an individual sample (i.e. plateauing of diversity with increasing sequencing depth). Although a higher rarefication sequencing depth could be more inclusive of rare bacteria, I chose to maintain a high percentage (95%) of samples. Any sample with fewer sequences than 3,000 were excluded from this point on. Additionally, since my samples were processed on 3 different Illumina sequencing runs, I ran a control sample where I used the same sample with the same barcode tag on multiple sequencing runs. The control sample ID was 23W16 and indicated as "Run 1" for the first sequencing run and "Run 2" for the second run. To visualize whether the resulting bacterial community composition was consistent across runs, the beta diversity PCoA plot of the weighted UniFrac distance matrix was viewed. Since the samples were extremely similar, as indicated in their immediate proximity on the PCoA plot (Figure 4), I concluded that there was no sequencing run bias and thus sample 23W16Run2 was excluded from further analysis. That sample was the only control to assess the differences of multiple runs. The sequences and table were then filtered for each frog species, so that statistics could be ran on both frog species separately. To analyze the effect of site, each frog species-specific dataset was further filtered to only include sites that had at least frogs prior to statistical analysis.

Statistical analyses

Alpha diversity, the diversity in an ecosystem (in this case, bacterial diversity on the skin of a single frog), and beta diversity, community similarity between two ecosystems (in this case, between different frogs), were assessed to gain an understanding of the microbiome diversity and composition. The following alpha diversity metrics were

quantified in this study using QIIME2: Shannon diversity, Faith's phylogenetic diversity, Evenness, and Observed Features. Shannon diversity measures the relative abundances of bacterial species on an individual frog's skin. Faith's phylogenetic diversity measures the diversity of bacteria based on their phylogeny. Evenness was assessed to see how even the relative abundances of each bacterial species are within the microbiome. Lastly, observed features measures the bacterial species richness based on the ASV.

To compare alpha diversity between frog species, the *Bd* infection status (indicated by presence or absence), and across wetland sites, a non-parametric Kruskal-Wallis test was performed and visualized through a box and whiskers plot using QIIME2. In addition, to test for a correlation between *Bd* infection intensity and alpha diversity measures of frog skin bacteria, a Spearman's rank correlation was performed and visualized with a scatter plot.

The following beta diversity metrics were quantified in this study using QIIME2: Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac. The Bray-Curtis Dissimilarity was used to quantify the dissimilarity of microbes between frogs based on the abundance of each ASV in each sample. The Jaccard Similarity measure is incidence-based and thus did not consider the abundances or phylogeny of ASVs. The Weighted UniFrac metric is the most comprehensive metric that measures both the phylogeny and the relative abundances of the ASVs. The Unweighted UniFrac measures phylogeny and incidence-based measures. Beta diversity was compared between frog species, *Bd* infection status (indicated by presence or absence), and across wetland site using the non-parametric PERMANOVA (Permutational Multivariate Analysis of Variance) and visualized by a Principal Coordinates Analysis (PCoA) ordination plot using QIIME2. In

addition, to test for a correlation between *Bd* infection intensity and beta diversity of frog skin bacteria, a Mantel test was performed.

To identify bacteria associated with certain groups (frog species, *Bd* presence/absence, wetland site), Indicator Species Analysis was performed in R (version 4.1.1) using the indicspecies function. *Bd* presence and wetland site were analyzed separately for each frog species.

Culture-Dependent Methods

To evaluate the ability of the skin bacteria to inhibit the growth of *Bd,* CSF and PCF were sampled for skin bacterial culturing in 2020 and 2021. In addition to skin swabs, the following abiotic environmental variables were recorded at each sampling site and event in 2021 using a YSI meter (model number: YSI 556 MPS): water temperature, pH, dissolved oxygen, and conductivity, were recorded using a YSI meter.

Frog skin swabs for this analysis were collected in Sept. 2020 (CSF, n=11) and June/July 2021 (CSF, n=4; PCF, n=2), from 5 wetland sites at TNWR in Cheney, WA. Frog sample collection was completed the same way as stated in the culture-independent portion of this Thesis. Swabs were placed in sterile 1.5 mL microcentrifuge tubes containing TSYE (Tryptone Yeast Soy Extract) +30% Glycerol cryoprotectant and stored in the lab at -80°C until isolation (Walke et al. 2015a). The swabs were grabbed with sterile forceps, rubbed along the side of the microcentrifuge tube to drain liquid, then rubbed directly onto the R2A plates to inoculate them (resulting in mass culture plates), which were incubated at room temperature for 3-14 days. Bacterial colonies from those plates were isolated by their morphology until pure (Bell et al. 2013, Walke et al. 2015a). After pure isolation, each sample was assigned an individual Sample ID, then

cryopreserved by placing a loop full of bacteria into a 1.5 microcentrifuge tube of 1 mL of TYSE + 30%glycerol, then vortexed gently and incubated at room temperature for 30 minutes, and stored at -80 degrees C.

Co-culture assays

To test the growth ability of *Bd* in the presence of bacterial metabolites over time, *Bd* and the bacterial metabolite are put together in a co-culture assay. Each bacterial isolate (125 µL of TSYE-glycerol bacteria) was regrown in a 1% tryptone broth (1 mL) with 7 day old *Bd* (125 µL) for 3 days so that the bacteria had time to produce metabolites and for the isolates to grow into late log or stationary phase (Bell et al. 2013). After that, 1 mL of the cultures were vortexed briefly, transferred to a sterile 1.5 microcentrifuge tube, and centrifuged for 5 minutes at 10,000 rpm to pellet the cells. The supernatant was syringe-filtered (0.22 µm pore size) for each isolate, resulting in a cell-free supernatant (CFS) containing bacterially-produced metabolites (Walke et al. 2015a). Four controls (*Bd* positive, *Bd* negative, heat killed (HK) negative, and an inhibition positive) were created using the CSF method. The HK and *Bd* positive control inoculated 125 µL of 7 day old *Bd* with 1 mL of 1% Tryptone broth and grown for 3 days. The negative control contained 125 µL of sterile TSYE-glycerol solution inoculated into the 1 mL of 1% Tryptone broth and growth for 3 days. Lastly, *Janthinobacterium lividum* (*J. liv*) (a bacteria known to produce anti-*Bd* metabolites (Brucker et al. 2008) was treated like a bacterial sample where 125 µL of cryopreserved *J. liv* was incubated for 3 days in a 1% Tryptone broth. To produce the *Bd* zoospores for the co-culture assays, 1.5 mL of 7 day old *Bd* was grown on 1% Tryptone agar plates for 3 days, the plates were flooded with 3 mL of 1% Tryptone broth for 5 minutes, then the medium was vacuum filtered

using a 20 µm nylon filter to remove the *Bd* zoosporangia. The resulting *Bd* zoospore solution was quantified using a hemocytometer and diluted (if necessary) to obtain a $2x10^6$ zoospore/mL concentration (Walke et al. 2015b).

Challenge assays were conducted in sterile 96-well microplates. Each well contained 100 µL of *Bd* zoospores (2×10^6 zoospore/mL) and 100 µL of the isolate metabolites (i.e. CSF). Each 96-well plate contained a positive *Bd* control (containing 100 µL of *Bd* zoospores and *Bd* metabolites/CSF), a negative *Bd* control (containing only 100 µL of 1% Tryptone and 100 µL of negative metabolites), a heat killed (HK) *Bd* negative control (containing 100 µL of HK *Bd* and 100 µL of negative metabolites) that was heated at 60 degrees C for 1 hour, and a positive inhibition control (containing 100 µL of *Bd* zoospores and 100 µL of *J. liv* CSF). Each sample and all controls were completed in triplicates. The 96-well plates were incubated for at 23°C for 10 days until the positive control wells with *Bd* grew. Each well's absorbance values were measured using a spectrophotometer read at 492 nm. Plates were read on days 0, 1, 4, 7, and 10. The wells were also observed visually to exclude wells with fungal or bacterial contamination. Each triplicate on each day for each sample had a standard deviation calculated. If the standard deviation was too high (threshold at 0.02), then one of the triplicates was excluded. If excluding a triplicate did not correct the standard deviation, then the sample was repeated in another co-culture assay. Five bacterial isolates were excluded from analysis due to standard deviation (between triplicates) being too high in multiple plate runs.

Classifying Inhibitory Bacteria

Each well's absorbance readings were corrected using the HK control by subtracting the average HK absorption for each day from the absorption readings from each isolate and each triplicate on each day. That was completed to account for absorbance readings of *Bd* zoospores that are intact but dead. After that, every triplicate was log transformed, and a slope was calculated to determine the growth rate of the samples with *Bd* as well as *Bd* only using the positive *Bd* control. The slope of each bacterial isolate was then compared to the slope of the positive *Bd* control to create an inhibition score by using the formula (1-(slope of bacterial isolate/slope of the *Bd* positive control)) (Becker et al. 2015c). The Hartigan's dip test was used with the diptest package in (R version 4.1.1) to check for multimodality. The samples were not multimodal using the Hartigan's dip test, but they were bimodal after using the normalmixEM in the mixtools package (Figure 5) (Becker et al. 2015c). The means of each mode were calculated using the mu and sigma. The mean of the first mode using the mu (green portion Figure 5) was 0.89 and the mean of the second mode using sigma (red portion Figure 5) was 0.11. The lambda (0.24, 0.75), mu (0.89, 0.17), and sigma (0.11, 0.2) values (values taken from the normalmixEM) were used to calculate the mean and standard deviation of each model. The 95% confidence interval between the two groups was calculated by subtracting 1.96 multiplied by the sigma from the mu, which resulted in a cutoff of 0.68, so any isolate with an inhibition score over 0.68 (in the first mode) was considered Inhibitory. Isolates with an inhibition score between 0.67 and 0 (in the second mode) were considered not inhibitory. Any isolate that had a higher growth rate than the positive *Bd* control (resulting in a negative inhibition score) were considered

facilitative. Once all inhibition categories were calculated, the inhibition scores were visualized on a cumulative frequency graph with their interaction with *Bd* to see the categories (Figure 6).

DNA Sequencing

The DNA of the anti-*Bd* bacterial isolates were extracted using a freeze-thaw method (Lauer et al. 2007) or, for isolates that failed using the freeze-thaw method (N=27), using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) following the gram-positive extraction procedure. The freeze-thaw method uses a colony of bacteria in 200 µL of TE buffer placed in a sterile 1.5 mL microcentrifuge tube. The mixture is vortexed, then placed through cycles of freezing and thawing. The freeze thaw steps were 1) 99 degrees C for 1 minute, 2) -80 degrees C for 3 minutes, 3) 99 degrees C for 2 minutes 4) steps 2 and 3 repeated two more times. Samples were then centrifuged at 10,000 rpm for 5 minutes, and 100 µL of supernatant was transferred into a new sterile 1.5 microcentrifuge tube. For samples extracted using the GenElute kit, 200 µL of elution solution was used to elute the DNA. DNA samples were stored at -80 degrees C.

Anti-*Bd* isolates were identified by sequencing the full 16S rRNA gene using Sanger Sequencing (primers 8F and 1492R) (Lane et al. 1991). Total volumes of PCR reagents were: 11 µL Qiagen UltraClean PCR grade H2O, 10 µL QuantaBio 5 Prime Hot Master Mix, 1 µL 10 mM Forward primer 8F, 1 µL 10mM Reverse primer 1492R, and 2 µL of DNA template. Reactions were run in a thermocycler set for 25 µL samples and for the following conditions: 1) 94°C for 2 min, 2) 94°C for 30 sec to denature the DNA, 3) 50°C for 30 seconds to anneal the DNA, 4) 65°C for 1.5 min to Extend the DNA, 5) 65°C for 10 min, and 6) held at 4°C. Steps 2-4 were repeated for 35 cycles. After each sample's

PCR was confirmed using 10% agarose gel electrophoresis, they were cleaned using the Exo-SAP-IT enzymatic PCR product clean-up kit, quantified using Qubit 4.0 fluorometer, and diluted to 2.8 ng/µL and for Sanger sequencing at Genewiz. BLAST was used to obtain the identity of each bacterial isolate that was inhibitory. The sequence of each isolate was entered into the Query Sequence box of BLAST, which resulted in percent identification of the species. The top 3 species ID, max score, total score, query cover, and percent ID were recorded.

Linking culture and culture-independent DNA sequences

Culture-dependent anti-*Bd* bacterial isolate Sanger DNA sequences identified to be anti-*Bd* bacteria in the lab were directly compared to the culture-independent bacterial Illumina DNA sequences. The two sequences were aligned and matched using the bioinformatics software Geneious (version 2019.1.1). The Illumina V4 and V5 regions of the 16S rRNA Sanger sequences from the culture-independent portion were set as the reference database in Geneious and the full 16S rRNA sequences from the culture-dependent portion were compared against those. I used the Geneious 'Map to Reference' method of alignment which considers sequences "matched" so long as they do not differ by more than one nucleotide. It is important to note that bacterial sequences can have variance outside of the V4 and V5 regions of the 16S rRNA that matched, but we considered them the same since those were the regions that we used to identify bacteria in the culture-independent portion of this project. To view potential differences in relative abundances of anti-*Bd* microbe ASVs between frog species and between frogs that were infected or uninfected, the number of reads of one bacterial sequence was divided by all of the reads from each frog sample. The relative abundances of the anti-*Bd* bacteria were

visualized using a stacked bar plot created in Excel. Indicator Species Analysis was run again with R using the indicspecies function with the ASVs instead of the genera taxonomy, and the ASVs that aligned with the anti-*Bd* bacterial species were searched for specifically. To understand if the frog species had different sums of anti-*Bd* bacteria, the relative abundances of the aligned anti-*Bd* bacteria were summed for each bacterial species and separated by frog species, then statistically compared in R using the function wilcox.test.

GIS Methods

GIS methods were introduced to integrate and spatially analyze data previously collected showing potential spatial patterns of *Bd* infection intensity across TNWR with data collected from this thesis. For the first step, multiple basemap layers (including TNWR boundaries, wetlands, streams, public/TNWR roads, and TNWR access gates) were obtained from online resources. Some were used with no modifications, and others were edited first (Table 1). The TNWR boundaries layer, for example, had to be edited due to topological inconsistencies in the source polygons resulting in overlap and gaps that did not belong. In this case, a new polygon of the TNWR boundary was created by tracing over the one obtained online. The main driving roads and TNWR access roads were also obtained online and added as layers (Table 1). The TNWR gates were created by georeferencing a scanned paper map that I was given created by TNWR biologists and digitizing the location of all gates visible on that map. Wetlands were obtained online and each wetland I had permission to sample at was selected and placed into a new layer. These basemap layers were also added to the EWU Data Warehouse on ArcGIS Online

and made public under the name “Walke Lab TNWR” so that it could be freely accessed by anyone.

The next step was to create a geodatabase with frog *Bd* infection data. Drawing together previous research from 2019 (Campos 2020) and 2020 with my current samples, a total of 72 PCFs and 48 CSFs in 17 wetland sites was converted to three feature classes (split by species and year) in a geodatabase. The environmental variables collected that were described in the culture-dependent section (air temperature, water temperature, water pH, dissolved oxygen, conductivity, specific conductivity, and date) were added as attribute fields to the feature classes. In total, the geodatabase contained 70 PCFs and 31 CSFs from 2019; 11 CSFs from 2020; and 2 PCFs and 4 CSFs from 2021. Each frog had *Bd* zoospore equivalent data (number of zoospores found on the frog swab sample) which was input into the dataset. The frog infection intensity per wetland site (separated by frog species) was averaged to give an overall *Bd* infection intensity per site. Once the geodatabase was complete, I was able to display the average *Bd* infection data (obtained by the number of zoospores per swab or zoospore equivalents). I created a histogram of the distribution of the *Bd* zoospore equivalents and chose categories based on that distribution. The *Bd* zoospore equivalents were not normally distributed and positively skewed. The infection categories were 0, 1-1,000; 1,000-5,000; 5,000-8,000; 8,000-10,000; and 10,000-245,802. The highest infection intensity in TNWR was 245,802 zoospores. Maps were generated displaying average infection intensity at each site for each frog species and year. Infection intensity patterns were then overlayed with the hydrology layer showing interconnecting streams between wetland sites to visualize any

possible patterns between the interconnectivity between wetland sites and *Bd* infection intensities.

To further explore differences seen by visualizing the average *Bd* infection data per site and per frog species, I explored whether location was a significant predictor of the differences in zoospore equivalence. To determine if site was a significant factor in the difference of *Bd* infection intensity, the Shapiro-Wilks test for normality was used to determine if the Zoospore Equivalences were normally distributed (p=0.0001, Shapiro-wilk test). The data were not normally distributed. Next, the Levene's test for equal variance, using the car package, was ran to see if the data had equal variance. Due to the different number of frogs collected and the range of *Bd* infection, the data did not have equal variance (p=0.67, Levene's test). Previous research conducted by Campos and Walke indicated that the PCF and CSF had significantly different infection intensities with the PCF having significantly higher infection intensities (p=0.01, ANOVA, Figure 7). Since the data were not normally distributed, did not have equal variance, and the PCF and CSF had different infection intensities, the Kruskal-Wallis non-parametric test was used to test whether sampling site (or wetland) was a significant predictor for *Bd* zoospore equivalents. This was visualized using box and whisker plot separated out by frog species.

RESULTS

Bd Infection Data (2019-2021)

In 2019, *Bd* prevalence and infection intensity was assessed for the first time in Turnbull National Wildlife Refuge indicating that *Bd* was highly prevalent (84.16% of frogs tested as *Bd* positive) (Campos 2020).Previous research (2019) showed that the

CSF and PCF *Bd* infection intensities were significantly different (Campos 2020) (Figure 7). The prevalence of *Bd* infection for the PCF was 91.43% (64 out of 70) and prevalence among the CSF was 67.74% (21 out of 31) (Campos 2020). All 15 wetland sites sampled at contained at least one frog testing positive for *Bd*. For the round of sampling that was conducted in 2020, all 3 of the wetland sites sampled at had at least one frog testing positive for *Bd.* The prevalence in this year for the CSF (which was the only frog species collected in 2020), 72.27% (8 out of 11). From the sampling efforts in 2021, one wetland site out of two contained at least one frog that tested positive for *Bd.* For the CSF prevalence, 25% (1 out of 4) tested positive for *Bd* and none of the PCF (N=2) tested positive for *Bd.* My thesis and the remaining analyses focus on the 2019 *Bd* infection data in relation to the skin microbiome.

Culture-Independent Results

Amphibian Species

The two frog species had significantly different microbiome compositions with all beta diversity metrics analyzed [Figure 8; Bray-Curtis Dissimilarity (pseudo-F=17.13, p=0.001, PERMANOVA), Jaccard Similarity (pseudo-F=3.15, p=0.001, PERMANOVA), Weighted UniFrac (pseudo-F=19.07, p=0.001, PERMANOVA), and Unweighted UniFrac (pseudo-F=5.45, p=0.001, PERMANOVA)]. Since species was found to be a significant predictor of the microbiome diversity, the two frog species were evaluated separately for all other analyses.

The two frog species had significantly different microbiome compositions with the Evenness metric for alpha diversity but was not significantly different for the other metrics [Shannon Diversity (H=29.48, p=0.095, Kruskal-Wallis), Faith's Phylogenetic

Diversity (H=1.95, p=0.16, Kruskal-Wallis), Evenness (H=5.88, p=0.015, Kruskal-Wallis), and Observed Features (H=.124, p=0.27, Kruskal-Wallis).

The indicator species analysis showed that 122 genera were associated with the PCF and 12 genera were associated with the PCF. In the top six most abundant bacterial genera, the Verrucomicrobiales was significantly associated with the CSF (p=0.005) (Figure 9).

Bd Presence and Intensity

The relationship between *Bd* and the skin microbiome varied across amphibian host species, with significant interactions for the PCF, but not the CSF. In the analysis of alpha diversity, *Bd* presence was a predictor of the alpha diversity metrics, Shannon diversity index (Figure 10; H= 3.67, p= 0.055, Kruskal-Wallis) and Evenness (H=4, p=0.045, Kruskal-Wallis) with diversity and evenness higher with *Bd* present but not Observed ASVs (H=17.9, p-0.64) and Faith's Phylogenetic Diversity (H=0.49, p=0.48). CSFs with and without *Bd* had the same alpha diversity levels (Figure 11; p>0.05 for all alpha diversity metrics).

Additionally, for PCF, *Bd* infection intensity (zoospore equivalents) was significantly negatively correlated with skin microbial diversity for the Shannon diversity index (H=-0.415, p=0.0007, Spearman's Rank Correlation) and Evenness (Figure 12; H= -0.4901, p=0, Spearman's Rank Correlation). In other words, PCFs with increasing bacterial diversity had fewer *Bd* zoospore equivalents. This pattern was not observed for CSFs (Figure 13; p>0.05 for all alpha diversity metrics).

In the analysis of beta diversity for PCFs only, frogs with *Bd* had significantly different skin microbiome compositions than frogs without *Bd* (Figure 14), using the

abundance-based beta diversity metrics Bray-Curtis Dissimilarity (pseudo-F=2.37, p=0.002, PERMANOVA) and Weighted UniFrac (pseudo-F=3.67, p=0.021, PERMANOVA). On the other hand, CSFs with and without *Bd* had the same microbiome compositions (Figure 15; p>0.05 for all diversity metrics).

Additionally, only for the PCF, *Bd* infection intensity (zoospore equivalents) was significantly correlated with the microbiome between frogs (Figure 16) using Bray-Curtis Dissimilarity (Spearman's rho=0.332, r=0.001, Mantel) and Weighted UniFrac (Spearman's rho=0.32, p=0.001, Mantel) distance matrices. There was no correlation between *Bd* infection intensity and the skin microbiome in CSF (Figure 17; p>0.05 for all diversity metrics).

Indicator species analysis resulted in 23 genera significantly associated with the CSF *Bd* negative group and 32 genera significantly associated with the PCF *Bd* negative group. None of the genera that were significantly associated to the *Bd* negative group for either species were the top seven most abundant genera.

Wetland Site

Alpha diversity varied significantly across wetland sites for both the PCF and CSF (Figure 18-19) for Shannon Diversity (CSF; H=10.86, p=0.028, Kruskal-Wallis, PCF; H=10.97, p=0.013, Kruskal-Wallis), Faith's Phylogenetic Diversity (CSF; H=12.56, p=0.014, Kruskal-Wallis, PCF; H=18.75, p=0.027, Kruskal-Wallis), and Evenness (CSF; H=10.5, p=0.033, Kruskal-Wallis, PCF; H=10.51, p=0.029, Kruskal-Wallis). Bacterial richness (observed features) varied across site for the CSF (H=12.17, p=0.016, Kruskal-Wallis), but not PCF (H= 16.35, p=0.06, Kruskal-Wallis).

In the analysis of beta diversity, skin microbiome composition was significantly different across wetland sites for both the CSF and the PCF for Bray-Curtis Dissimilarity (CSF; pseudo-F=1.46, p=0.047, PERMANOVA, PCF; pseudo-F=2.37, p=0.002, PERMANOVA), the Jaccard Similarity (CSF; pseudo-F=1.43, p=0.001, PERMANOVA, PCF; pseudo-F=1.46, p=0.001, PERMANOVA), and Unweighted UniFrac (CSF; pseudo-F=1.53, p=0.007, PERMANOVA, PCF; pseudo-F=1.7, p=0.001, PERMANOVA). Additionally, site was also a predictor for Weighted UniFrac for the PCF (Figure 20, pseudo-F=2.45, p=0.002, PERMANOVA), but not for CSF (Figure 21, pseudo-F=1.36, p=0.17, PERMANOVA).

The indicator species analysis was used to identify bacteria at the genus level that may be associated with specific wetland sites. There were 16 genera significantly associated with 6 wetland sites for the CSF and 44 genera significantly associated with 5 wetland sites (Table 2).

Culture-Dependent Results

Inhibitory Bacteria and Sequences

After the isolates' inhibition score was calculated, 23% (35 out of 148) of the samples were inhibitory against *Bd*, 60% (89 out of 148) of the samples were not inhibitory, and 14% (22 out of 148) of the samples were facilitative (Figure 6). The CSF had 26% (34 out of 130) that were inhibitory and PCF 5% (1 out of 18) that were inhibitory, but the two frog species did not significantly differ in the proportion of inhibitory bacteria (p=0.77, Fisher's exact test). Of the bacteria that were inhibitory, the CSF had 12% (4 out of 34) that had a lower growth rate than the negative control (resulting in an inhibition score over 1) and the PCF had 0% (0 out of 3) that had a lower

growth rate than the negative control, but the two proportions were not significantly different between frog species (p=1, Fisher's exact test). The CSF had 16% (21 out of 130) of the isolates that were facilitative, and the PCF had 11% (2 out of 18) that were facilitative, and the two frogs were not significantly different (p=1, Fisher's exact test). The CSF had 58% (76 out of 130) isolates that were not inhibitory, and the PCF had 72% (13 out of 18) that were not inhibitory, and the two frog species did not significantly differ in the proportion of non-inhibitory bacteria (p=0.69, Fisher's exact test).

Bacterial Identification

All of the bacterial isolates that had an inhibition score above 0.68 (for both frog species) were sequenced using Sanger sequencing of the full length 16S rRNA gene (Table 3). A total of 35 bacterial isolates were sequenced.

Culture-Independent & Culture-Dependent Results Combined

The sequences of the bacterial sequences that were considered inhibitory were aligned with the ASV sequences from the culture-independent portion. Some of the isolates did not align with any ASV and some bacterial isolate sequences aligned with the same ASV. Of the forward and reverse reads that I sent for Sanger sequencing, 74 out of 76 reads assembled to DNA sequences through Geneious. 23 ASVs were matched by sequences from the culture-dependent Sanger sequences (Table 4). The CSF and PCF had significantly different sums of the anti-*Bd* bacteria with the PCF having significantly more (Figure 22, p=0.0018, Wilcox). A stacked bar plot of the anti-*Bd* bacterial species relative abundances and the frog species present and absent for *Bd* was created to visualize the data (Figure 23). Indicator species analysis was analyzed for the anti-*Bd* bacteria using the ASVs. One bacterial species (*Pseudomonas silesiensis*) was

significantly associated with the PCF *Bd* absent group, which is most likely attributed to the relative abundance of that one being so high (Figure 23, p=0.04). One bacterial species (*Flavobacterium succinicans*) was significantly associated with the CSF but was not associated to one group (*Bd* Present/Absent) or the other (p=0.01).

GIS Results and Deliverables

TNWR Bd Frog Dataset Results, 2019-2021

Bd infection intensities (average per site and per frog species) were displayed in ArcGIS Pro (Figures 24-27). After visualizing the frog *Bd* data, it appeared that CSF had an overall lower infection intensity than the PCF when the two frog species were separated. More of the sites were in the higher infection categories (red) for the PCF than for the CSF which had more sites in the lower infection categories (green). Not only was this visually apparent, but previous research indicated that the PCF had a significantly higher infection intensity than the CSF (Campos 2020) (Figure 7). Additionally, when the two frog species were visualized together along with the interconnecting streams, it appeared that there may be a connection between the sites with a high infection intensity and streams throughout the central-eastern of TNWR. When the *Bd* infection levels and location data were compared statistically, there was no significance difference of zoospore equivalence among sites for the CSF, but there was a significant difference for the PCF in the Kruskal-Wallis test (p=0.038). The Dunn's test, using the dunn.test package, was used to assess a pairwise comparison using the Bonferroni method to see if there was significance between specific sites, and though there was an overall significance, there was no significance found between specific sites.

Deliverable #1: TNWR Field Site Basemap Layers (geodatabase suitable for ArcGIS Pro)

A file geodatabase was completed representing a total of 72 PCF and 64 CSF. The years 2019 and 2021 also had environmental data capture for each frog. Frogs are divided by species and year into separate layers. All field data was collected using a GPS devise (eTrex) with approximately 15 feet accuracy and recorded with the spatial reference GCS NAD83.

The basemap layers (boundaries, public roads, TNWR roads, gates, permissible wetlands, and flow streams between the wetlands) were added to the geodatabase (Figures 28-30). The gates and permissible wetlands were labeled according to the Turnbull paper map that was georeferenced.

Deliverable #2: TNWR Field Site Interactive Map on ArcGIS Online

For use in the Walke lab and these layers were added to ArcGIS online to create a web map named Walke Lab TNWR. Each layer was made public, so that anyone could access these layers online. If future researchers wanted to use data from the online map, they could download layers directly to ArcGIS Pro as feature classes or shapefiles. Future researchers could also either modify the Walke Lab map from data existing on ArcGIS Online or upload their own layers from ArcGIS Pro. In addition, I created a web map app site to add interactive features to the web map under the name, “Walke Lab TNWR Interactive Map” and was made available to the public (Figures 31-33). The interactive map allows future researchers to search for gate numbers or wetland sites and the map will zoom to that specific point. Additionally, a measurement widget was added so that future researchers could measure the distance from the TNWR access roads to a

wetland or site location. On the right side, there are two informative bubbles (Figure 12). The first information bubble is about the Walke Lab, and the second information bubble is about TNWR. Future researchers are able to edit the interactive web map app itself if they want to add more tools or widgets or customize information bubbles.

DISCUSSION

Culture-Independent

The culture-independent portion of my thesis was important in evaluating the merit of my hypotheses 1, 2, 3 and 4. The two frog species (*Rana luteiventris,* Columbia Spotted Frog, CSF; & *Pseudacris regilla,* Pacific Chorus Frog, PCF) differed in their skin microbiomes regarding composition, diversity and species relative abundances (Hypothesis 4), which was apparent from comparing the two frog species using alpha and beta diversity. The two species differed in their bacterial community evenness (alpha diversity) and differed in all four diversity metrics for beta diversity, suggesting a significantly different microbiome composition and structure. The two frog species had different microbiomes in terms amplicon sequence variants (ASV; ~bacterial strains) relative abundance, presence/absence (composition), and phylogeny. Amphibian species differences in the skin microbiome has been observed in other studies (McKenzie et al. 2012, Walke et al. 2014); however, to my knowledge, this study is the first study comparing the skin microbiomes of the CSF and PCF in the Inland Northwest region. These host species differences in the skin microbiome could explain variation in *Bd* susceptibility among host species, as some members of the skin microbiome can compete with *Bd* for resources or inhibit *Bd* growth (Walke et al. 2015d).

Not only did the two frog species have different skin microbiomes from each other, but the two frog species had different interactions between the skin microbiome and factors including *Bd* infection and wetland location. For hypothesis 1, I did find that *Bd* presence/absence and *Bd* infection intensity (zoospore equivalents) as significant predictors for the differences in bacterial communities, but this pattern was only observed for the PCF. Considering the PCF had a higher *Bd* infection intensity than the CSF, and the infection presence and intensity is a predictor of differences in bacterial communities, it is clear that either *Bd* is impacting the microbiome for the PCF, and/or it could be that the microbiome is serving as an aid in protection against *Bd* infection with the CSF. Hypothesis 2 was also supported with evenness and Shannon diversity, as bacterial community evenness and diversity was negatively correlated with *Bd* infection intensity for the PCF, but not the CSF. This means that higher bacterial species diversity and evenness of the microbiome seem to be important for the PCF in having a lower infection or even no infection at all, although manipulative experiments would need to be performed to determine causality of this relationship.

Wetland site also seemed to play a role in the differences seen in the bacterial communities on both frog species. Wetland site was a significant predictor of the differences in *Bd* infection intensity and prevalence for the PCF. Since location is a predictor of the microbial and *Bd* infection intensity differences, the microbiomes of the location may be playing a role in the relationship between *Bd* and the microbiome. Similar to variation in skin microbiome among host species, my study is also consistent with others in that the skin microbiome varied among amphibian host populations of the same species (Walke et al. 2014). The observed variation in *Bd* susceptibility among host

populations could also be explained by observed variation in the interacting skin microbiome among populations.

Culture-Dependent

From previous research finding a connection between the anti-*Bd* microbes and *Bd* infection, I expected that the PCF with higher infection intensity would have lower relative abundances and a lower sum of anti-*Bd* bacteria (hypothesis 3), but the results here did not support my hypothesis. The PCF had a higher amount of anti-*Bd* bacteria and had higher relative abundances of those than the CSF, despite PCF having higher *Bd* infection levels. Other research has found that the PCF is a more tolerant frog species to *Bd* infection, meaning that it could have higher infection intensities than other frog species and still survive (Pope et al. 2016). It is possible that the tolerance of this frog species could come from its microbiome. Since PCF have a higher abundance and sum of anti-*Bd* bacteria, they may be able to withstand higher infection intensities compared to the CSF.

Additionally, since the highly inhibitory bacterial species *Pseudomonas silesiensis* was found to be significantly associated with the *Bd* absent frogs at a high abundance (mean relative abundance: 10%), this bacterial species may prevent the PCF from getting infected with *Bd.* Another possible relationship between *Bd* and the microbiome of the PCF is that *Bd* is altering the microbiome. It could be that *Pseudomonas silesiensis* is at a high abundance on frogs without *Bd* because it has nutrients and space to grow on the skin, but once the frog becomes infected, then that bacterial species may not be able to survive on the skin after infection due to competition for nutrients and space on the skin.

The relative abundances and sums of anti-*Bd* bacteria for the PCF did not differ between frogs with and without *Bd,* but these measures did differ between the PCF and CSF, which also differ in *Bd* infection levels. Since the PCF had higher infection intensities than the CSF, the PCF having a higher sum of anti-*Bd* bacteria may suggest that the PCF and microbiome are actively fighting high *Bd* infection. The microbiome may be adapting to *Bd* infection by increasing the amount of anti-*Bd* bacteria on the skin, which would fit the hologenome theory that the microbiome is able to adapt quicker than the host because of the faster generational time (Zilber-Rosenberg and Rosenberg 2008). Lastly, mass die-offs of frogs have not been observed so far in TNWR. This may suggest that *Bd* and the skin microbiome are in an evolutionary arms race where *Bd* is attempting to infect the skin, but the skin and its microbes are attempting to limit *Bd* from taking over and killing the host. If we assume that *Bd* has been in TNWR for 10 years or longer, these results would support the idea that chytridiomycosis in TNWR is enzootic (the disease dynamic that has been endemic in animals and present for a long time and not epizootic (the disease dynamic of a newly introduced disease leading to many deaths due to hypervirulence of the pathogen (Farrer et al. 2011) or a lack of evolutionary host defenses (Bates et al. 2018). The relationship between the microbiome and *Bd* infection in TNWR seems to be less devastating than infections seen in other geographical locations on other frog species, such as the Panamanian Golden Frog in Central America (Becker et al. 2015a). This relationship is more akin to the *Bd*-microbiome dynamic seen in places like Asia where it was first discovered. *Bd* is likely to be endemic to Asia (Bates et al. 2018) and a lack of declines of amphibians in Asia suggest that amphibians were adapted to the disease (Scheele et al. 2019b).

GIS

Hypothesis 5 (wetland location will impact *Bd* and the microbiome) was supported in this thesis. The differences seen in the microbiome due to wetland location was discussed earlier, but wetland location also impacted *Bd* infection intensity for the PCF. The two frog species had significantly different *Bd* infection intensities, which the maps I created helped illustrate. Site was a predictor of variation seen in *Bd* infection intensity with the PCF, but not the CSF. I expected that wetland site would impact *Bd* and the microbiome based on the first map that was created by Philip Campos showing the differences in infection intensity across TNWR, but without examining that, I would've expected the infection intensities to be similar across wetland site. I was surprised that wetland site was a significant predictor of *Bd* infection and the microbiome because 1) *Bd* spreads by direct contact, moving zoospores and between host life stages (Berger et al. 2005). 2) the two frog species can move throughout the connected wetlands of TNWR, and 3) the microbiome can be transmitted by horizontal transmission (between frogs touching each other or picking up microbes from the field) or by vertical transmission (parent to offspring) (Lauer et al. n.d., Lam et al. 2010, Walke et al. 2014). Since TNWR is interconnected by streams, there are over 130 wetlands, and the area is relatively small, location was not expected to impact *Bd* distribution or the skin microbiome, but it did for the PCF. The results of this thesis indicate that there is a definite relationship between the microbiome, *Bd,* and microhabitats, but that relationship hasn't been researched to the fullest extent in TNWR with the PCF.

Concluding Remarks

To address the mystery of microhabitats impacting *Bd* and the microbiome, research on the location and *Bd* infection intensities should be continued, especially for the PCF. Water samples should be taken from each location to see if *Bd* zoospore equivalents are different in the water of different wetlands or just on the frog species. An environmental DNA (eDNA) approach would be useful for sampling wetland water for *Bd*. A population assessment of both frog species in each of the wetlands surveyed would help understand if the *Bd* infection intensities are higher with larger and/or more dense populations due to the frogs interacting with each other (transmission by direct contact).

Since site was found to be a significant predictor of zoospore equivalents with the PCF, it should be included as an environmental factor in future analyses. Other environmental factors such as the flow of interconnecting streams, vegetation cover, physical barriers should be assessed using GIS to analyze whether those factors influence *Bd* spread in TNWR. In the future, assessing these spatial patterns may lead to an understanding of how *Bd* is spreading in TNWR. The addition of mapping *Bd* infection data over time opens possibilities of tracking *Bd* infection data and predicting the spread. Additionally, if the anti-*Bd* bacteria identified in this study are developed into probiotics prove to be beneficial for helping amphibians in TNWR fight *Bd* infection, then the geographical information could help future researchers target areas that were predicted to be highly infected in the future. Once those areas are identified, the probiotic could be used to bathe frogs for the purpose of bioaugmentation for conservation. This would be

helpful not only as a continuation of this project but for projects in other geographical locations hoping to target highly infected areas to protect threatened species.

While conducting my research, I found it very imperative that lethal loads of *Bd* have not been assessed for either frog species that I was researching, as it has been determined for some species (Vredenburg et al. 2010). It may not matter that the PCF has a higher infection intensity than the CSF if the two frog species may tolerate different loads of *Bd.* If the PCF's lethal load of *Bd* was higher than what we found in TNWR, then that could help affirm my hypothesis that the microbiome and *Bd* are in an evolutionary arms race.

Environmental factors were previously not found to be a significant predictor of *Bd* infection for either frog species in TNWR (Campos 2020). The environmental variables measured as part of this study, including pH, water temperature, air temperature, humidity, and dissolved oxygen should be explored in further detail to evaluate whether those factors influence the composition of the microbiome for each frog species.

This study was an observational study where frog skin swabs were taken directly from the field and assessed. Observational studies are a basis for manipulative studies because they assess what the relationship is like in the field, but they have limitations. A few of the limitations in this study were that the number of CSFs caught were lower than the number of PCF. Additionally, for the PCF, it was rare to find frogs that were not infected by *Bd*, which made evaluating the presence/absence of *Bd* more complicated. There were only 3 PCFs (out of 70) that were found to not have *Bd*, but those three frogs did have a higher evenness than the one that did not have *Bd.* That discovery was consistent with the infection intensity and microbiome evenness (as infection intensity

increased, the evenness of the microbiome decreased). In an observational study, it is difficult to get an even number of frogs caught per site, per species, and for frogs with and without *Bd.* In a manipulative study, a certain number of frogs could be infected, the infection intensities could be evaluated over time, the microbiome could be manipulated, and an even number of frogs for both frog species could be assessed, which would help explore one variable at a time. On the other hand, it is valuable to see how *Bd* and the microbiome interact in the field with multiple factors at play due to the interconnectivity of these factors.

For the indicator species analysis, there were so many genera of bacteria that were found to be significantly associated with particular groups, that led to only the top seven most abundant or ones that were anti-*Bd* to be explored. Further exploration of this data should be completed to see if the 122 genera significantly associated with the CSF are important in diversifying the microbiome. Additionally, more research on *Pseudomonas silesiensis* that was significantly associated with the *Bd* absent PCF group and *Flavobacterium succinicans* that was significantly associated with the CSF and with wetland site 7B should be done to see where the frog species are likely to acquire these bacterial species (i.e. environment or frog species). This discovery would aid in future probiotic work. Since both of these bacterial species were found on both frog species, they both would be good probiotic candidates for either species. I would suggest testing the *Pseudomonas silesiensis* first, since it was significantly associated with *Bd* absent PCFs. This probiotic could be developed and tested to see if increasing the relative abundance of this microbe would prevent the CSFs or PCFs from contracting disease and if they contract that disease if that *Pseudomonas* species would help decrease the

infection intensity for both frog species. The *Flavobacterium* species would be interesting to use as a probiotic for the PCF to see if a higher relative abundance of that bacterial species would help lower the infection intensities seen in the PCF.

Another limitation to this study is that not all of the culture-dependent isolates were sequenced. This could limit the understanding of inhibitory bacteria in the context of the culturable bacteria. Since some of the bacteria that were inhibitory had the same bacterial sequence ID as bacteria with unknown inhibition, there may be some bacteria that were not sequenced that also shared the same bacterial sequence ID. Even though samples were run in triplicate for the co-culture assay, not sequencing the rest of the culturable microbes may limit our understanding of the inhibition variation that could be seen with bacterial sequence data. There may be variation of the sequences that are outside of the 16S rRNA gene that could lead to the culturable bacteria having different morphologies and phenotypes. To fully explore the culture-dependent aspect of this project, it would be helpful to sequence the full genome of the isolates that had different morphologies but the same bacterial sequence ID to assess if there is variation outside of the 16S rRNA gene that could contribute to differences seen in inhibition score. Lastly, previous research not done in this lab has indicated that *J. liv* produces violacein, which has been found to be inhibitory against *Bd* (Brucker et al. 2008). Many studies use this bacterium as a probiotic (e.g. Harris et al. 2009), so we used the metabolites from this bacterium as an inhibition control in the 96-well plates. The inhibition score was not what we expected. This could've been due to the incubation time. In the future, I would suggest incubating *J. liv* for a longer period of time so that it has more of an opportunity to produce the anti-*Bd* metabolite violacein. Additionally, we were able to culture a purple bacterium from the

CSF, so I decided to sequence it to see if *J. liv* was on the skin of the amphibians in TNWR. The bacterial sequence ID of that isolate was *J. liv*. Not only was the inhibition score lower for the inhibitory control we used, but it was deemed "not inhibitory" after calculating and categorizing the inhibition score. It is possible that *J. liv* is not inhibitory against the strain of *Bd* that we used in the lab, but it is more likely that it did not grow for long enough and did not produce enough violacein. After *J. liv* had been cultured with *Bd* for 3 days, it did not appear as purple as expected.

Compared to the rest of the United States, the Inland Northwest has a lower amount of research done on *Bd* and frogs in this area. Luckily, previous research competed in this lab was conducted in Northern Idaho which allows for comparison in this area (Campos 2020). Infection intensity was lower in Northern Idaho than what was found in TNWR. The alpha diversity between *Bd* infected and uninfected frogs did not significantly differ using any of the diversity metrics in Northern Idaho but did differ in TNWR with the PCF. The diversity of the microbiome was similar in Northern Idaho and TNWR with increasing infecting intensity correlating with lower evenness of the microbiome (Campos 2020). Wetland site accounted for the differences seen in the microbiome in Northern Idaho and TNWR (Campos 2020).

My results are a solid foundational basis for the relationship between pathogens and symbiotic microbiomes and warrant additional research to be done in TNWR. The results of this observational study have generated further hypotheses on host-microbiome-pathogen relationships in this region and beyond and is an amazing start to the continuation of amphibian conservation in the Inland Northwest.

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TABLES AND FIGURES

Table 1. A description of each layer added to the basemap in ArcGIS Pro, its source, the modification of the layer, and a description

TNWR Layers			
Layer Name	**Source**	**Preparation**	**Description**
TNWR Boundaries	US Fish and Wildlife Service GIS Data https://www.fws.gov/refuge/turnbull	Manually digitized outer TNWR boundary as a single polygon from the USFW layer	The boundary of the area of land owned by TNWR
TNWR Roads	US Fish and Wildlife Service https://www.fws.gov/refuge/turnbull	Clipped national wildlife refuge roads layer to TNWR boundaries	Dirt access roads that lead to different locations within TNWR
Public Roads	WSDOT GIS Data https://www.wsdot.wa.gov/mapsdata/geodatacatalog/default.htm	Clipped Washington state Spokane country roads to TNWR boundaries	Roads that are freely drivable by the public
TNWR Gates	TNWR Walke-EWU sampling wetlands	Manually digitized points from paper map	Gates with codes that lead to the TNWR roads

TNWR Permissible Wetlands	US Fish and Wildlife Service https://www.fws.gov/program/national-wetlands-inventory/data-download	Clipped national NWI wetlands to TNWR boundaries, selected out wetlands with permits	Wetlands within TNWR that I we were able to sample at according to the permit we attained
TNWR Streams	National Hydrography Dataset https://www.usgs.gov/national-hydrography/national-hydrography-dataset	Clipped original dataset (national NHD) to TNWR boundaries	Streams and other drainage features from the national hydrographic dataset that go through TNWR

Table 2. Indicator species analysis groups and the number of genera associated with that group from the culture-independent taxonomy genera assigned through QIIME2.

Group	Number of Genera Associated
CSF Wetland Site 98B	2
CSF Wetland Site 42	4
CSF Wetland Site 62	5
CSF Wetland Site 75	1
CSF Wetland Site 7B	3
CSF Wetland Site 97A	8
PCF Wetland Site 23B	4
PCF Wetland Site 42	1
PCF Wetland Site 54A	1
PCF Wetland Site 62	23
PCF Wetland Site 75	15

Table 3. ASV ID that was assigned in QIIME2 from the culture-independent Illumina sequencing and the bacterial species that matched that ID from the culture-dependent Sanger sequencing.

Bacterial Species	**#ASV ID**
Curtobacterium herbarum	33d5a2c7d222f0b503a88a922c1312e1
Macrococcus goetzii	aa9148b274b6c30e19fd08f7071b0363
Chryseobacterium carnipullorum	55221c27739a619ec307f19252473eab
Pantoea agglomerans	6ba560a76661744bc65eed04953c1314
Shewanella xiamenensis	f152563f7ee6b624c355a373025d5bf9
Pseudomonas orientalis	8f09bac714c02dc9415770271349dc99
Pseudomonas umsongensis	dc5030eb18fe395a50b8235f2e583dbd
Pseudomonas peli	c2d4d5c46e3c67b2aeb67d0635c45962
Flavobacterium succinicans	7d9f90a81cc4cd3f78117b0b87025a54
Streptomyces rishiriensis	54ea0db20a51922444a6cfd8403a3122
Streptomyces praecox	5183b5b73de1ecce749af92acdb90f8c
Paenibacillus nebraskensis	9718f2a8eed150f0cc5afac193adfe37
Paenibacillus endophyticus	677929eac52be081cdae08a0b9c70eb2
Pseudomonas alcaligenes	763b8e619996f3062d095a14e8087225
Bacillus paramycoides	0d87b112bc336b97248bf4757a668689
Lysinibacillus parviboronicapiens	49a361dc4554ca6f03bc8d8bf47e329c
Microbacterium oxydans	dd240dc95518ae32a95c2cdbdf225771
Pseudomonas silesiensis	b2169de7b5980a96680c0a9cff5fbe7a
Kocuria rhizophila	e6f6be4ccfb278fe562ca7dffb543652
Pseudarthrobacter enclensis	4bf03fa8fb8cd66f79240593a6defce9
Jeotgalibacillus campisalis	02be30e5c5a21a948ab5a2fad97561bc
Paracoccus yeei	3aecfdb2f359cc71dab3837d7d008602

Table 4. Culture-Dependent sequence IDs from Sanger sequencing. Each isolate has the Isolate ID that was used to identify the isolates in the lab, the frog species the isolate came from, the top three hits from BLAST for the bacterial species ID, and other sequencing information for each of those top hits.

Isolate ID	Inhibition Score	Frog Spcecies	Bacterial Species ID	Max Score	Total Score	Query Cover	Percent ID
2W16_21H	0.73	CSF	*Chryseobacterium carnipullorum*	2525	2525	99	99.71
			Chryseobacterium shigense	2523	2523	100	99.57
			Chryseobacterium shigense	2490	2490	100	99.13

2W16_21A	0.81	CSF	*Chryseobacterium carnipullorum*	2529	2529	99	99.78
			Chryseobacterium shigense	2529	2529	100	99.57
			Chryseobacterium shigense	2495	2495	100	99.13
3W42_21I	0.78	CSF	*Pseudomonas peli*	2470	2470	100	98.5
			Pseudomonas anguilliseptica	2453	2453	97	99.19
			Pseudomonas guineae	2399	2399	99	97.84
3W42_21O	0.87	CSF	*Curtobacterium herbarum*	2555	2555	100	99.86
			Curtobacterium herbarum	2555	2555	100	99.86
			Curtobacterium ammoniigenes	2403	2403	99	98.19
3W42_21R	0.98	CSF	*Streptomyces rishiriensis*	2547	2547	100	99.78
			Streptomyces rishiriensis	2547	2547	100	99.78
			Streptomyces humidus	2508	2508	100	99.28
4W42_21C	0.79	CSF	*Aeromonas veronii*	2593	2593	100	99.93
			Aeromonas veronii	2593	2593	100	99.93
			Aeromonas veronii	2593	2593	100	99.93
5W42_21A	0.93	PCF	*Pantoea agglomerans*	2580	2580	100	99.72
			Enterobacter ludwigii	2575	2575	100	99.65
			Leclercia adecarboxylata	2547	2547	100	99.29
5W42_21C	0.75	PCF	*Pantoea agglomerans*	2580	2580	100	99.72
			Enterobacter ludwigii	2575	2575	100	99.65
			Leclercia adecarboxylata	2547	2547	100	99.29
6W42_21A	0.83	PCF	*Lysinibacillus parviboronicapiens*	1613	1895	73	98.99
			Lysinibacillus parviboronicapiens	1604	1871	73	98.99

			Lysinibacillus sphaericus	1598	1791	73	98.77
W64-1I	1.05	CSF	*Microbacterium oxydans*	2534	2534	100	99.35
			Microbacterium maritypicum	2518	2518	99	99.57
			Microbacterium liquefaciens	2516	2516	100	99.14
W64-2F	0.96	CSF	*Paenibacillus nebraskensis*	2514	2514	100	98.51
			Paenibacillus chondroitinus	2447	2447	100	97.59
			Paenibacillus aceris	2438	2438	100	97.59
W64-3J	0.73	CSF	*Shewanella xiamenensis*	2516	2516	100	98.79
			Shewanella putrefaciens	2477	2477	100	98.3
			Shewanella hafniensis	2460	2460	100	98.08
W66-4C	0.92	CSF	*Bacillus paramycoides*	2590	2590	99	99.44
			Bacillus tropicus	2584	2584	99	99.37
			Bacillus nitratireducens	2584	2584	99	99.37
W66-4I	0.98	CSF	*Flavobacterium succinicans*	2435	2435	97	99.11
			Flavobacterium succinicans	2431	2431	97	99.04
			Flavobacterium granuli	2342	2342	97	97.78
W66-4J	1.05	CSF	*Shewanella xiamenensis*	2560	2560	100	99.3
			Shewanella putrefaciens	2516	2516	100	98.73
			Shewanella hafniensis	2505	2505	100	98.59
W66-4K-2	0.83	CSF	*Jeotgalibacillus campisalis*	2567	2567	100	99.3
			Jeotgalibacillus marinus	2521	2521	97	99.57
			Jeotgalibacillus marinus	2516	2516	97	99.49

W112-2E	0.73	CSF	*Macrococcus goetzii*	2627	2627	100	99.93
			Macrococcus epidermidis	2627	2627	100	99.93
			Macrococcus bohemicus	2621	2621	100	99.86
W112-3C	0.95	CSF	*Paracoccus yeei*	2418	2478	100	99.4
			Paracoccus lutimaris	2266	2266	100	97.37
			Paracoccus laeviglucosivorans	2259	2259	100	97.31
W112-3E	0.83	CSF	*Kocuria rhizophila*	2564	2564	100	99.79
			Kocuria tytonis	2538	2538	99	99.5
			Kocuria salsicia	2483	2483	100	98.71
W64-2C	1.08	CSF	*Pseudomonas silesiensis*	2584	2584	99	99.86
			Pseudomonas mandelii	2580	2580	99	99.79
			Pseudomonas mandelii	2579	2579	99	99.79
W64-3F	0.95	CSF	*Streptomyces praecox*	2555	2555	100	99.78
			Streptomyces cyaneofuscatus	2555	2555	100	99.78
			Streptomyces pratensis	2555	2555	100	99.78
W66-3D	0.74	CSF	*Pseudomonas alcaligenes*	2569	2569	100	99.71
			Pseudomonas alcaligenes	2569	2569	100	99.71
			Pseudomonas alcaligenes	2567	2567	100	99.71
1W16_21G	0.95	CSF	*Paenibacillus taichungensis*	2590	2590	99	99.51
			Paenibacillus pabuli	2555	2555	99	98.87
			Paenibacillus pabuli	2553	2553	99	98.94
2W16_21B	0.98	CSF	*Pseudomonas baetica*	2551	2551	100	99.5
			Pseudomonas helmanticensis	2545	2545	100	99.43
			Pseudomonas umsongensis	2529	2529	100	99.22

2W16_21D	0.94	CSF	*Pseudomonas koreensis*	2531	2531	100	99.22
			Pseudomonas vancouverensis	2519	2519	100	99.07
			Pseudomonas moraviensis	2516	2516	100	99
2W16_21E	0.9	CSF	*Pseudomonas orientalis*	2564	2564	100	99.79
			Pseudomonas marginalis	2547	2547	100	99.57
			Pseudomonas antarctica	2547	2547	100	99.57
2W16_21J_1	0.91	CSF	*Pseudomonas helmanticensis*	2531	2531	100	99.22
			Pseudomonas baetica	2523	2523	100	99.14
			Pseudomonas jessenii	2510	2510	100	98.86
3W42_21G	0.88	CSF	*Pseudomonas koreensis*	2560	2560	100	99.57
			Pseudomonas moraviensis	2547	2547	100	99.43
			Pseudomonas reinekei	2540	2540	100	99.29
3W42_21J	0.91	CSF	*Pseudarthrobacter chlorophenolicus*	2464	2464	99	99.56
			Pseudarthrobacter equi	2460	2460	99	99.56
			Pseudarthrobacter defluvii	2453	2453	100	99.34
3W42_21K	0.87	CSF	*Pseudomonas koreensis*	2560	2560	100	99.57
			Pseudomonas moraviensis	2547	2547	100	99.43
			Pseudomonas reinekei	2540	2540	100	99.29
3W42_21M	0.91	CSF	*Pseudarthrobacter enclensis*	2435	2435	99	98.34
			Arthrobacter humicola	2429	2429	100	98.2
			Pseudarthrobacter siccitolerans	2410	2410	100	97.99

3W42_21Q	0.96	CSF	*Paenarthrobacter nicotinovorans*	2536	2536	100	99.71
			Paenarthrobacter histidinolovorans	2523	2523	100	99.49
			Paenarthrobacter nitroguajacolicus	2466	2466	100	98.77
W64-1D	1.05	CSF	*Pseudomonas silesiensis*	2579	2579	100	99.79
			Pseudomonas mandelii	2575	2575	100	99.72
			Pseudomonas mandelii	2573	2573	100	99.72
W64-2D	0.99	CSF	*Paenibacillus endophyticus*	2532	2532	100	98.73
			Paenibacillus castaneae	2510	2510	97	99.35
			Paenibacillus prosopidis	2412	2412	100	97.19
W64-3B	0.51	CSF	*Janthinobacterium lividum*	2547	2547	100	99.5
			Janthinobacterium lividum	2542	2542	100	99.43
			Janthinobacterium rivuli	2542	2542	100	99.43

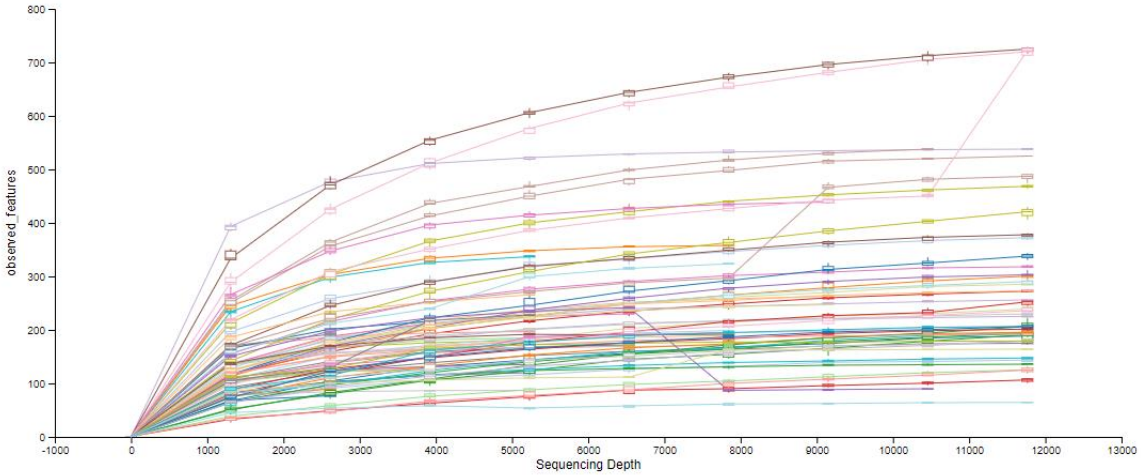


Figure 1. Alpha Rarefaction plot of Observed features and the sampling depth of each swab.

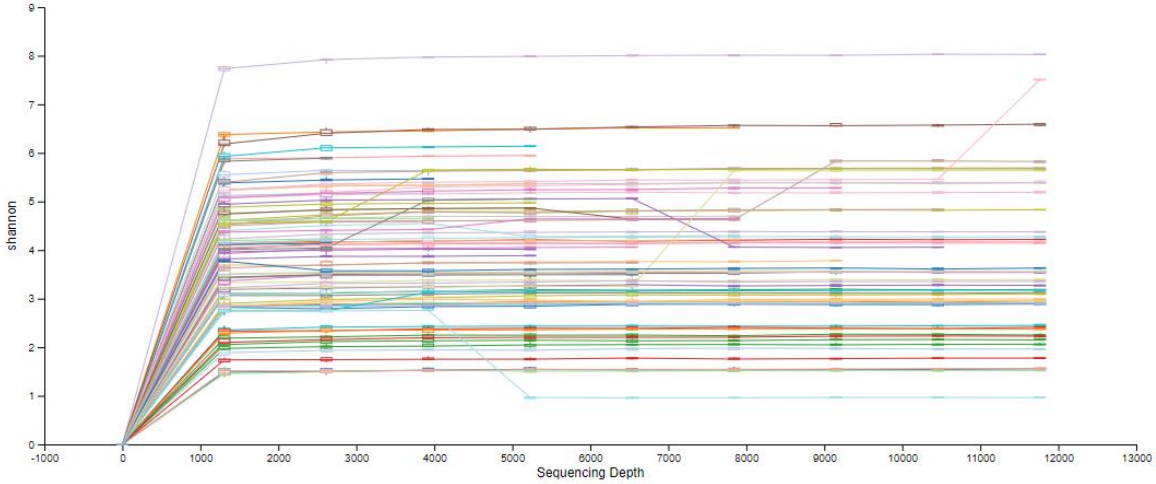


Figure 2. Alpha Rarefaction plot of Shannon's index and the sampling depth of each swab.

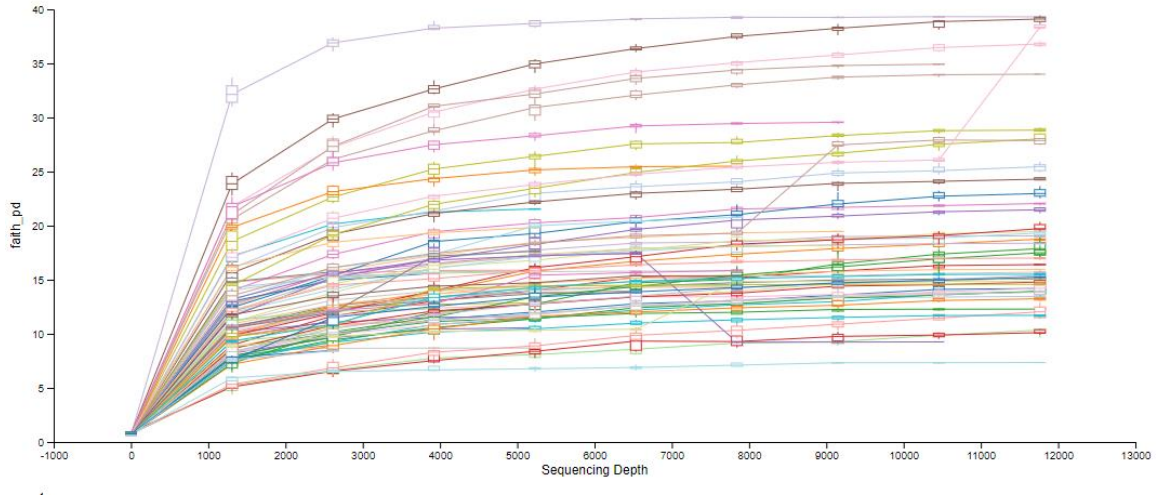


Figure 3. Alpha Rarefaction plot of Faith's phylogenetic diversity and the sampling depth of each swab.

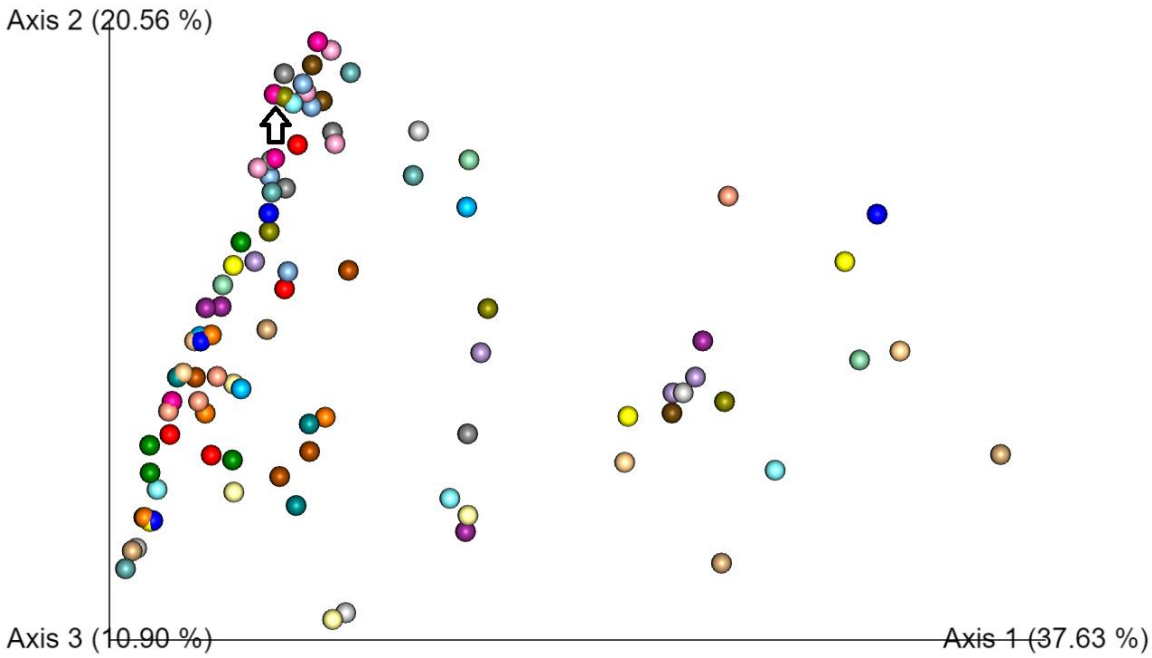


Figure 4. Beta Weighted UniFrac emperor plot. Each dot represents a unique sample ID. The closer the dots are to each other, the more similar the microbiome relative abundance of bacterial species between samples. 23W16Run1 and 23W16Run2 samples are indicated with a black arrow. The dots are very close together indicating they have a very similar microbiome relative abundance composition.

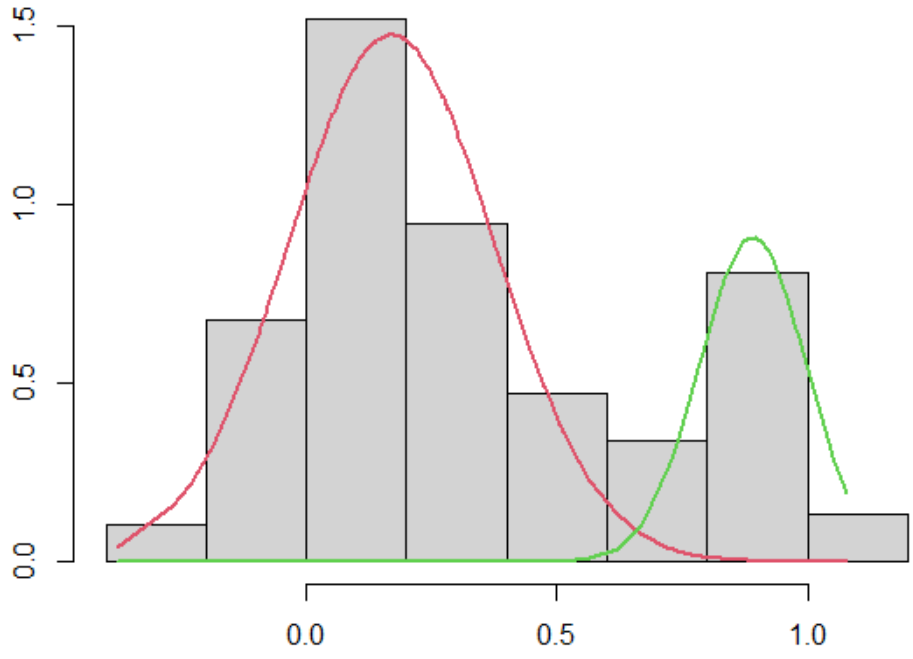


Figure 5. Distribution of the inhibition scores for the entire dataset. The x-axis is the inhibition score that was calculated, and the y-axis is the number of isolates that had that

score. The 95% confidence interval that was discovered was the portion where the green and red line cross.

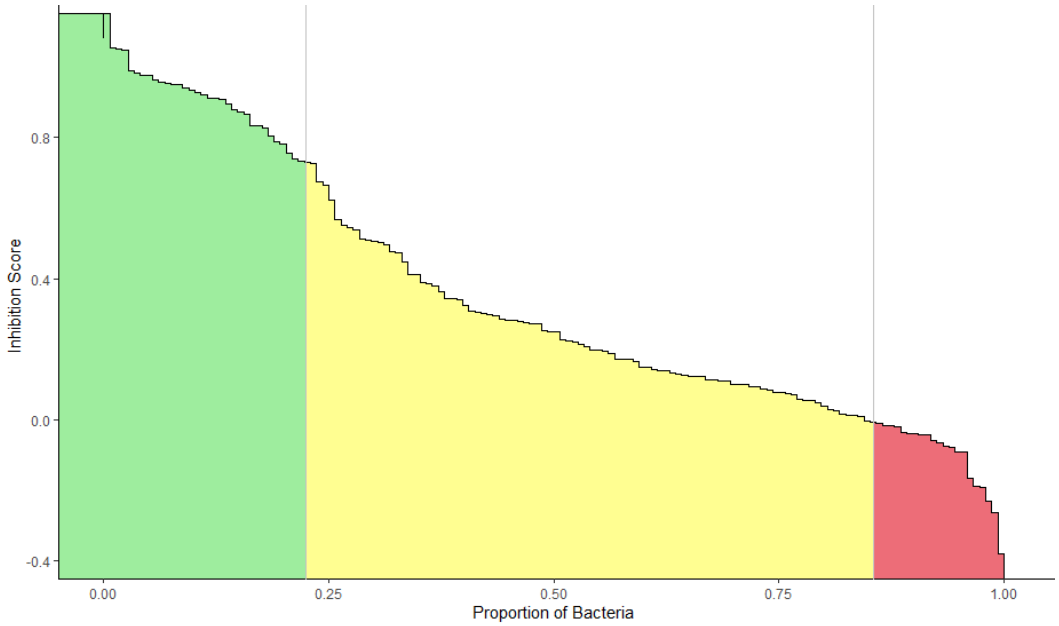


Figure 6. Cumulative frequency graph showing the culturable bacteria and their inhibition score. The green portion of the graph indicates the portion of bacteria that had an inhibition score over 0.68, the yellow portion is bacteria classified as not inhibitory that had an inhibition score between 0.67 and 0, and the red portion is bacteria that were considered facilitative with an inhibition score below 0.

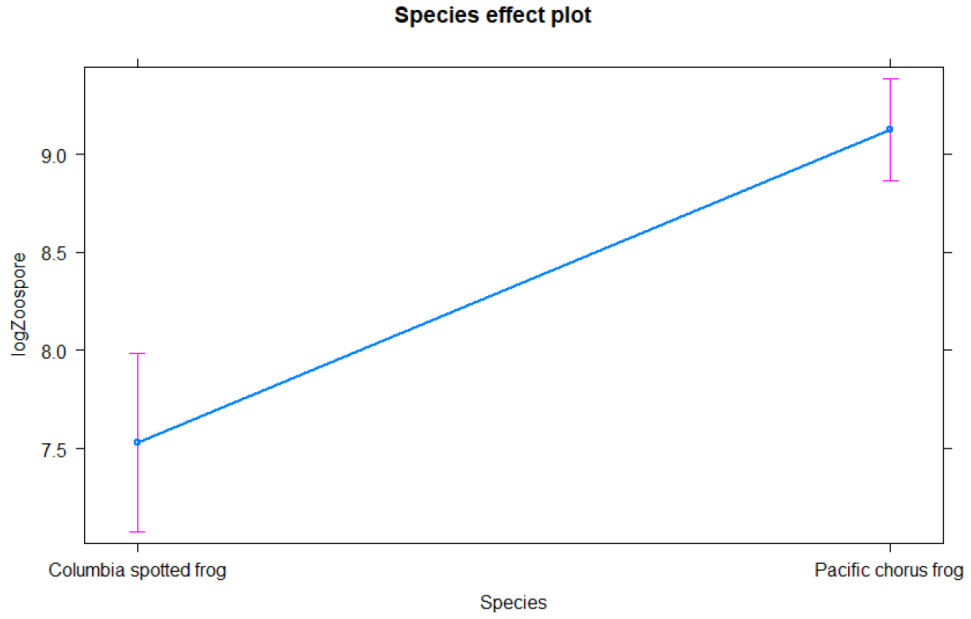


Figure 7. Effect plot of Bd infection intensity quantified by log scale of zoospore equivalents of the two frog species. The pink lines indicate the 95% confidence intervals.

This plot shows that the infection intensity is significantly higher in the PCF than the CSF (p=0.01, ANOVA). Taken from Philip Campos' Thesis (2020).

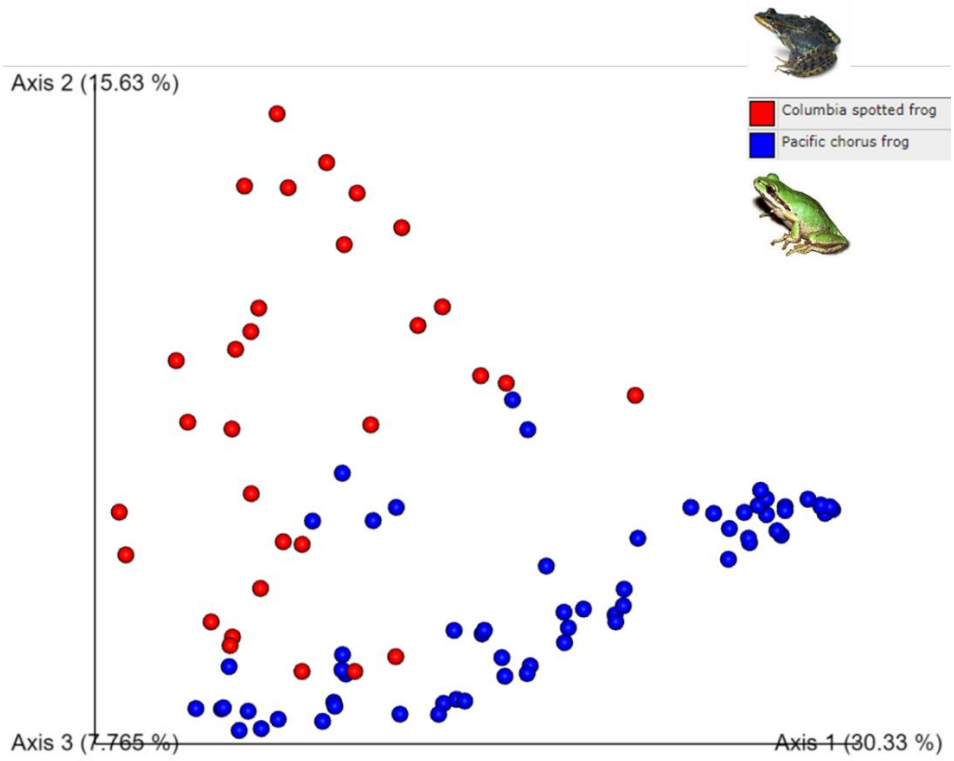


Figure 8 Principal Coordinates Analysis (PCoA) ordination plot of Bray-Curtis Dissimilarity matrix. Each dot represents a single frog and the color of the dot represents the frog species. The CSF is indicated by red dots and the PCF is indicated by blue dots. Dots that are closer together have more similar microbiomes, and dots that are further apart have more dissimilar microbiomes. This plot shows that the CSF and PCF have significantly different microbiome abundances (p=0.001, PERMANOVA).

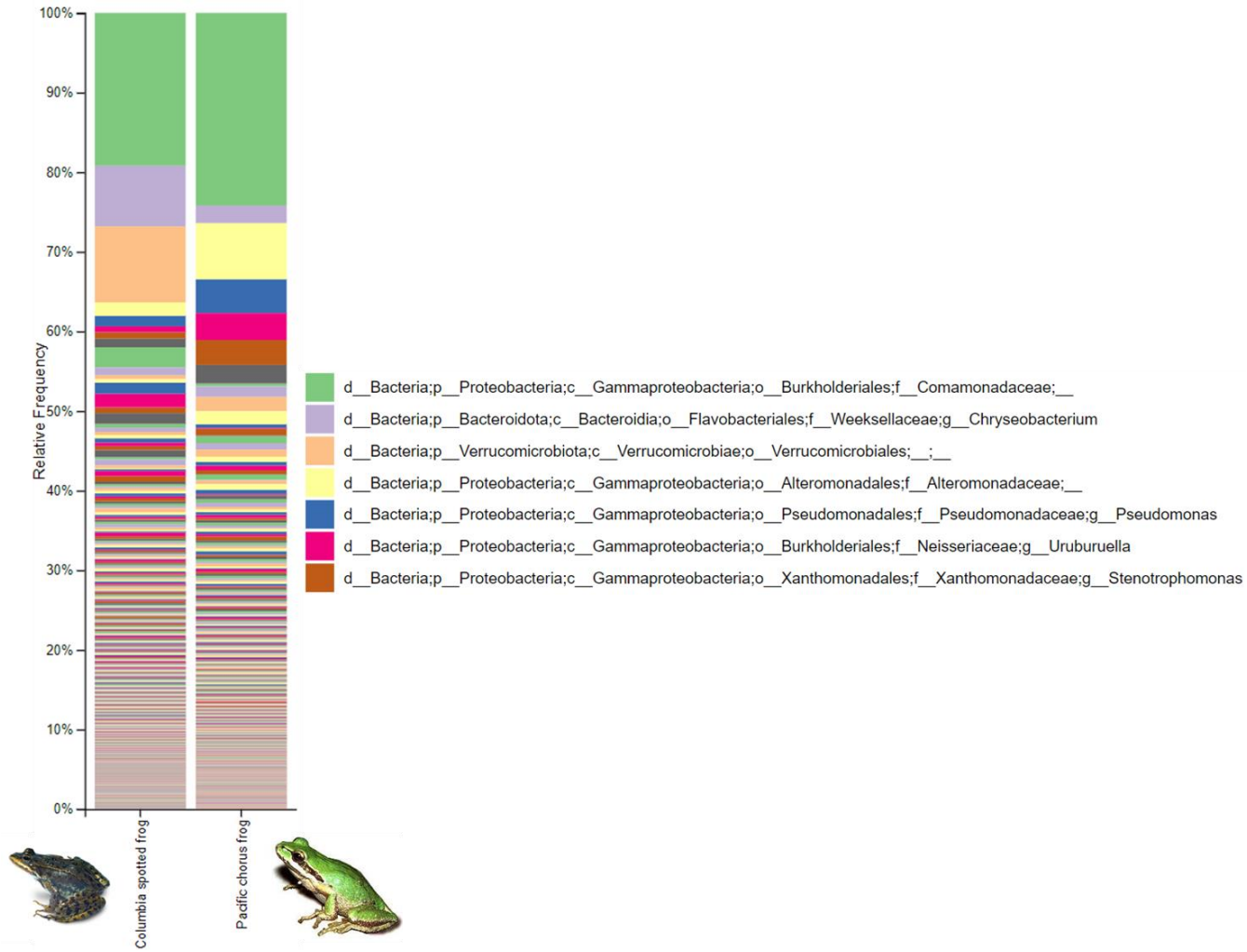


Figure 9. Stacked bar plots of the relative abundances of bacterial genera in the microbiome for the CSF and PCF. The CSF mean relative abundances of genera is on the lefthand side, and the PCF is on the righthand side of the stacked bar plot. The legend of the top seven genera of these two frog species is listed to the right.

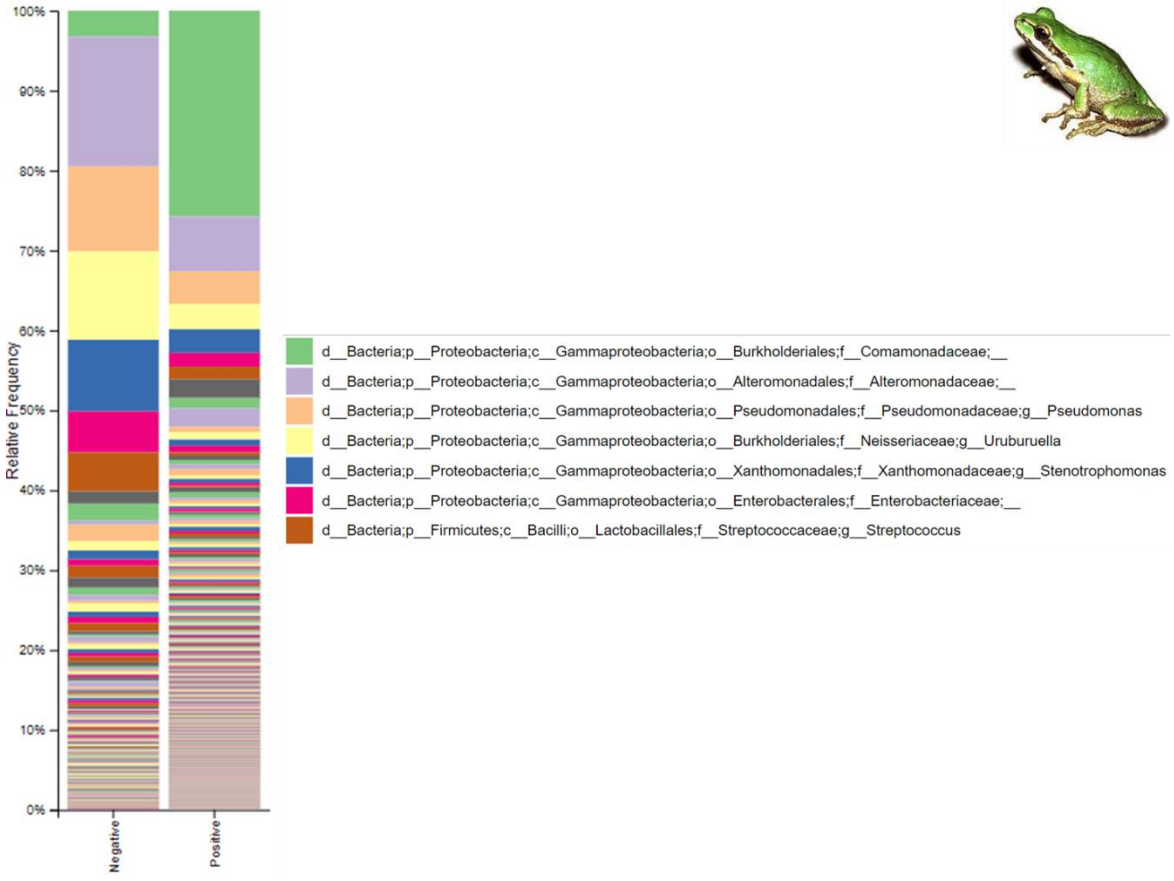


Figure 10. Stacked bar plot for the PCF mean relative abundances of bacterial genera of frogs without Bd on the left (negative) and frogs with Bd on the right (positive). The legend of the top seven genera is indicated to the right. The PCF had significantly different microbiomes between the *Bd* negative and *Bd* positive groups (p=0.021, PERMANOVA)

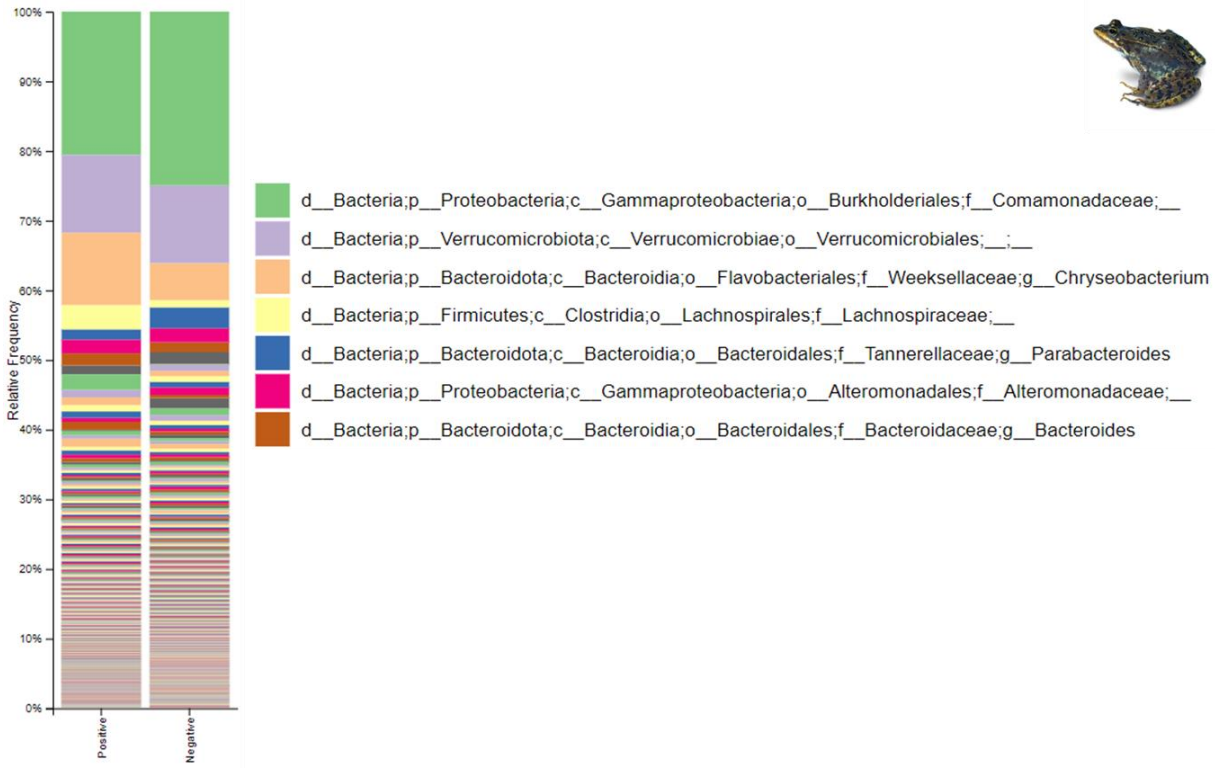


Figure 11. Stacked bar plot for the CSF mean relative abundances of bacterial genera of frogs without Bd on the left (positive) and frogs with Bd on the right (negative). The legend of the top seven genera is indicated to the right. The CSF did not have significantly different microbiomes between the *Bd* negative and *Bd* positive groups (p=0.74, PERMANOVA)

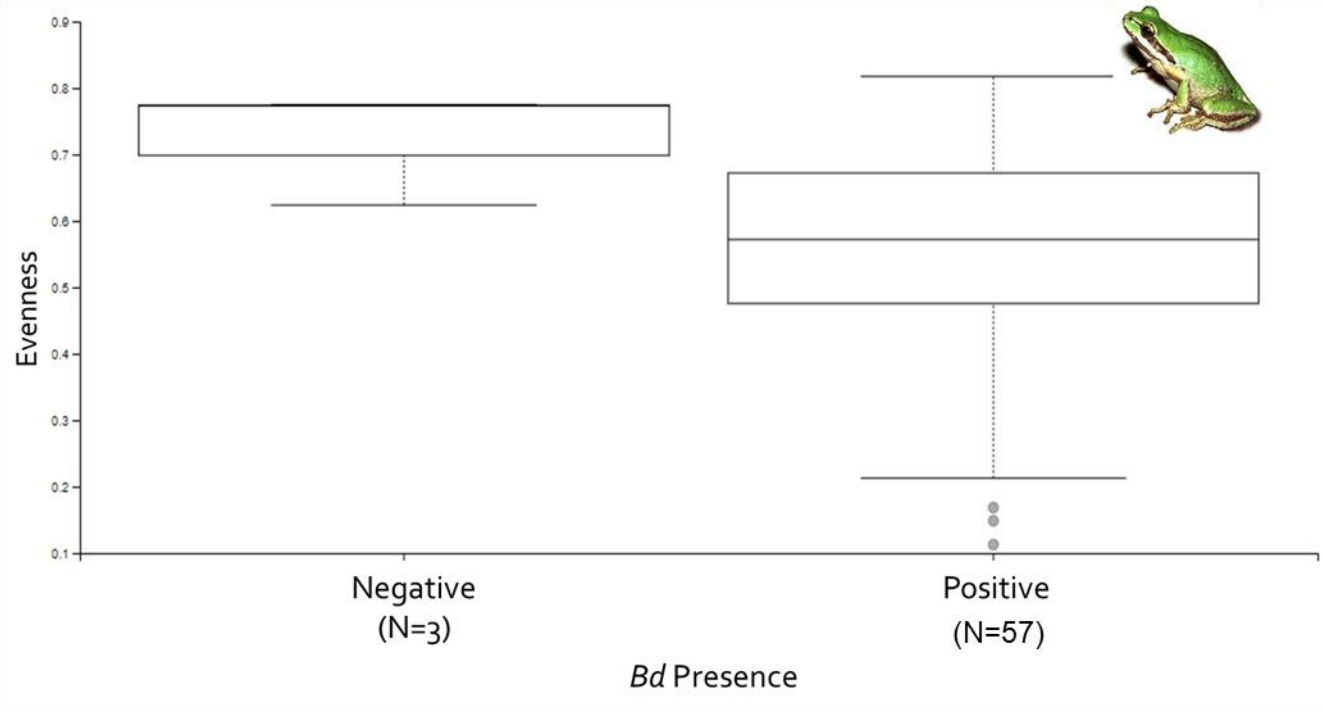


Figure 12. Box and whisker plot of the PCF indicating *Bd* Presence on the x-axis (Negative on the left and Positive on the right) and bacterial community evenness on the y-axis. PCFs without *Bd* had a higher evenness than PCFs with *Bd.* PCFs without *Bd* had a higher evenness than PCFs with *Bd* (p=0.045, Kruskal-Wallis).

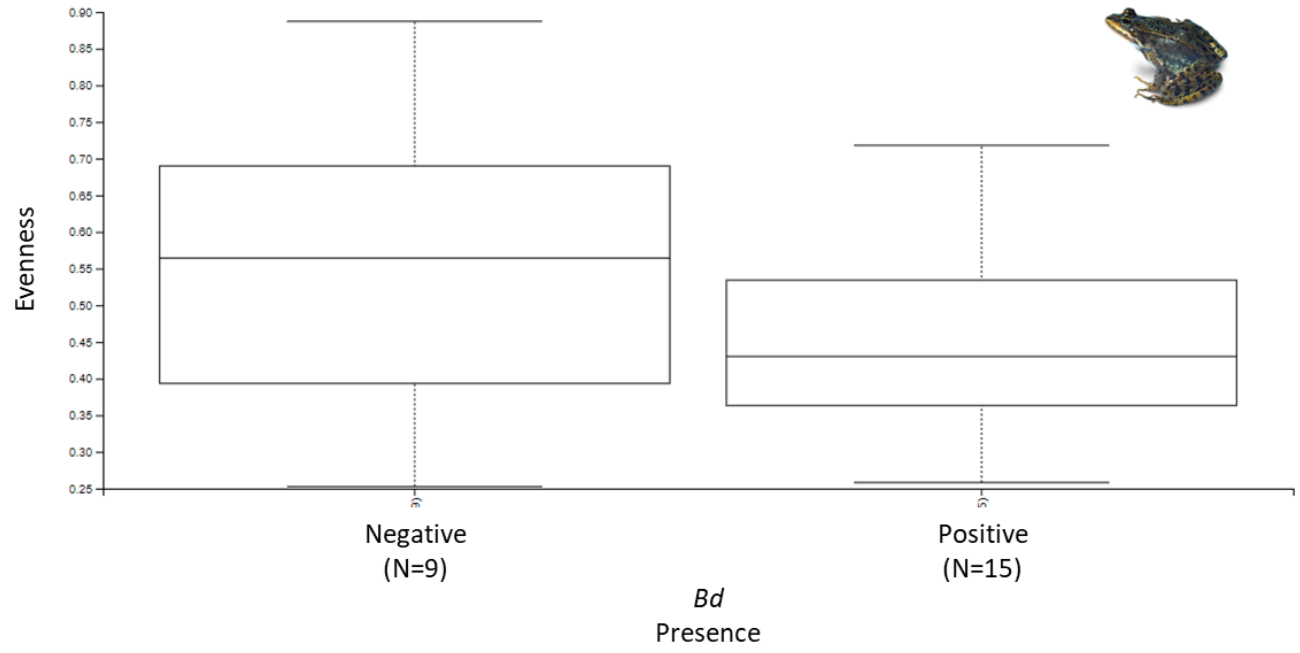


Figure 13. Box and whisker plot of the CSF indicating *Bd* Presence on the x-axis (Negative on the left and Positive on the right) and bacterial community evenness on the

y-axis. PCFs without *Bd* had a higher evenness than PCFs with *Bd.* CSFs without *Bd* did not have a higher evenness than CSFs with *Bd* (p=0.18, Kruskal-Wallis).

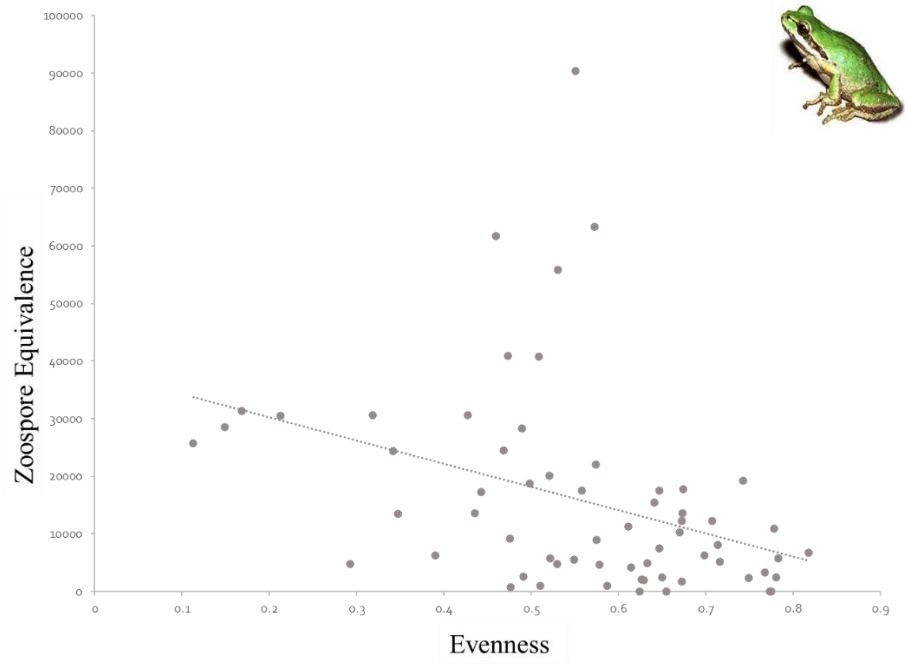


Figure 14. Scatter plot of the PCF with bacterial evenness on the x-axis and *Bd* zoospore equivalence on the y-axis. The trendline indicates that there is a negative relationship as evenness of the microbiome increases, zoospore equivalence decreases (p=0.00, Spearman's Rank Correlation).

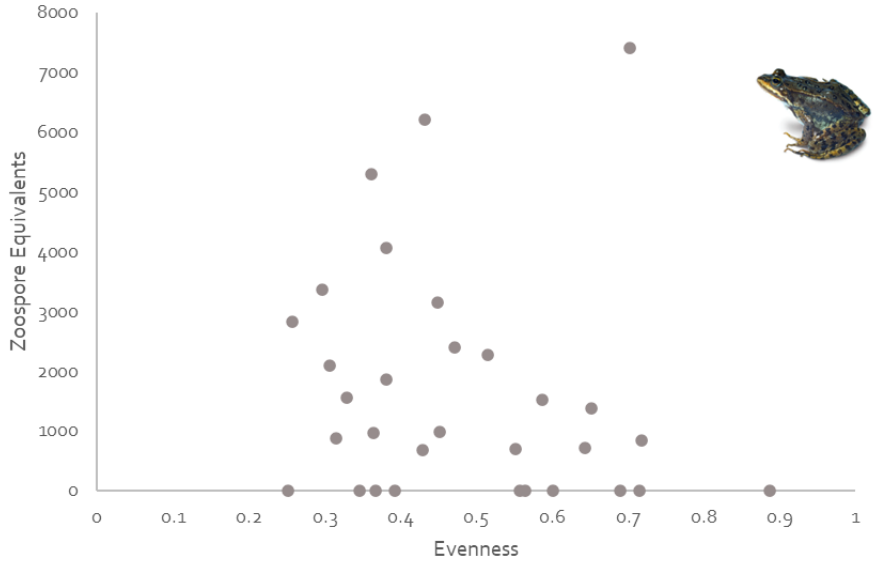


Figure 15. Scatter plot of the CSF with evenness on the x-axis and zoospore equivalence on the x-axis showing that there is no significant relationship between Zoospore Equivalents and evenness (p=0.16, Spearman's Rank Correlation).

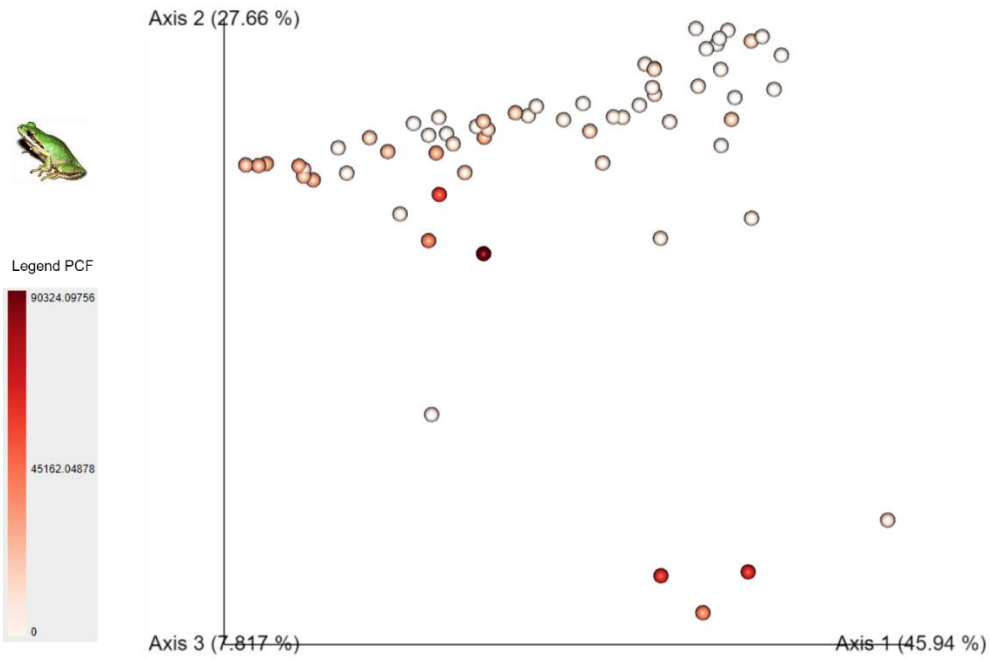


Figure 16. Principal Coordinates Analysis (PCoA) ordination plot of Weighted UniFrac distance. Each dot represents an individual PCF sample. The color of the dots represents *Bd* infection intensity, with white dots having low or not infection and the deep red having high infection. Dots that are close together have similar microbiomes, whereas dots that are farther apart have dissimilar microbiomes. On this plot and based on the associated statistical analysis, there was a clear relationship between *Bd* infection intensity and the microbiome between PCFs (p=0.001, Mantel).

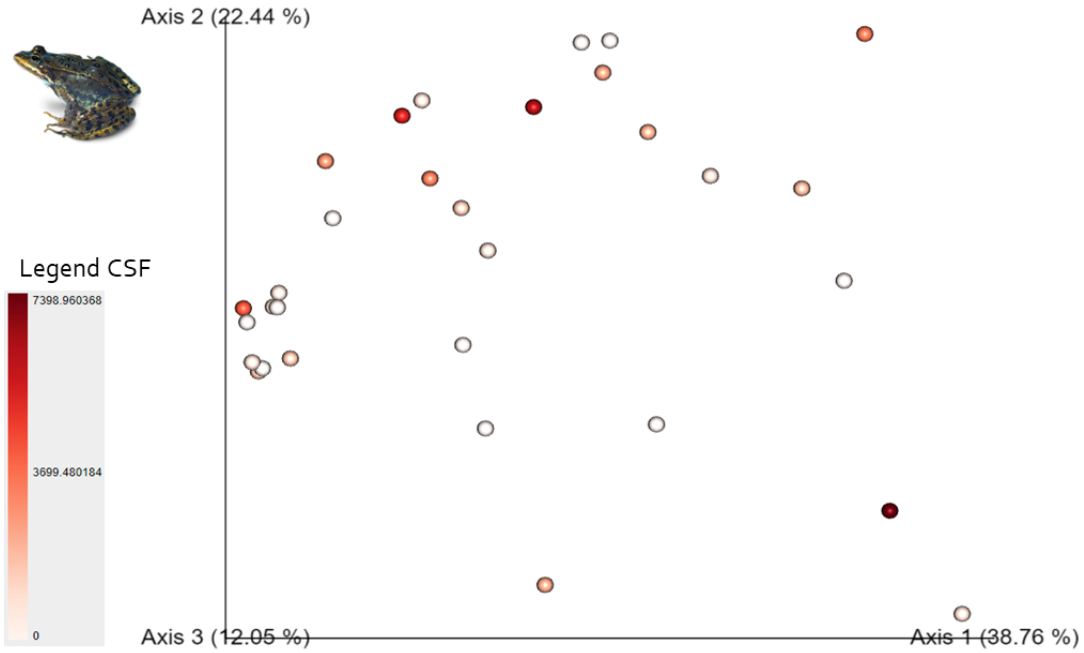


Figure 17. Principal Coordinates Analysis (PCoA) ordination plot of Weighted UniFrac distance. Each dot represents an individual CSF sample. The color of the dots represents *Bd* infection intensity, with white dots having low or not infection and the deep red having high infection. Dots that are close together have similar microbiomes, whereas dots that are farther apart have dissimilar microbiomes. On this plot, there is no significant relationship between *Bd* infection intensity and the microbiome between CSFs (p=0.93, Mantel).

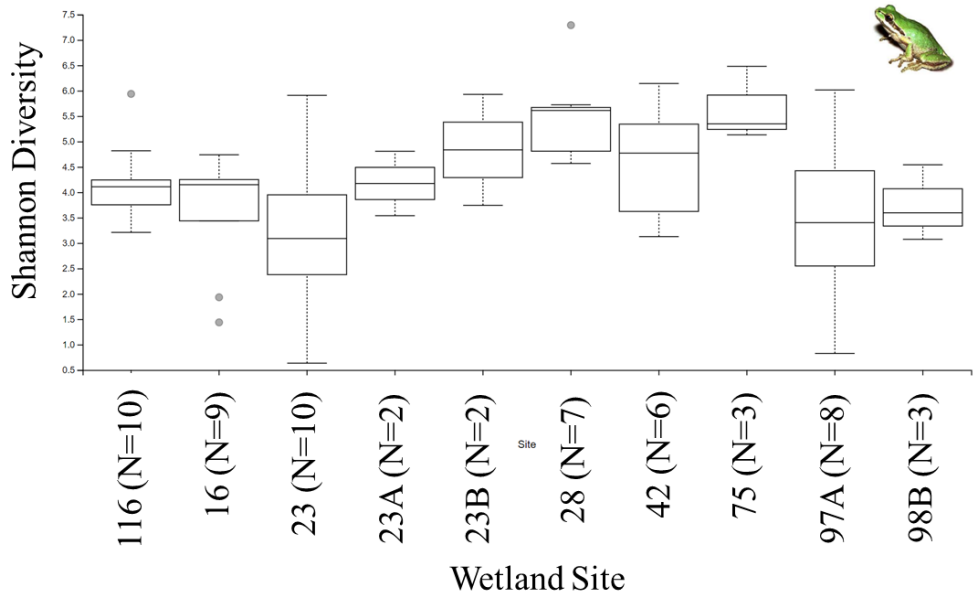


Figure 18. Box and Whisker plot of the wetland sites (2019) that the PCF skin swab samples were collected at (x-axis) and the Shannon diversity of the microbes from the

frogs collected at that wetland site (y-axis) which were significantly different overall (p=0.027, Kruskal-Wallis).

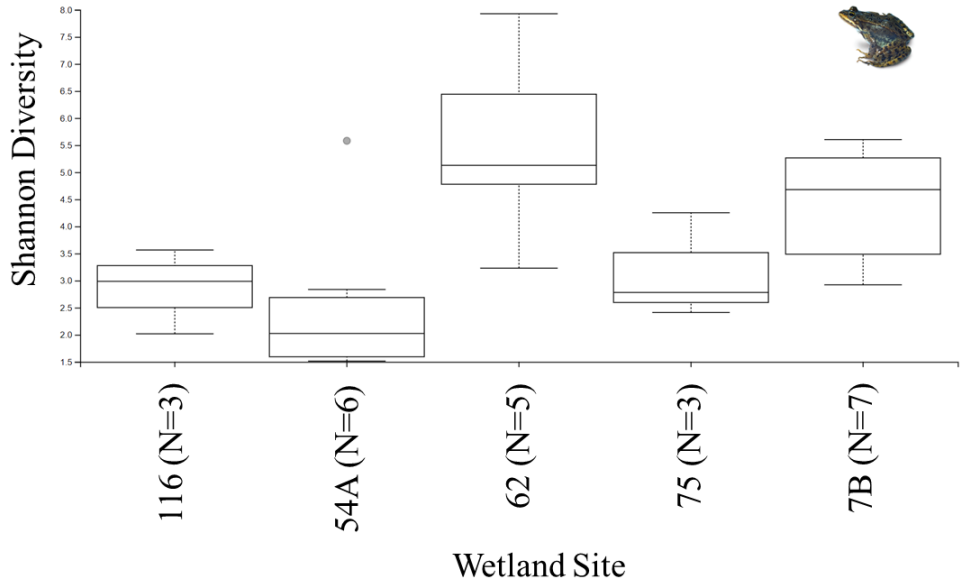


Figure 19. Box and Whisker plot of the wetland sites (2019) that the CSF skin swab samples were collected at (x-axis) and the Shannon diversity of the microbes from the frogs collected at that wetland site (y-axis) which were significantly different overall (p=0.028, Kruskal-Wallis).

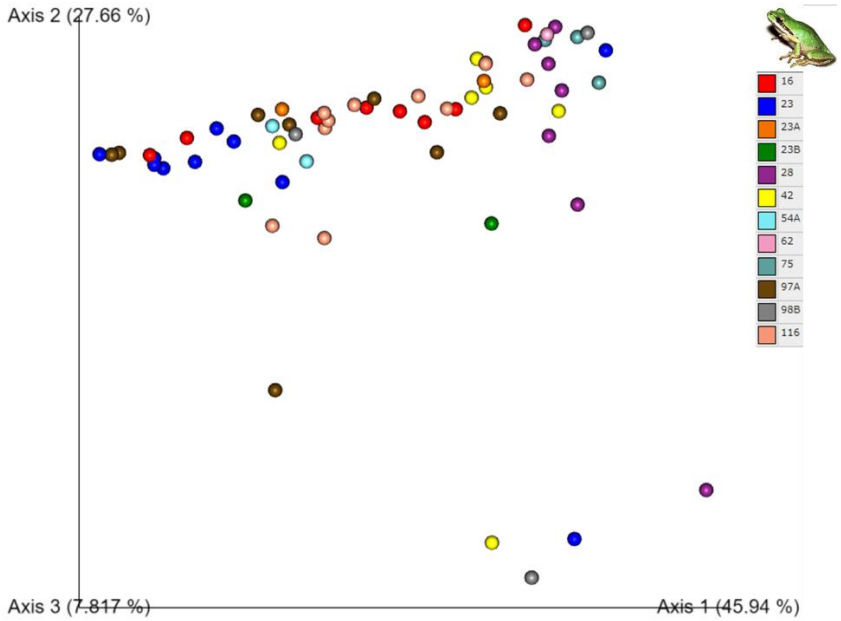


Figure 20. Principal Coordinates Analysis (PCoA) ordination plot of Weighted UniFrac distance for the PCF with each dot representing a single frog and the color of that dot representing the wetland site where the frog skin swab sample was taken. Dots that are closer together have a more similar microbiome and dots that are further apart have a

more dissimilar microbiome. This figure shows that frogs collected from the same wetland site have similar microbiomes (p=0.002, PERMANOVA).

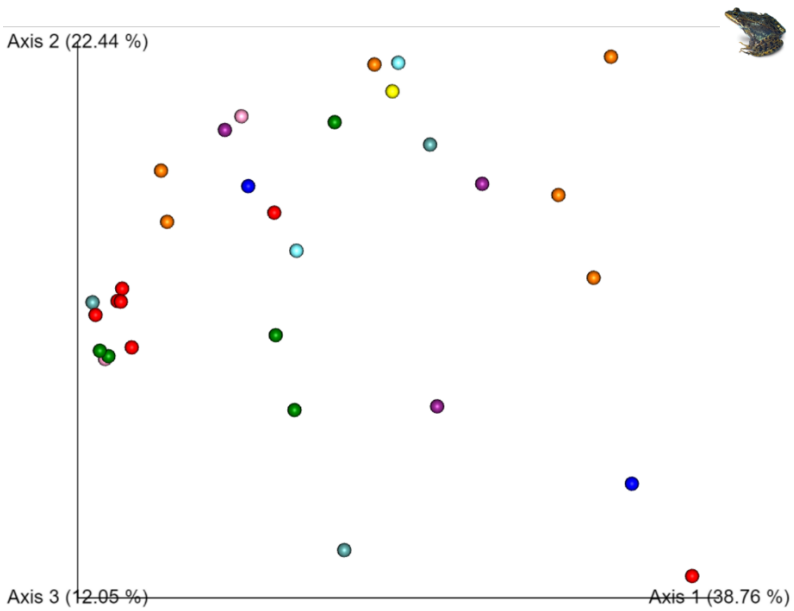


Figure 21. Principal Coordinates Analysis (PCoA) ordination plot of Weighted UniFrac distance for the CSF with each dot representing a single frog and the color of that dot representing the wetland site where the frog skin swab sample was taken. Dots that are closer together have a more similar microbiome and dots that are further apart have a more dissimilar microbiome. This figure shows that frogs collected from the same wetland site do not have the same microbiome diversity (p=0.17, PERMANOVA).

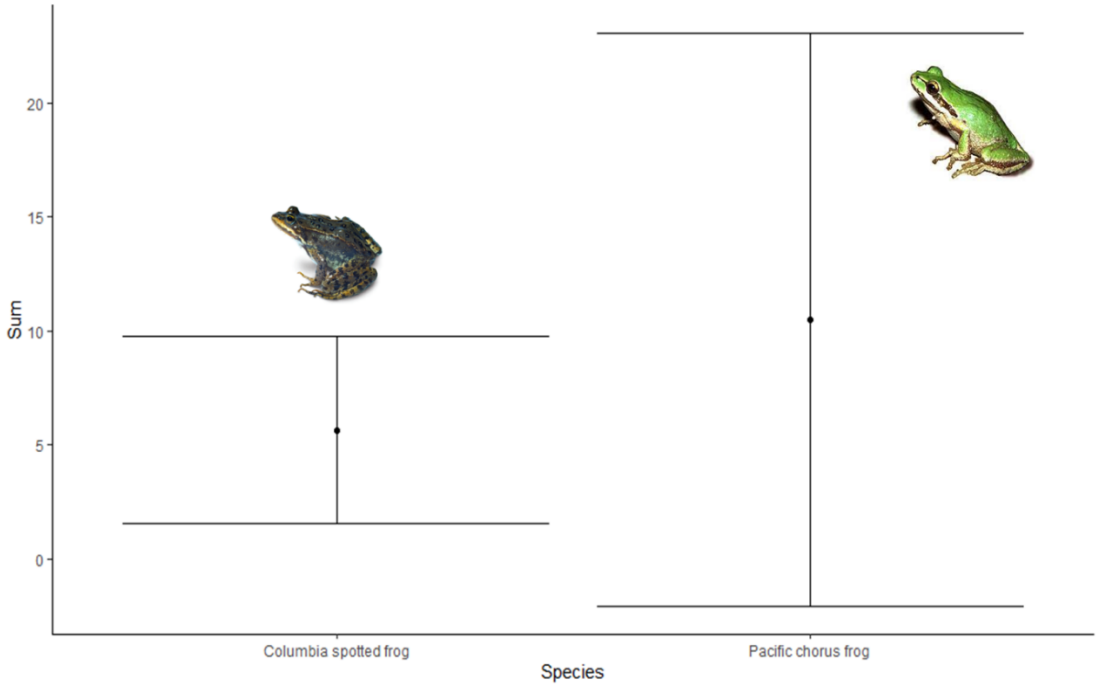


Figure 22. Ggplot of the means of the sum of the relative abundances of culturable anti-*Bd* bacteria for each frog species with the standard deviation. The PCF (n=31) had significantly more anti-*Bd* bacterial species than the CSF (n=70) (p=0.0018, Wilcox)

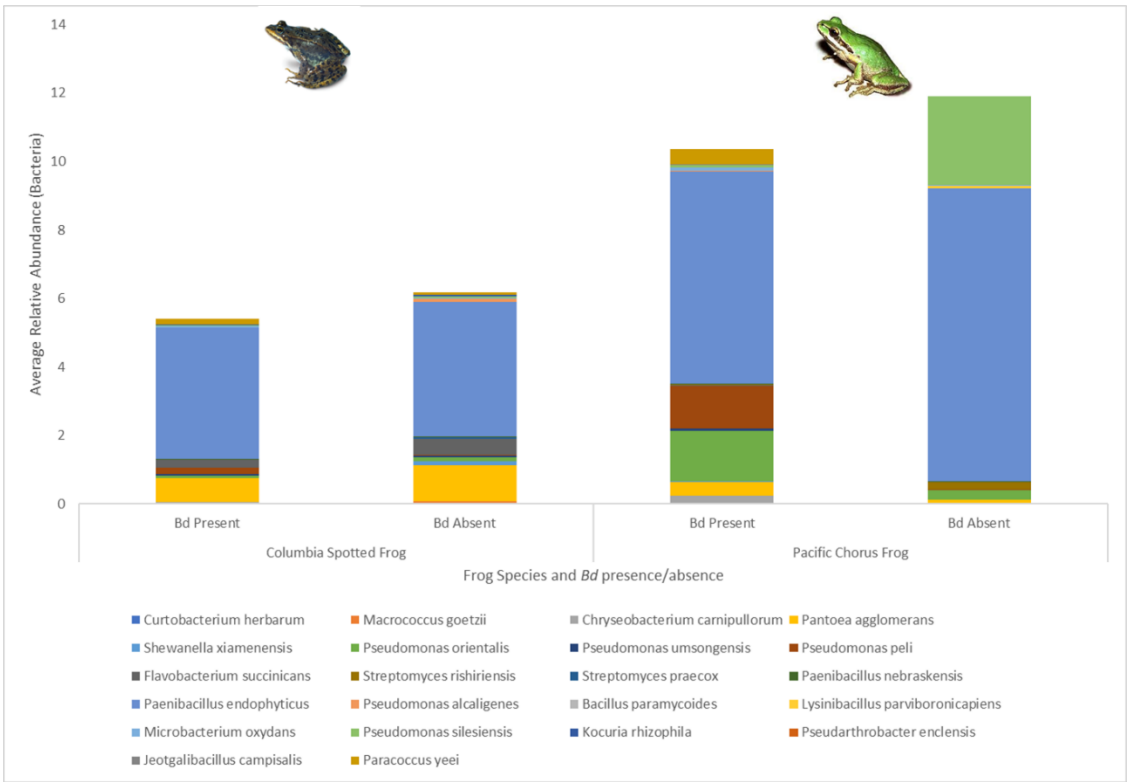


Figure 23. Stacked bar plot with the frog species separated by *Bd* presence on the x-axis and average relative abundance of culturable anti-*Bd* bacteria on the y-axis, based on

comparing culture-dependent and -independent DNA sequences. The CSF is on the left side and the PCF is on the right side.

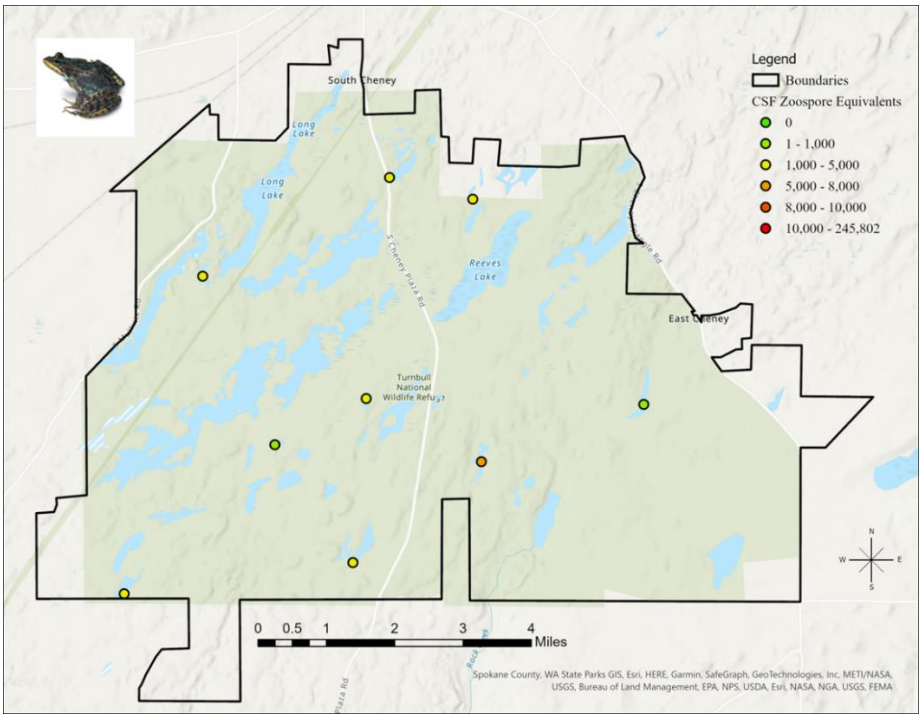


Figure 24. 2019 CSF Bd infection data visualized. The circles on the map indicate locations where the CSF samples were collected. The color of the circle indicates what the average Bd infection was at that location.

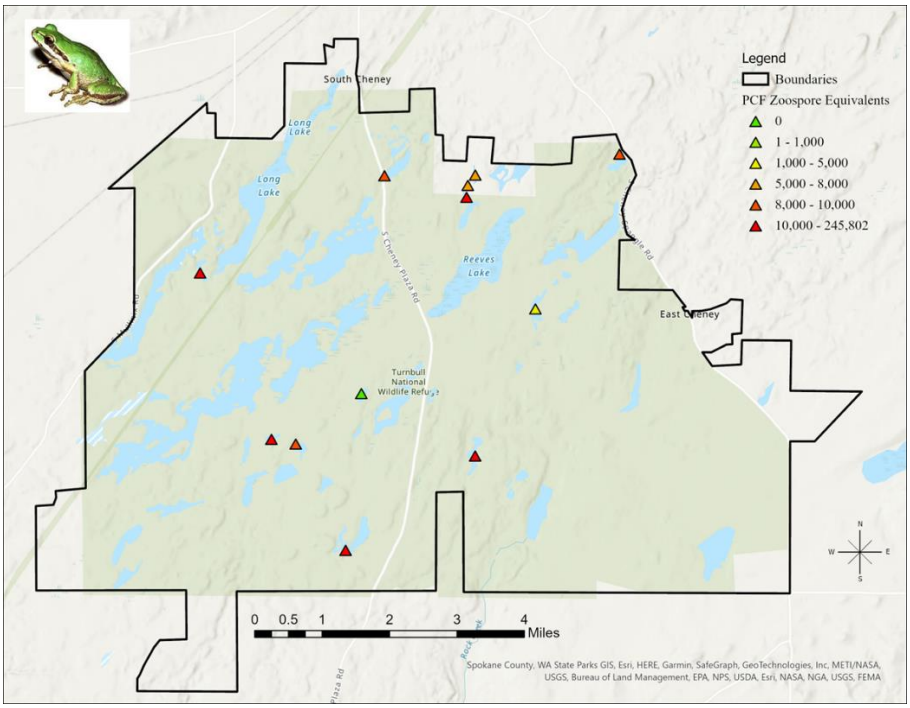


Figure 25. 2019 PCF Bd infection data visualized. The circles on the map indicate locations where the PCF samples were collected. The color of the circle indicates what the average Bd infection was at that location.

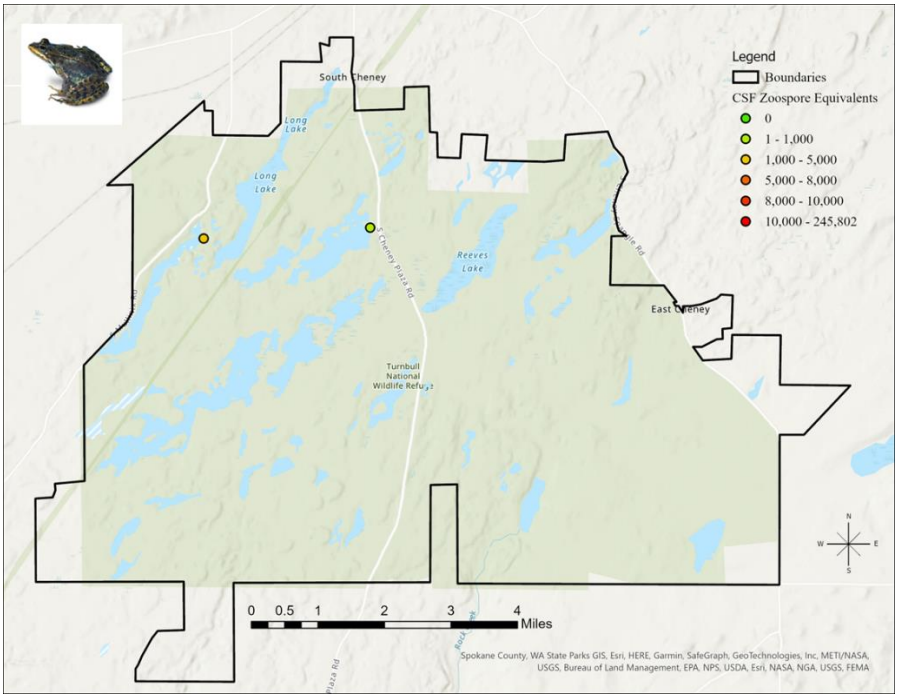


Figure 26. 2020 CSF Bd infection data visualized. The triangles on the map indicate locations where the CSF samples were collected. The color of the triangles indicates what the average Bd infection was at that location.

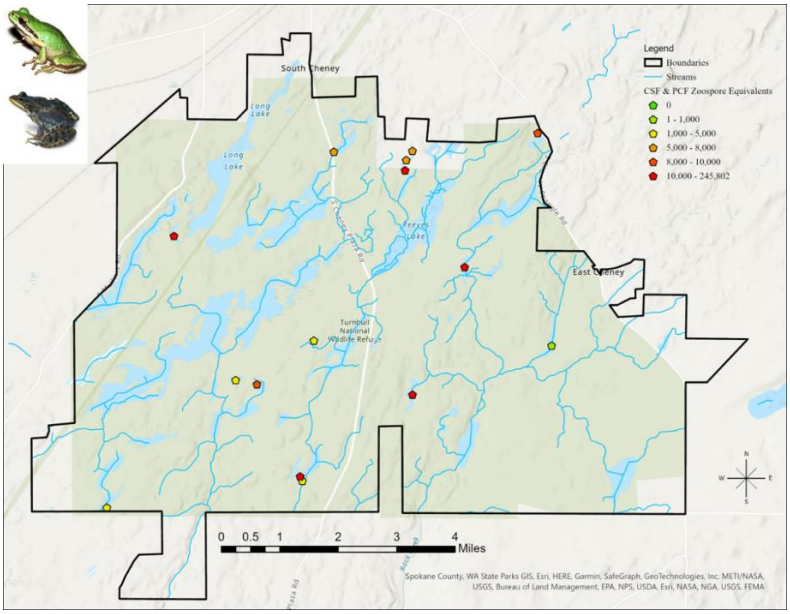


Figure 27. 2019 CSF and PCF Bd infection data visualized with streams. The hexagons indicated where the CSF and PCF samples were collected. The colors of those hexagons indicate the site's average Bd infection intensity. The interconnecting streams are

indicated with lines of a darker blue color. Depending on the flow of the stream, there could be Bd spreading between sites by these interconnecting streams.

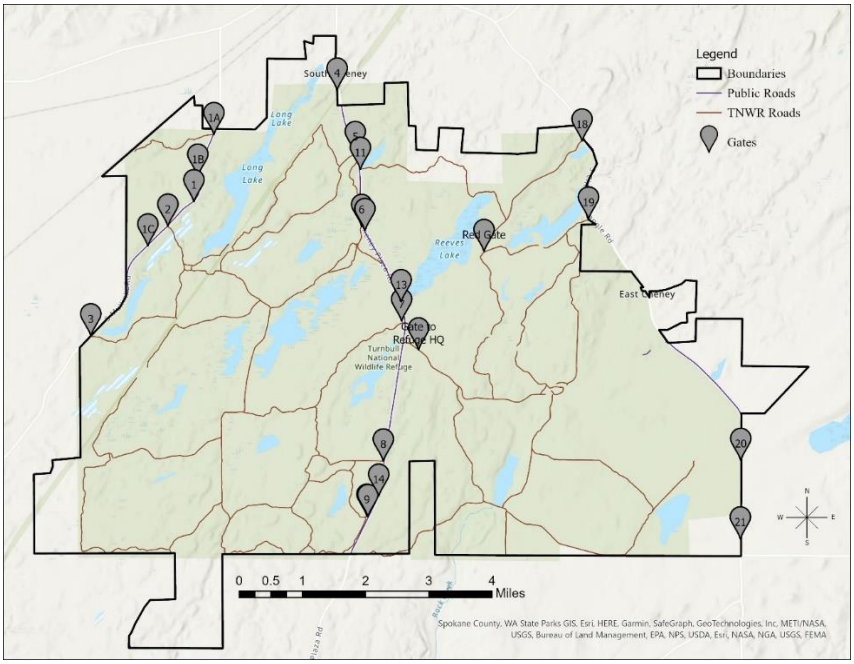


Figure 28. Basemap of Turnbull National Wildlife Refuge (TNWR). This basemap includes the public roads into TNWR, the TNWR access roads, and the main boundaries of TNWR.

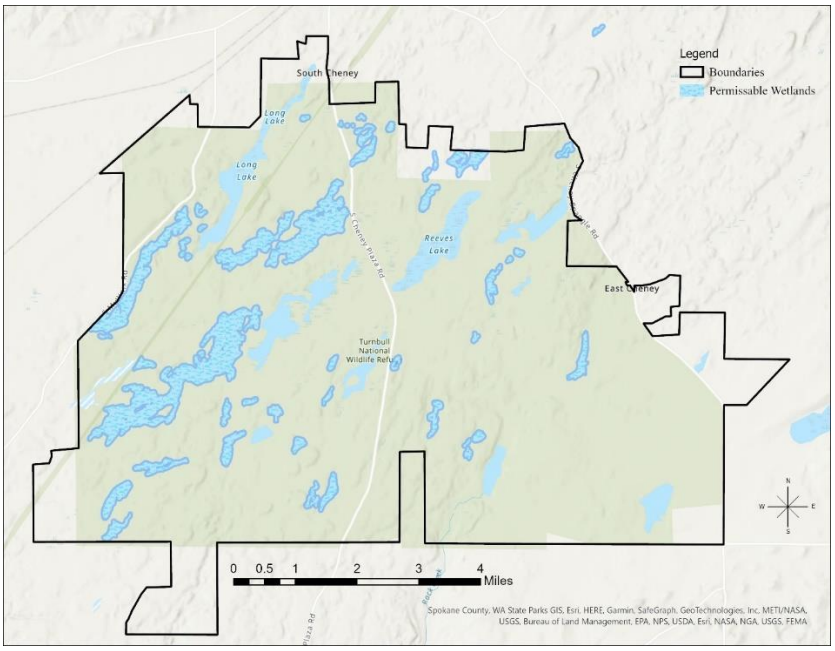


Figure 29. Wetlands available to sample on the TNWR permit that was attained. The wetlands that were outlined are the wetlands that were permissible for sampling in 2019-2021.

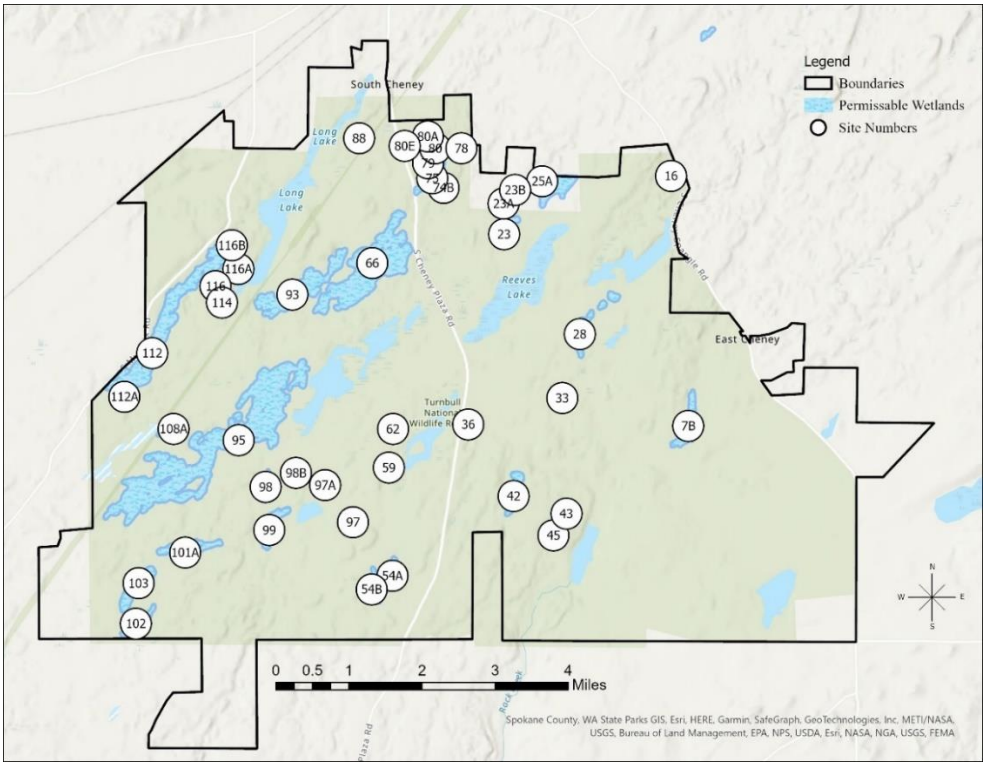


Figure 30. Labeled wetlands. The circles with numbers inside indicate the wetland number that I was able to sample at according to the permit that was attained from 2019-2021. This layer was made so that each wetland could be identified by its labeled number (assigned by TNWR).

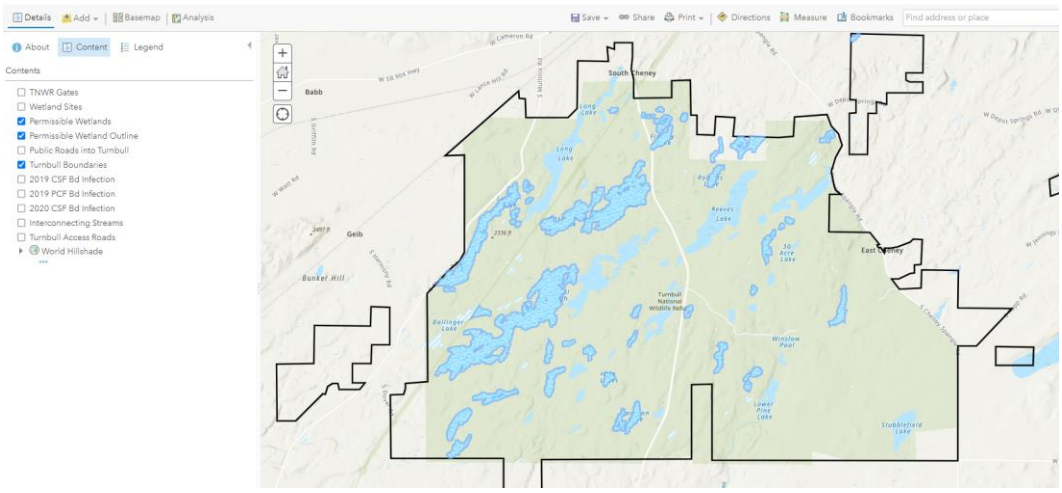


Figure 31. TNWR geodatabase with each layer uploaded to ArcGIS Online. The map displayed is the permissible wetlands (wetlands Walke lab could sample at according to

the permit). On the lefthand side is an example of how the layers display on ArcGIS Online. The layer boxes clicked on at this point are TNWR boundaries, permissible wetlands, and permissible wetlands outline.

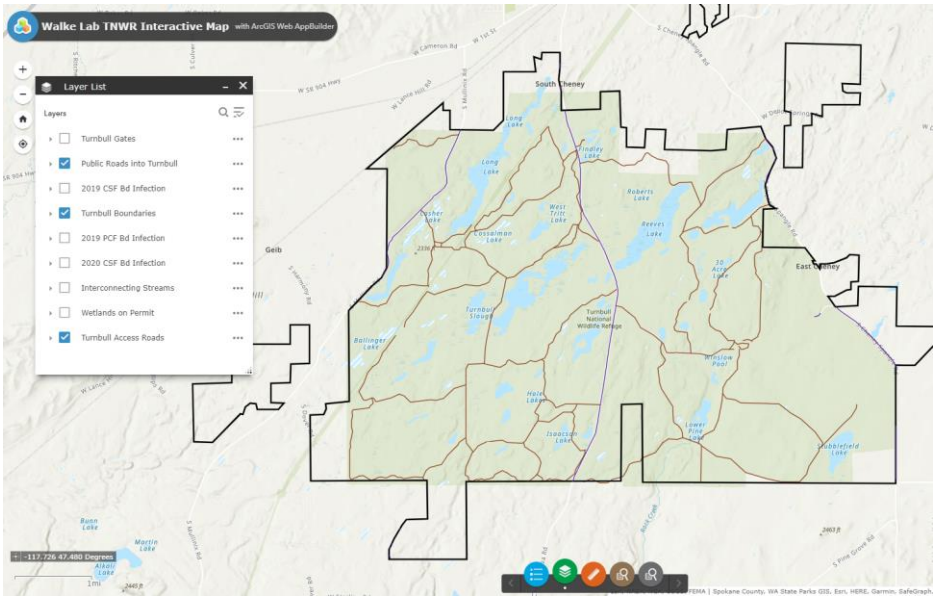


Figure 32. TNWR ArcGIS Online Interactive Map. The layers can be clicked on and off to view different data similar to ArcGIS Pro and ArcGIS Online. The layers clicked on are TNWR boundaries, Public roads, and TNWR access roads. The widgets used to explore layers, searching tools, and the measurement tool are lined up along the bottom.

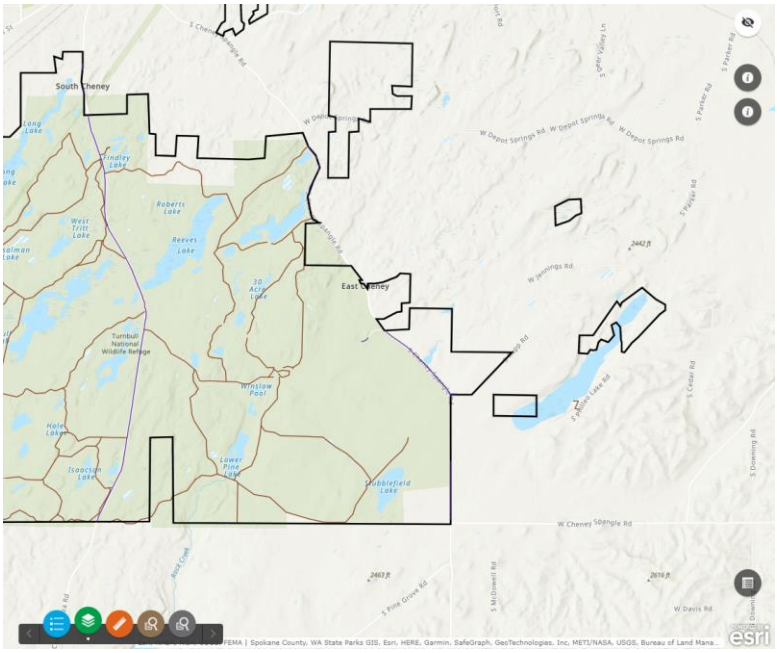


Figure 33. The righthand side of the interactive map indicating the two information bubbles on the righthand side. The top information bubble shows information about the Walke Lab and the second information bubble shows information about TNWR.

APPENDIX

Protocol for Frog Swabbing

A. Prior to going into the field:

1. Gather and organize supplies

2. Charge batteries (if using rechargeable batteries)

3. Sterilize centrifuge tubes, DI water

4. Label tubes (you can also do this in the field, depends on if you know what you are sampling ahead of time)

5. Clean boots and field gear with bleach and Lysol

B. Supply list:

___sterile whirlpack bags

___nonsterile plastic bags for weighing and measuring frogs

___gloves

___Lysol

___Sterile DI water

___50 ml Falcon tubes + rack

___Swabs

___1.5 ml centrifuge tubes

___centrifuge tube racks or plastic freezer boxes

___sharpies

___pencils

___70% ethanol + bottle

___notebook

___trash bags

___cooler/ice

___headlamps/flashlights

___paper towels

___GPS

___Batteries

___Camera

___Thermometer or YSI meter

___Air temperature & humidity gauge

___Scales/Balance for weighing frogs

___Ruler or calipers for measuring frogs

___watch

C. Sampling protocol:

1. Site-level environmental data

- *Air temperature*
- *Water temperature (water temp for stream and pond)*
- *Water quality (PH, DO, Salinity, Conductivity)*
- *Humidity*
- *Description of habitat*
- *Elevation*
- *Latitude, Longitude (take lat and long at stream, pond, forest transects)*

2. Individual frog data

- *Frog ID, Sex, SVL, Weight, anything unusual about the animal*

3. Swabbing

- For microbes, use sterile rayon swabs (from Medical Wire and Equipment). For metabolite analysis using LCMS/UV-vis, use sterile methanol-treated foam swabs.

- If individuals are spread out (i.e., along stream or in the forest), can swab them as you find them. Otherwise, you can place frogs in sterile whirl-pak bags as you find them and swab all frogs at once to make sure you don't re- capture and sample same individuals.

Methods:

Catch frog with clean gloves on. Place in a sterile whirlpack bag if not swabbing immediately. Always change gloves between frogs.

Duties: Person 1 holds frog, Person 2 rinses, swabs, records data

Person 1: Put on fresh gloves, unless you just caught and are still holding the frog. Remove frog from bag if you collected the frog earlier. We recommend swabbing within an hour or so of catching a frog.

Person 2: Put on gloves. “Wash” gloved hands with ethanol frequently to sterilize. Make sure ethanol is evaporated prior to handling frog. Changing gloves is only necessary if you come in contact with a frog or otherwise contaminate your gloves.

Person 2: Prior to swabbing, rinse frog with 50 mL sterile DI water to remove any dirt and transient bacteria while Person 1 is holding frog.

Person 2: Remove swab from package. Swab the ventral surface 10 times (up and down = 1 time), each thigh 5 times in a single direction, and each hind foot 5 times in a single direction. Place swab in sterile, empty 1.5 mL centrifuge tube, and break off excess handle. Place metabolite swabs in sterile 15ml tubes. Close and label tube appropriately. Record Site, Date, Species, Individual #, Individual data, and Label in notebook. Immediately place samples on ice and get to a freezer as soon as possible (record date and time samples collected, as well as when sample was placed in -80 freezer).

Weigh frog in a new, clean plastic bag. Remove frog from bag and weigh bag. Record bag + frog weight and bag weight. Measure SVL. Record in notebook.

Release frog near where collected.

D. Site-level swabbing of the environmental bacteria

Three swabs each of:

- stream water
- pond water
- leaf litter
- soil

Swab areas of the habitat representing each of the above.

Illumina MiSeq Sequencing Protocol

Adapted from the Earth Microbiome Project (EMP, http://www.earthmicrobiome.org/emp-standard-protocols/)

Edited in August, 2017 by: Jeni Walke, Angie Estrada, Daniel Medina, Jessica Hernandez and Lisa Belden.

Reagents:

UltraClean PCR grade H2O

5 Prime Hot Master Mix

Forward primer IL 515F

Reverse primer + barcode IL 806R

Before beginning:

- Sterilize workspace with 10% bleach solution followed by 70% ethanol. If possible, perform in a hood dedicated to PCR set up. UV hood before using.
- Sterilize pipettors (use pipettors dedicated for PCR reagents and use a separate pipettor for the DNA) with bleach and ethanol or with DNA away.
- Clean and sterilize with bleach 1 large centrifuge tube rack and several small PCR tube racks. Rinse and allow to dry.
- Prepare new labels for all of your tubes if necessary.

- Locate samples and barcodes. Assign samples to barcodes. Keep both in fridge until ready to use.

Step 1: Make your PCR reactions

A) For each sample, you will run triplicate PCR reactions plus a negative control with just water = 4 PCR tubes per sample.

Per sample	5X volume (extra for pipetting)
12 µL UltraClean PCR grade H2O	65 µL
10 µL 5 Prime Hot Master Mix	50 µL
0.5 µL Forward primer IL 515F	2.5 µL
0.5 µL Reverse primer + barcode IL 806R	2.5 µL

B) For samples that might have LOW DNA CONCENTRATIONS, the PCR reactions could be prepared with the same method as above, but with a small change in the volume of the reagents and DNA, aditionally BSA could be added to increase PCR yield as follows:

Per sample	4X Volume
12 µL Ultra Clean PCR grade H20	48 µL

10 µL	5Prime Hot Master Mix	40 µL
0.5 µL	Forward Primer IL 515F	2.0 µL
0.5 µL	Reverse Primer + barcode IL 806R	2.0 µL
2.0 µL	DNA	6.0 µL (in triplicate)

1. Add all reagents, except DNA, to the each PCR tube in the first row of the plate.
2. Pipette 23 µL from the first row of PCR tubes, with every reagent listed above except DNA, into the negative PCR tubes.
3. Add DNA (6µL) to first replicate. Vortex gently, then centrifuge briefly
4. Take 25 µL from the first row of PCR tubes and add into replicate rows #2 and #3.
5. Centrifuge each PCR tube briefly to eliminate any bubbles.

Step 2: Run reactions in thermocycler

1. Make sure machine is set for 25 µL samples.
2. Thermocycler conditions:

Temp	Time	
94°C	3 min	
94°C	45 sec	
50°C	1 min	35 cycles
72°C	1.5 min	
72°C	10 min	
4°C	hold	

You can maintain your PCR product in the fridge overnight if you need to wait until the next day to run your gel.

Step 3. Run gels to check amplification and negative controls

1. Combine your three separate PCR reactions into a single PCR tube. Use post-PCR pipettors and tips.

2. Make a 1% gel. Combine 100 ml 1X TBE and 1 g agarose in a small Erlenmeyer flask. Microwave until just boiling. Swirl. Continue boiling/swirling until solution is clear.

3. Once the solution has cooled slightly, add 10 µL gel red stain. Note: Gel red stain is light sensitive--keep away from light as much as possible.

4. Pour gel into mold and allow to cool completely.

5. Combine 4 µL PCR product and 2 µL loading dye Pipette up and down to combine.

6. Reset pipettor to 7 or 7.5 µL. Pipette each sample into gel well. As the amount of solution decreases (due to evaporation), you may need to reset your pipette µL setting. Avoid air bubbles in the pipette tip as this will cause the DNA to leak out. Gently pipette solution into wells.

7. Load your ladder. You can use a broad range 50-10,000 bp ladder.

8. Run gel at a voltage of ~160 for approximately 20 minutes, until dye is about halfway.

9. Visualize gels. Bands will be at ~ 300-350 bp. Sample bands may be a little smeary, but there should not be multiple bands. No bands should be visible for the negative controls.

NOTE: If sample bands are very faint (indicating too low or too high DNA content), try the following alternatives (see table):

a. Modify the starting DNA concentration with 1:10 or 1:50 dilutions. Or use ½ of the DNA volume. Dilute in PCR water.

b. Reduce de volume of water (for example: 4ml/sample) and replace with BSA which increases PCR yield (also usefull when bands are not amplyfing) .

c. If the previos does not work, is possible that DNA is too low in which case duplicate the volume of DNA samples (to 4ml) or try to duplicate DNA + BSA

	Original Reaction		BSA only		½ DNA		2XDNA + BSA	
	Per sample	4X Vol	Per sample	4X Vol	Per sample	4X Vol	Per sample	4X Vol
PCR grade H20	12	48	10	40	13	52	9	36
5Prime Hot MasterMix	10	40	10	40	10	40	10	40

Forward IL 515F	0.5	2	0.5	2	0.5	2	0.5	2
Reverse barcode	0.5	2	0.5	2	0.5	2	0.5	2
DNA (3x)	2	6	2	6	1	3	4	12
BSA			2	8			1	4
Total	25	98	25	98	25	98	25	98

NOTE: If there are bands in the negative control for a sample, redo the PCR

Store PCR products at -20 C until you've accumulated all of the samples that you are going to run on a single Illumina plate before moving on to Step 4.

Step 4: Quantifying the DNA

We use a Qubit 2.0 Florometer and the dsDNA High Sensitivity assay kit. Readings can be a bit fickle, so it is better to do all of your samples on the same day at the same time with the same working solution and standards. This can be done on the countertop. Use post-PCR pipettors and tips.

Before beginning:

- Organize your samples in a single PCR tube rack on ice.
- Label florometry tubes supplied by Qubit in a tube rack with sample names, in the same order as they occur in the PCR tube rack.

1. Combine in a 50 ml falcon tube:

Per sample (so multiply by the number of samples you are quantifying, plus your 2 standards, plus a little extra for pipetting.

1 µL Qubit reagent

199 µL Qubit buffer

Vortex. This is your working solution.

2. Make your standards. Combine 10 µL of each standard with 190 µL working solution. Make a separate solution for each standard and combine in the tubes supplied by Qubit.

3. For your samples: Combine 2-5 µL sample with 198-195 µL working solution. Total solution volume should be 200 µL. Make a separate solution for each sample and combine in the florometry tubes that you labeled already. To get the most accurate measurements, it is very important that you get the precise amount of your entire sample into the working solution. Try 2 µL of sample first. If the readings are too low (there's too little DNA), then redo, increasing the amount sample.

4. Vortex and briefly centrifuge all tubes. Drops of liquid stuck on the sides or lids of tubes can mess up the readings.

5. Incubate at room temperature for 2 min.

6. Read tubes in the Florometer. Specify the amount of sample you used (i.e., 2-5 µL). Record reading in ng/µL.

Step 5: Combine equal amounts of amplicons into a single tube

1. Based on the concentration determined by the Florometer, determine how much of each sample you need to add. The goal is to to add the same amount of ng of DNA per sample (~180 ng) into a single, 1.5 ml centrifuge tube.

Example: If Sample 1 has a concentration of 38 ng/µL, you should add 200/38 = 5.3 µL to the pool.

2. Add the appropriate volume of each sample to a single centrifuge tube. This is your pooled sample. Compute the volume of the pooled sample.

Step 6: Clean up pooled sample.

We use the Qiagen QIAquick PCR Clean Up Kit.

If this is the first time you are using the kit, make sure you add ethanol and the PH indicator to the appropriate buffers as described in the manufacturer's instructions.

1. Vortex the pooled sample to thoroughly mix it. Pipette 100 µL of the pooled sampled into a new, clean 1.5 ml centrifuge tube. **Store the remaining, uncleaned pooled sample in storage box in -20C.

2. Add 500 µL of Buffer PB to the 100 µL of your pooled sample. Vortex. Check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a Qiaquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s at 13,000 rpm.

5. Discard flow-through. Place the QIAquick column back into the same tube.

6. Wash the pooled sample. Add 0.75 ml Buffer PE to the QIAquick column, let the buffer sit on the filter for 2 min, then centrifuge for 30–60 s at 13,000 rpm.

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at 13,000 rpm.

8. Place the QIAquick column in a new, clean 1.5 ml centrifuge tube.

9. To elute the DNA, add 50 µL Buffer EB to the QIAquick column, let the buffer sit on the filter for 3 min, then centrifuge for 1 min at 13,000 rpm.

10. Measure the concentration of the cleaned, pooled sample using the Qubit Florometer (as above, but with only one sample) and the 260/280 using the Nanodrop. 260/280 should be between 1.8-2.0.

Step 7: Add PhiX

For running these libraries in the MISeq and HiSeq, you may need to make your sample more complex by adding 30-50% PhiX to your run.

However, the sequencing facility may add PhiX for you. Check with the particular sequencing facility you are using for information about adding PhiX. The sequencing facility that we use (listed below) adds PhiX for you.

Step 8: Send for sequencing!

Keep cleaned, pooled sample frozen until ready to send. Send sample on dry ice.

Sequencing Facility and contact info:

Zach Herbert <zherbert@research.dfci.harvard.edu>

Molecular Biology Core Facilities

Dana Farber Cancer Institute at Harvard

http://mbcf.dfci.harvard.edu/

DNA Extraction Culture-Dependent Freeze Thaw Protocol

Freeze-Thaw Extraction

1. Sterilely pipette 200µL of buffer TE or **AE** into a microcentrifuge tube
2. Add a colony of bacteria
3. Vortex
4. Place in heat block (99C) for 1 minute
5. Place in -80C freezer for 3 minutes
6. Place in heat block (99C) for 2 minutes
7. Repeat 5 and 6 two more times
8. Centrifuge at 10,000 for 5 minutes
9. Pipette out 100µL of supernatant into a new centrifuge tube
10. Store at -20 to -80C until use

Sigma-Aldrich: GenElute Bacterial Genomic DNA Kit Protocol

A. Gram-Negative Bacterial Preparation

1a. Harvest Cells	Pellet 1.5 mL of an overnight bacterial broth culture by centrifuging for 2 minutes at 12,000-16,000 X g. Remove the culture medium completely and discard. **Note:** Skip if bacteria were grown on agar plates.
2a. Resuspend Cells	Resuspend the pellet thoroughly (or suspend a loop full of bacteria) in 180 µL of Lysis Solution T/Buffer STL for GenElute Mammalian Genomic DNA Kit. If residual RNA is not a concern, continue with step 3a. Optional RNase A treatment**:** If RNA-free genomic DNA is required, add 20 µL of RNase A Solution, mix, and incubate for 2 minutes at room temperature, then continue with step 3a.
3a. Prepare for Cell Lysis	Add 20 µL of the Proteinase K solution to the sample. Mix and incubate for 30 minutes at 55 °C.
4a. Lyse Cells	Add 200 µL of Lysis Solution C, vortex thoroughly (about 15 seconds), and incubate at 55 °C for 10 minutes. A homogeneous mixture is essential for efficient lysis. Continue with step 5.

B. Gram-Positive Bacterial Preparation

1b. Prepare Lysozyme Solution	Prepare a 2.115 x 10^6 unit/mL (45 mg/mL) stock solution of lysozyme as described under Preparation Instructions. For each DNA preparation to be performed, 200 µL of Lysozyme Solution is required. Prepare extra solution to account for pipetting error.
2b. Harvest Cells	Pellet 1.5 mL of an overnight bacterial broth culture by centrifuging for 2 minutes at 12,000-16,000 x g. Remove the culture medium completely and discard. **Note:** Skip if bacteria were grown on agar plates.
3b. Resuspend Cells	Resuspend the pellet thoroughly in 200 µL of Lysozyme solution (prepared in step 1b) (or suspend a loop full of bacteria) and incubate for 30 minutes at 37 °C.
4b. Lyse Cells	Add 20 µL of the Proteinase K solution to the sample, followed by 200 µL of Lysis Solution C. Vortex thoroughly (about 15 seconds) and incubate at 55 °C for 10 minutes. A homogonous mixture is essential for efficient lysis. Continue with step 5.

DNA Isolation from Gram-Positive and Gram-Negative Bacteria

This is a continuation of the procedure from the lysates prepared in steps 1-4a and/or 1-4b.

5. Column Preparation	Assemble a binding column with a 2 mL collection tube. Add 500 µL of Column Preparation Solution to the binding

	column and centrifuge at 12,000 X g for 1 minute. Discard the flow-through liquid but retain the collection tube.
6. Prepare for Binding	Add 200 µL of ethanol (95%-100%) to the lysate from step 4a or 4b and mix thoroughly by vortexing for 5-10 seconds. A homogenous mixture is essential.
7. Load Lysate	Transfer the entire contents of the tube to the treated binding column from step 5. Use a wide bore Pipette tip to reduce shearing the DNA when transferring contents into the binding column. Centrifuge at >6500 X g for 1 minute. Discard the collection tube containing the flow-through liquid and place the binding column in a new 2 mL collection tube.
8. First Wash	Add 500 µL of Wash Solution 1 to the column and centrifuge for 1 minute at > 6500 X g. Discard the flow-through liquid, but retain the collection tube.
9. Second Wash	Add 500 µL of Wash solution Concentration to the column and centrifuge for 3 minutes at maximum speed (12,000-16,000 X g) to dry the column. The column must be free of ethanol before eluting the DNA. Centrifuge the column for an additional 1 minute at maximum speed if residual ethanol is seen. You may empty and re-use the collection tube if you need additional centrifugation step. Finally,

	discard the collection tube containing the flow-through and place the binding column in a new 2 mL collection tube.
10. Elute DNA	Pipette 200 µL of the Elution solution directly to the center of the column; centrifuge for 1 minute at >6500 X g to elute the DNA. To increase the elution efficiency, incubate for 5 minutes at room temperature after adding the Elution Solution, then centrifuge.

Store the DNA at -20 °C.

PCR of 16S rRNA gene for Sanger Sequencing Protocol

Protocol: PCR of 16S rRNA gene for Sanger Sequencing [Updated Oct. 2021 MM]

Reagents:

- Nuclease-free PCR grade H_20
- AccuStart Master Mix
- Forward primer 8F (10 µM)
- Reverse primer 1492R (10 µM)

Before beginning:

- Sterilize workspace with RNA away. If possible, perform in a hood dedicated to PCR set up.

  UV hood for at least 15 minutes before using; UV open PCR tubes for additional 15 minutes.
- Sterilize pipettes with bleach and ethanol, or with RNA away

  (use pipettors dedicated for PCR reagents and use a separate pipettor for the DNA).
- Clean and sterilize with 5% bleach: 1 large centrifuge tube rack and several small PCR tube racks.

  Rinse and allow to dry before use.
- Locate samples and reagents. Keep both in the fridge until ready to use.

Step 1: Make your PCR reactions

A. For each sample, you will run one PCR reaction

B. You will run one negative control each PCR run

C. For samples that might have LOW DNA CONCENTRATIONS, the PCR reactions could

be prepared with the same method below, but with a small change in the volume of the reagents and DNA; additionally, BSA could be added to increase PCR yield.

- If using BSA, the volume of nuclease-free H_2O will need to be adjusted.

"Master-Master Mix" / "Cake Batter"

Per sample	"Cake batter" for N = 8
11 µL Nuclease-free PCR grade H_20 x N (# of samples + 1 extra for pipetting)	88 µL
10 µL 5 Prime Hot Master Mix	80 µL
1 µL Forward primer 8F	8 µL
1 µL Reverse primer 1492R	8 µL
23 µL Total (before adding DNA)	
+ 2 µL DNA (or water for negative control)	
= 25 µL Total (after adding DNA, or water for negative control)	

1. Add all reagents EXCEPT DNA into a 1.5 ml centrifuge tube. This is your "cake batter".

2. Pipette 23 µL of "cake batter" into each of your sample PCR tubes.
3. Add 2 µL of DNA to each tube EXCEPT the negative control tube.
4. Add 2 µL of water (or "cake batter") to the negative control tube.
5. Vortex gently and briefly centrifuge each PCR tube, including negative control.

For samples that need **BSA** added during PCR:

1. Make a working stock of BSA to ensure the concentration is 0.1 µg/µL per PCR reaction
    - If stock BSA = 50 mg/ml, then dilute to a working stock of 10 µg/µL by adding 20 µL of stock BSA + 80 µL of molecular water to a sterile 1.5 ml tube and vortex briefly
    -

1. Add 0.25 µL of the BSA working stock to your PCR tubes and subtract that amount from the

   amount of water in your "cake batter". So, if adding 0.25 µL of working stock BSA to a single PCR reaction, you'd add 10.75 µL of water instead of 11 µL of water in the "cake batter".

    - Example: If 18 samples need BSA: 18 samples x 11 µL nuclease-free H_2O = 198 µL H_2O

      0.25 µL working stock BSA x 18 samples = 4.5 µL BSA

      198 µL H_2O – 4.5 µL BSA = 193.5 µL H_2O

- The rest of the “cake batter” ingredients are NOT changed, only the water is adjusted!

Step 2: Run PCR reactions in thermocycler

1. Make sure the machine is set for 25 µL samples.
2. Thermocycler conditions:

	Temp.	Time	
	94 °C	2 min	
	94 °C	30 sec	Denaturing
	50 °C	30 sec	Annealing
	65 °C	1.5 min	
			Elongation
Repeat cycles 2 – 4		34x	
	65 °C	10 min	
	4 °C	Hold	

1. You can maintain your PCR product in the fridge overnight if you need to wait until the next day to run your gel.

Step 3: Run gel electrophoresis to check amplification and negative controls

1. Make a 1% or 1.5% gel:
    - Combine 1X TBE and agarose in a small Erlenmeyer flask
    - Microwave until just boiling, swirl, continue boiling/swirling until solution is completely clear. Be sure liquid does not overboil—use appropriate size flask for volume of liquid to prevent this from happening.

a. Mini-gels:

.1% = 40 mL buffer + 0.4 g agarose

i.1.5% = 40 mL buffer + 0.6 g agarose

b. Big gels:

.1% = 140 mL buffer + 1.4 g agarose

i.1.5% = 140 mL buffer + 2.1 g agarose

1. Once the solution has cooled slightly, add GreenGlo stain:
    a. Mini gels: 0.4 µL GreenGlo
    b. Big gels: 1.4 µL GreenGlo

NOTE: GreenGlo is the dye that stains your DNA for visualization.

NOTE: GreenGlo is light-sensitive—keep away from light as much as possible!

1. Pour gel into mold and add combs; allow to cool completely.
1. On a strip of parafilm (waxy side), combine 4 µL of PCR product and 1 µL of loading dye.

Pipette up and down to combine.

NOTE: loading dye is the dye that is used to view how far your samples have traveled in the gel during electrophoresis.

1. Reset pipettor to 5 µL and pipette each sample into gel well.

NOTE: As the amount of solution decreases (due to evaporation), you may need to

reset your pipette µL setting. Avoid air bubbles in the pipette tip as this will cause the DNA to leak out. **Gently** pipette solution into the wells.

1. Load 5 µL of DNA ladder into gel. You can use a broad range 50-10,000 bp ladder.

2. Run gel at a voltage of ~160V for approximately 20 minutes, until dye is about halfway across
   gel and each of the three colored bands has separated. Longer time for larger DNA fragments.

1. Visualize gels using ImageLab software. Do not touch the computer, gel imager, or handle on a gel tray with gloved-hands to avoid getting sticky buffer on equipment.

1. Bands for this primer set will be between 1200 and 1500 bp when compared to DNA ladder. Sample bands may be a little smeary, but there should not be multiple bands.
   No bands should be visible for the negative controls.

   NOTE: If sample bands are very faint (indicating low or too high DNA content), try the following alternatives (see table):

   a. Modify the starting DNA concentration with 1:10 or 1:50 dilutions. Or use ½ of the DNA volume. Dilute in PCR water.

   b. Reduce the volume of water and replace with BSA which increases PCR
   yield (also useful when bands are not amplifying).

   c. If the previous troubleshooting methods do not work, it's possible that DNA is too

low in which case double the volume of DNA (to 4 µL) or try to duplicate DNA + BSA

NOTE: If there are bands in the negative control for a sample, redo the PCR

1. Store PCR products at -20°C until you've accumulated all of the samples that you are going to send for sequencing.

    - If sending **pre-cleaned** products for sequencing, continue to Step 4: Quantifying DNA using Qubit

    - If sending **un-cleaned** products for sequencing, determine appropriate amounts of PCR products and primers to be sent according to the company's sequencing guidelines, see "Protocol: Preparing PCR products for Sanger sequencing"

Step 4: Quantifying DNA using Qubit

We use a Qubit 2.0 Florometer and the dsDNA High Sensitivity assay kit.
Readings can be a bit fickle, so it is better to do all of your samples on the same day at the same time with the same working solution and standards. This can be done on the countertop. Use post-PCR pipettes and tips.

Before beginning:

- Organize your samples in a single PCR tube rack on ice.

- Label florometry tubes supplied by Qubit in a tube rack with sample names, in the same order as they occur in the PCR tube rack.

1. Make your standards:

    - Combine 10 µL of each standard with 190 µL of working solution.

    - Make a separate solution for each standard and combine in the tubes supplied by Qubit.

1. For your samples:

    - Combine 2 µL sample with 198 µL working solution.

        - Total solution volume should be 200 µL.

    - Make a separate solution for each sample and combine in the florometry tubes that you labeled already. To get the most accurate measurements, it is very important that you get the precise amount of your entire sample into the working solution.

        - If the readings are too low (there's too little DNA), then redo, increasing the amount sample and adjusting the working solution volume to ensure the total volume is 200 µL.

2. Vortex and briefly centrifuge all tubes.

    - Drops of liquid stuck on the sides or lids of tubes can mess up the readings.

3. Incubate at room temperature for 2 min.

4. Read tubes in the Florometer:

    - Select "dsDNA", then assay "1X dsDNA high sensitivity"

    - Specify the amount of sample you used (2 µL)

- Record the RFU for each standard, then record the readings of your samples
    - Record reading in ng/µL
        - For sequencing, the sample must be at least 2.8 ng/µL

Chytrid-Bacteria 96-well Plate Assay Protocol

Based on Bell et al. 2013

Adapted procedure from Becker et al. (2015) and Walke et al. (2017)

Supply list:

- 200 µL Pipettor
- Multi-tip pipettor
- 1000 µL pipettor
- 200 µL filtered tips
- 1000 µL filtered tips
- Culture flasks
- 16 mm culture tubes
- 1.5 ml microcentrifuge tubes
- 50 ml falcon tubes
- 96-well plates and lids (USA Scientific # cc7672-7596)
- Petri-plates
- 1% tryptone (broth and plates)
- TSYE-glycerol
- 3 ml syringes
- 13 mm syringe-filter holders (Millipore #SX0001300)
- 13 mm, 0.22 um filters (Millipore #GSWP01300)
- 47 mm filter holder (Millipore #XX1104700)
- 47 mm, 20 um filter (Millipore #NY2004700)

1% Tryptone Broth (1 L)

- 10 g Tryptone Powder
- 1 L diH_2O
- Autoclave

1% Tryptone Plates (~20 Plates)

- 10 g Tryptone Powder
- 10 g Agar
- 1 L diH_2O
- Autoclave

TYSE + Glycerol (250 mL)

- 0.5 g Trypticase Soy Broth
- 0.25 g Yeast Extract
- 50 mL Glycerol (Glycerin)
- 200 mL diH_2O
- Autoclave 20 min

Perform entire procedure in a biosafety cabinet, UV'ed for at least 20mins.

Day 1:

1. Set up new liquid culture of Bd from stock.
2. Plate bacterial cultures from glycerol stock onto R2A or 1% Tryptone plates using sterile yellow inoculating loop.

Day 7:

1. Autoclave 32 culture tubes or use 24 well plates.
2. Defrost 29 bacterial or fungal isolates to be tested from TSYE-glycerol stock.
3. Scrape the side of the 7-day-old Bd stock.
4. Set up culture tubes (50 mL sterile glass tubes):
    0. Positive control: 1000 µL 1% tryptone, 125 µL Bd, & 125 µL of TSYE-glycerol
    1. Negative control: 1125 µL 1% tryptone & 125 TSYE-glycerol
    2. Test samples: 1000 µL of 1% tryptone, 125 µL Bd, 125 µL TSYE-glycerol bacteria stock
5. Slant culture tubes in rack and put on shaker (100 rpm) for 3 days at Room Temp (RT).
6. Make 3 Bd plates:
    0. Pipette 1.5 ml Bd onto 1% tryptone plates.
    1. Swirl Bd so liquid covers plate evenly.
    2. Keep plates half-covered in hood for approximately 10 to 15 minutes. (you want the liquid to soak into the agar, but you don't want the plates to dry out)
    3. Wrap plates with parafilm and place in incubator (23 C)

Day 9:

1. Set up bacteria syringe-filters (13 mm, 0.22 um) and autoclave
2. Set up Bd filter (47 mm, 20 um) and autoclave

Day 10:

1. Prepare filtered Bd:
    0. Flood Bd plates with 3 ml of tryptone. Let sit for 3-5 minutes.
    1. Remove liquid and place into falcon tube labeled unfiltered Bd.
    2. Filter Bd into new falcon tube labeled filtered Bd with 20 um filter.
2. Dilute Bd to 2x10^6 zoospores/ml:
    0. Prepare dyed zoospore solution:

        0.50 µL filtered Bd

        1.49 µL tryptone

        2.1 µL iodine

a. Count zoospores using hemacytometer. Count four corner boxes and center box four different times. Average counts. **Avg x 50000 x 2**

b. C1V1= C2V2 (**found concentration * V = 2,000,000 * 30ml**)

c. Desired volume =30 ml (for 1 assay plate, this can be ~15ml)

Desired concentration = 2,000,000 zoosp/ml

Dilute using media.

a. Put into new falcon tube labeled 2x10^6 zoospores.

b. Put 2x10^6 zoospores into sterile, empty Petri plate (or multi-channel pipette reservoir).

c. Heat-kill ~500µL diluted Bd at 60C for 30-60 minutes. Cool completely.

1. Prepare bacterial extracts (i.e. Cell-free Supernatant, CFS):
    0. Confirm bacterial growth via visual turbidity check.

1. Vortex bacteria/Bd culture tubes for about 5 sec and then transfer 1ml to 1.5 ml microcentrifuge tube. Use new pipet for each sample.
2. Centrifuge bacteria/Bd for 5min at 10,000 rpm.
3. Filter bacteria/Bd supernatant into new microcentrifuge tubes using 0.22 um syringe-filter.
4. Place used filters in 70% ethanol.

2. Setup 96-well plate:
    0. Positive: 100 µL zoospores + 100 µL positive extract (this includes the Bd metabolites, or CFS)
    1. Bd-Negative: 100 µL heat-killed Bd + 100 µL positive extract
    2. Negative: 100 µL 1% tryptone + 100 µL positive extract
    3. Test samples: 100 µL zoospores + 100 µL filtered bacterial extract
3. Read plate using microplate reader at 492 nm (Day 0 reading).
4. Parafilm plate and incubate at 23 C

Day 11:

1. Read plate using microplate reader at 492 nm (Day 1 reading).

Day 14:

1. Read plate using microplate reader at 492 nm (Day 4 reading).

Day 17:

1. Read plate using microplate reader at 492 nm (Day 7 reading).

Day 20:

1. Read plate using microplate reader at 492 nm (Day 10 reading).

QIIME 2 Bioinformatics Processing Steps

QIIME2 Program and Version: qiime info
(qiime2-2021.11) qiime2@qiime2core2021-2:~$ **qiime info**

System versions
Python version: 3.8.12
QIIME 2 release: 2021.11
QIIME 2 version: 2021.11.0
q2cli version: 2021.11.0

Application config directory
qiime@qiime2core-2021-11:/media/sf_QiimeShare$

Getting help
To get help with QIIME 2, visit https://qiime2.org

Create Share Folder for QIIME2
The first thing I had to do was to create a share folder on my computer, so that when I opened QIIME2 terminal, it pulled up the same folder that I could pull up on my computer.

I created my manifest file for our single-end sequencing data and tried to import my data into QIIME2.

Creating your manifest file:

- The headers must be the way they are below, with "sample-id" and "absolute-filepath"
    - Also, QIIME doesn't like underscores (_), dashes (-) or spaces in sample IDs, so I replaced all of those with periods
- To get the absolute file path, I dragged and dropped my zipped fasta files into the QIIME2 terminal, and then copy-pasted those into my manifest for the corresponding sample
- ***The manifest file I am using is in the directory I am working from (my "QIIME" folder) not a subfolder within that directory folder – this is important or else it won't work for the import step!***
- My manifest file was saved as a .csv, as this seems to work well for PCs. It seems that Mac users have some more wiggle room in regards to what file type they save their manifest file as (i.e., .txt), but I've had the best success with a .txt.
- I had to pull sequences from three different files and put them into one file.
- First, I had to remove all single quotations (') around the file path names in excel. We think that these are added during the transfer between the two systems. So, the 'file paths' were changed to just file paths with no quotations

```
(qiime2-2021.11) qiime2@qiime2core2021-11:~$ '/media/sf_QiimeShare/Bd Data/20210
401_1W23_JW8816_S74_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_2W23
_JW8816_S75_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_3W23_JW8816_
S76_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_4W23_JW8816_S77_R1_0
01.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_6W23_JW8816_S78_R1_001.fastq
.gz' '/media/sf_QiimeShare/Bd Data/20210401_7W23_JW8816_S79_R1_001.fastq.gz' '/m
edia/sf_QiimeShare/Bd Data/20210401_9W23_JW8816_S81_R1_001.fastq.gz' '/media/sf_
QiimeShare/Bd Data/20210401_10W23_JW8816_S82_R1_001.fastq.gz' '/media/sf_QiimeSh
are/Bd Data/20210401_11W97A_JW8816_S83_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd
 Data/20210401_12W97A_JW8816_S84_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/
20210401_13W97A_JW8816_S85_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/202104
01_14W97A_JW8816_S86_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_15W
97A_JW8816_S87_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_16W97A_JW
8816_S88_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_17W97A_JW8816_S
89_R1_001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_18W97A_JW8816_S90_R1_
001.fastq.gz' '/media/sf_QiimeShare/Bd Data/20210401_19W97A_JW8816_S91_R1_001.fa
stq.gz' '/media/sf_QiimeShare/Bd Data/20210401_20W97A_JW8816_S92_R1_001.fastq.gz
' '/media/sf_QiimeShare/Bd Data/20210401_21W97A_JW8816_S93_R1_001.fastq.gz' '/me
dia/sf_QiimeShare/Bd Data/20210401_22W16_JW8816_S94_R1_001.fastq.gz' '/media/sf_
QiimeShare/Bd Data/20210401_23W16_JW8816_S95_R1_001.fastq.gz' '/media/sf_QiimeSh
are/Bd Data/20210401_24W16_JW8816_S80_R1_001.fastq.gz' q
bash: /media/sf_QiimeShare/Bd Data/20210401_1W23_JW8816_S74_R1_001.fastq.gz: No
such file or directory
(qiime2-2021.11) qiime2@qiime2core2021-11:~$ '/media/sf_QiimeShare/Bd Data JW702
```

sample-id	absolute-filepath	direction
23A.1	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23A_1_JW7027-2_S76_R1_001.fastq.gz	Single
23A.2	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23A_2_JW7027-2_S77_R1_001.fastq.gz	Single
23A.3	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23A_3_JW7027-2_S78_R1_001.fastq.gz	Single
23B.C1	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23B_C1_JW7027-2_S79_R1_001.fastq.gz	Single
23B.C2	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23B_C2_JW7027-2_S80_R1_001.fastq.gz	Single
23B.C3	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23B_C3_JW7027-2_S81_R1_001.fastq.gz	Single
23B.C4	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23B_C4_JW7027-2_S82_R1_001.fastq.gz	Single
23B.C5	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_23B_C5_JW7027-2_S83_R1_001.fastq.gz	Single
98B.CSF1	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_98B_CSF1_JW7027-2_S84_R1_001.fastq.gz	Single
98B.CSF2	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_98B_CSF2_JW7027-2_S85_R1_001.fastq.gz	Single
28.3.1	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_28_3_1_JW7027-2_S86_R1_001.fastq.gz	Single
28.3.2	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_28_3_2_JW7027-2_S87_R1_001.fastq.gz	Single
28.3.3	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_28_3_3_JW7027-2_S88_R1_001.fastq.gz	Single
28.3.4	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_28_3_4_JW7027-2_S89_R1_001.fastq.gz	Single
28.3.5	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_28_3_5_JW7027-2_S90_R1_001.fastq.gz	Single
28.3.6	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_28_3_6_JW7027-2_S91_R1_001.fastq.gz	Single
28.3.7	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_28_3_7_JW7027-2_S92_R1_001.fastq.gz	Single
98B.4.1	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_98B_4_1_JW7027-2_S93_R1_001.fastq.gz	Single
98B.4.2	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_98B_4_2_JW7027-2_S94_R1_001.fastq.gz	Single
98B.4.3	/media/sf_QiimeShare/Bd Data JW7027-2/20190531_98B_4_3_JW7027-2_S95_R1_001.fastq.gz	Single
1W23	/media/sf_QiimeShare/Bd Data JW7027-2/20210401_1W23_JW8816_S74_R1_001.fastq.gz	Single
2W23	/media/sf_QiimeShare/Bd Data JW7027-2/20210401_2W23_JW8816_S75_R1_001.fastq.gz	Single

1.) Importing Sequence Data into QIIME:

For single-end sequence data:

qiime tools import --type 'SampleData[SequencesWithQuality]' --input-path Manifest" "7027_2-new.txt --output-path manifesttest-single-end-demuxround6.qza --input-format SingleEndFastqManifestPhred33V2

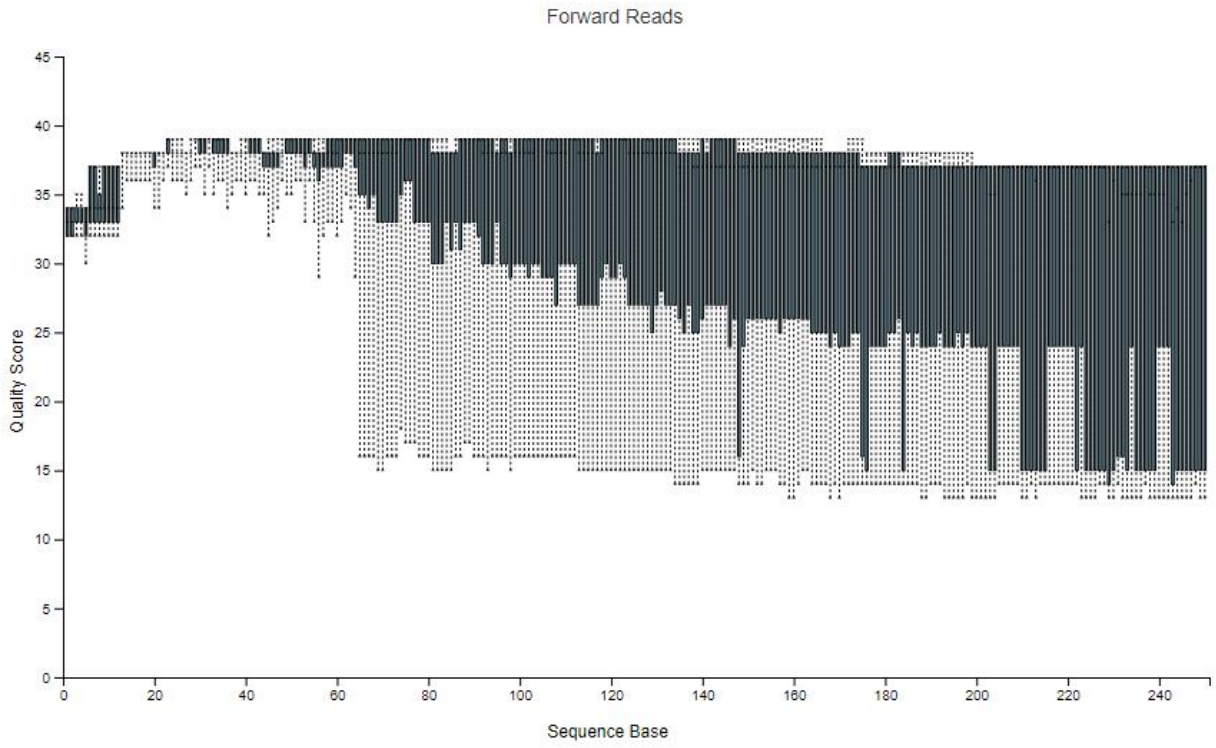
Imported Manifest 7027_2-new.txt as SingleEndFastqManifestPhred33V2 to manifesttest-single-end-demuxround6.qza

2.) Visualize the sequence data in QIIME:

qiime demux summarize --i-data '/media/sf_QiimeShare/manifesttest-single-end-demuxround6.qza' --o-visualization TurnbullFrogs2019-single-end-demuxround6.qzv
Saved Visualization to: TurnbullFrogs2019-single-end-demuxround6.qzv
Use the .qzv file from this step in qiime2view to visualize the sequence quality

3.) QZV File visualization via qiime2view: https://view.qiime2.org/

Quality plot:



- The sequence data quality drops off initially around 140 bases, although it drops off even more around 190 bases
- Total number of sequences: 8,607,133 sequences
- Number of reads per sequence: average of 84,383.656863

Where should I trim the sequences for the deblur step?

- **Visually:** No trimming due to multiple sequences falling under the “poor quality” category

- **Figaro: https://github.com/Zymo-Research/figaro**
    - Amplicon length = 250 (250 bp long total)
    - Forward primer length (515f) = 19, have barcode tag as well (Parada)
    - Reverse primer length (926r) = 20 (Quince)
    - Path to output → drag and drop a folder for a location, give it a unique name, specify location
    - Figaro ended up not working so we didn't bother with it

What is deblur and what does it do?
Deblur is a quality-control step used to trim and filter single end sequence data by their quality. Sequences that fall below a certain quality threshold are discarded, and low-quality reads are trimmed out.

After this, we will trim the sequences by sight (either no trimming at all because the quality of these sequences is not that bad). By sight, we looked at the dark bands coming down from the curve. Most people use a quality score of 25 as the cut-off (which most of ours are above), although using 30 if possible is even better.

Useful info about deblur and QIIME processing workflow in general:
https://awbrooks19.github.io/vmi_microbiome_bootcamp/rst/3_sequences_to_composition.html

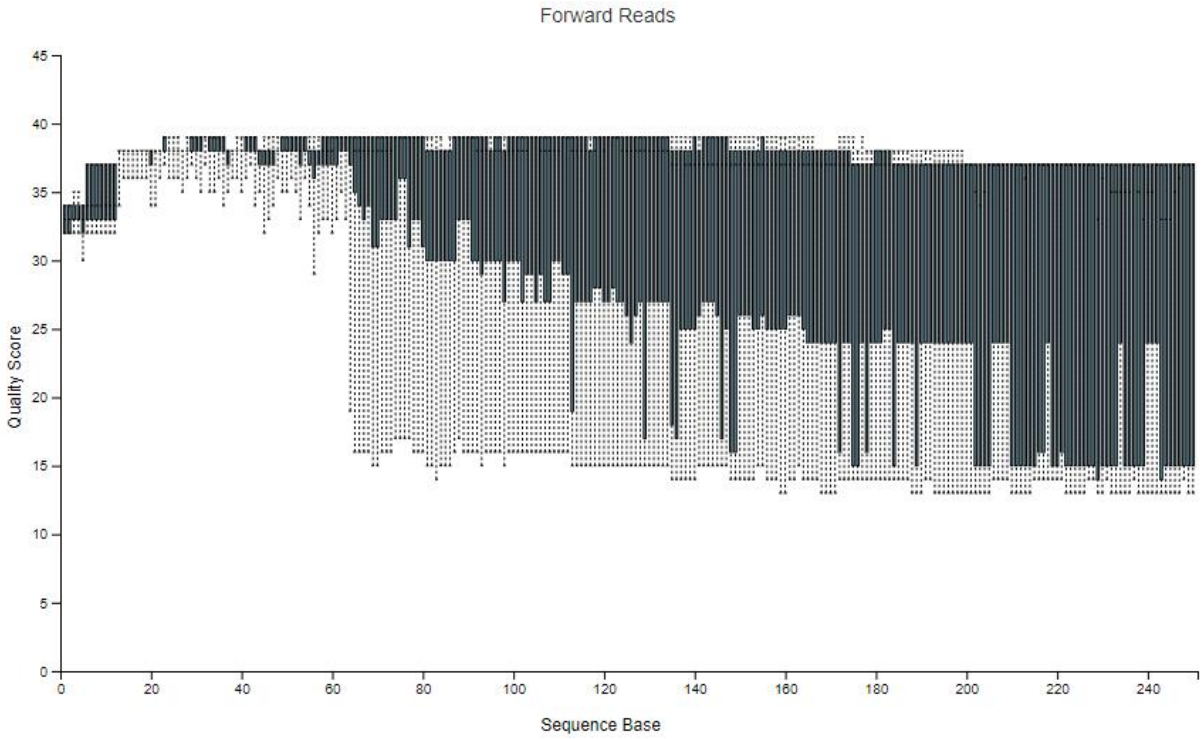
3.) Quality control using Deblur Filter by quality score:

qiime quality-filter q-score --i-demux manifesttest-single-end-demuxround6.qza --o-filtered-sequences TurnbullFrogs2019_demux-filtered.qza --o-filter-stats TurnbullFrogs2019_demux-filter-stats.qza
Saved SampleData[SequencesWithQuality] to: TurnbullFrogs2019_demux-filtered.qza
Saved QualityFilterStats to: TurnbullFrogs2019_demux-filter-stats.qza

4.) Visualize the output from the filter quality control step:

qiime demux summarize --i-data TurnbullFrogs2019_demux-filtered.qza --o-visualization TurnbullFrogs2019_demux-filtered-stats.qzv
Saved Visualization to: TurnbullFrogs2019_demux-filtered-stats.qzv

Filtered sequences (TurnbullFrogs2019_demux-filtered-stats.qzv) QZV visualization in qiime2 view:



- Total number of sequences: 8,606,252reads (vs. 8,607,133 unfiltered)
    - The filter step filtered out 881 sequences (lost ~1.0x 10^-4% of total sequences, kept 99.9% of sequences)
- Number of reads per sequence: average of 84,375.019608 reads (vs. 84,383.656863 unfiltered)

5.) Visualize the filtered stats - visualize the sample list, how quality filtering step worked (use this in addition to the quality plot comparisons)

qiime quality-filter q-score --i-demux manifesttest-single-end-demuxround6.qza --o-filtered-sequences TurnbullFrogs2019_demux-filtered.qza --o-filter-stats TurnbullFrogs2019_demux-filter-stats.qza
Saved SampleData[SequencesWithQuality] to: TurnbullFrogs2019_demux-filtered.qza
Saved QualityFilterStats to: TurnbullFrogs2019_demux-filter-stats.qza

qiime demux summarize --i-data TurnbullFrogs2019_demux-filtered.qza --o-visualization TurnbullFrogs2019_demux-filtered-stats.qzv
Saved Visualization to: TurnbullFrogs2019_demux-filtered-stats.qzv

6.) Run the deblur process: check sequence length summary in QIIME2 view
Deblur groups based on sequence similarity and an algorithm (figures out if sequence is real biological sequence vs. an error based on how present it is across the whole dataset).

qiime deblur denoise-16S --i-demultiplexed-seqs TurnbullFrogs2019_demux-filtered.qza --p-trim-length 250 --o-representative-sequences TurnbullFrogs2019_deblur_seq.qza --o-table Turnbull-table-deblur.qza --p-sample-stats --o-stats Turnbull-deblur-stats.qza
Saved FeatureTable[Frequency] to: Turnbull-table-deblur.qza

Saved FeatureData[Sequence] to: TurnbullFrogs2019_deblur_seq.qza
Saved DeblurStats to: Turnbull-deblur-stats.qza

7.) Visualize the deblur stats

qiime deblur visualize-stats --i-deblur-stats Turnbull-deblur-stats.qza --o-visualization Turnbull-deblur-stats.qzv
Saved Visualization to: Turnbull-deblur-stats.qzv

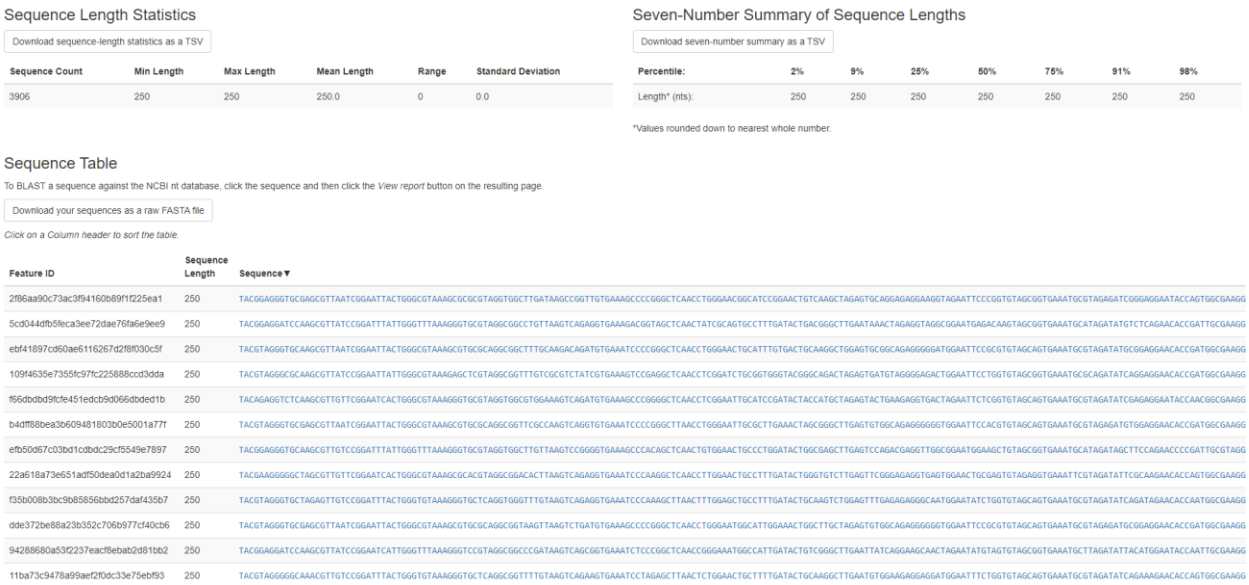
Deblur stats table in qiime2 view: visualize with the deblur-stats.qzv

	sample-id	reads-raw	fraction-artifact-with-minsize	fraction-artifact	fraction-missed-reference	unique-reads-derep	reads-derep	unique-reads-deblur	reads-deblur	unique-reads-hit-artifact	reads-hit-artifact	unique-reads-chimeric	reads-chimeric	unique-reads-hit-reference	reads-hit-reference	unique-reads-missed-reference	reads-missed-reference
0	23B.C2	20	1.000000	0.000000	NaN	0	0	0	0	0	0	0	0	0	0	0	0
1	23B.C3	170250	0.989480	0.097034	0.054902	5820	18311	329	1533	5428	16520	2	3	51	737	18	84
2	23B.C1	10589	0.927472	0.000189	0.000000	81	770	45	591	1	2	1	2	37	572	0	0
3	98B.4.2	73931	0.895484	0.011768	0.022539	1083	8597	133	5553	405	870	3	7	82	5317	9	125
4	23A.2	356350	0.889446	0.023951	0.002444	5978	47931	532	23639	3289	8535	62	318	220	22717	15	57
5	17W97A	39046	0.851201	0.000051	0.001447	467	5812	92	4155	1	2	6	8	68	4099	1	6
6	23A.3	62079	0.780699	0.002561	0.035142	947	13773	104	9675	78	159	0	0	79	9281	6	340
7	23W16Run1	130224	0.766656	0.000215	0.005450	1760	30415	197	19662	14	28	49	213	111	19247	3	106
8	28.3.3	237162	0.766147	0.012839	0.001806	4621	58506	427	34007	1326	3045	61	232	220	33388	9	61
9	24W16	104522	0.751009	0.001473	0.015362	1716	26179	215	17640	74	154	20	64	139	17136	6	270
10	15W97A	107402	0.726048	0.000093	0.065502	1973	29433	210	18998	5	10	40	113	125	17525	6	1237
11	52W42	108041	0.715469	0.000907	0.008244	2406	30839	458	19798	48	98	76	269	245	19089	5	161
12	71W75	118218	0.699530	0.001294	0.004052	2600	35674	390	22380	74	153	59	169	189	21789	9	90
13	29W16	115401	0.691432	0.001976	0.007171	2682	35837	388	22418	110	228	73	245	194	21758	2	159
14	51W42	122785	0.682087	0.003217	0.014169	3193	39430	503	24403	188	395	104	407	219	23330	3	340
15	47W42	119950	0.660417	0.002009	0.022987	2839	40974	162	24570	117	241	15	34	101	23870	3	564
16	59W54A	61531	0.603306	0.000975	0.008301	2083	24469	299	15283	29	60	45	105	149	14832	5	126
17	28.3.7	134923	0.586016	0.008731	0.002899	4038	57034	274	34865	537	1178	10	20	191	34542	10	101

- The unique sequences can act as preliminary indicators of how many unique bacterial species each sample will probably have
- Chimeric sequences are sequence hybrids
- Deblur uses the quality info to do the deblur step, so you can't see the quality plot after deblur runs, you only have your straight sequences without the quality information

8.) Visualize the representative sequences from the deblur step: To check and see if deblur did what it was supposed to do
qiime feature-table tabulate-seqs --i-data TurnbullFrogs2019_deblur_seq.qza --o-visualization TurnbullFrogs2019_deblur_seq.qzv
Saved Visualization to: TurnbullFrogs2019_deblur_seq.qzv

Deblur representative sequences qza visualization:



Sequence Length Statistics

Download sequence-length statistics as a TSV

Sequence Count	Min Length	Max Length	Mean Length	Range	Standard Deviation
3906	250	250	250.0	0	0.0

Seven-Number Summary of Sequence Lengths

Download seven-number summary as a TSV

Percentile:	2%	9%	25%	50%	75%	91%	98%
Length* (nts):	250	250	250	250	250	250	250

*Values rounded down to nearest whole number.

Sequence Table

To BLAST a sequence against the NCBI nt database, click the sequence and then click the *View report* button on the resulting page.

Download your sequences as a raw FASTA file

Click on a Column header to sort the table.

Feature ID	Sequence Length	Sequence▼
2f86aa90c73ac3f94160b89f1f225ea1	250	TACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAAGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGG
5cd044dfb5feca3ee72dae76fa6e9ee9	250	TACGGAGGATCCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCCTGTTAAGTCAGAGGTGAAAGACGGTAGCTCAACTATCGCAGTGCCTTTGATACTGACGGGCTTGAATAAACTAGAGGTAGGCGGAATGAGACAAGTAGCGGTGAAATGCATAGATATGTCTCAGAACACCGATTGCGAAGG
ebf41897cd60ae6116267d2f8f030c5f	250	TACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGCTTTGCAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGCAAGGCTGGAGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGG
109f4635e7355fc97fc225888ccd3dda	250	TACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTATCGTGAAAGTCCGAGGCTCAACCTCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGG
f66dbdbd9fcfe451edcb9d066dbded1b	250	TACAGAGGTCTCAAGCGTTGTTCGGAATCACTGGGCGTAAAGGGTGCGTAGGTGGCGTGGAAAGTCAGATGTGAAAGCCGGGGCTCAACCTCGGAATTGCATCCGATACTACCATGCTAGAGTACTGAAGAGGTGACTAGAATTCTCGGTGTAGCAGTGAAATGCGTAGATATCGAGAGGAATACCAACGGCGAAGG
b4dff88bea3b609481803b0e5001a77f	250	TACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGCCAAGTCAGGTGTGAAATCCCCGGGCTTAACCTGGGAATTGCGCTTGAAACTAGCGGGCTTGAGTGTGGCAGAGGGGGGTGGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGATGGCGAAGG
efb50d67c03bd1cdbdc29cf5549e7897	250	TACGGAGGGTGCAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTGCGTAGGTGGCTTGTTAAGTCCGGGGTGAAAGCCCACAGCTCAACTGTGGAACTGCCCTGGATACTGGCGAGCTTGAGTCCAGACGAGGTTGGCGGAATGGAAGCTGTAGCGGTGAAATGCATAGATAGCTTCCAGAACCCCGATTGCGTAGG
22a618a73e651adf50dea0d1a2ba9924	250	TACGAAGGGGGCTAGCGTTGTTCGGAATCACTGGGCGTAAAGCGCACGTAGGCGGACACTTAAGTCAGAGGTGAAATCCCAAGGCTCAACCTTGGAACTGCCTTTGATACTGGGTGTCTTGAGTTCGGGAGAGGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGG
f35b008b3bc9b85856bbd257daf435b7	250	TACGTAGGGTGCTAGAGTTGTCCGGATTTACTGGGTGTAAAGGGTGCTCAGGTGGGTTTGTAAGTCAGAGGTGAAATCCCAAAGCTTAACTTTGGAGCTGCCTTTGATACTGCAAGTCTGGAGTTTGAGAGAGGGCAATGGAATATCTGGTGTAGCAGTGAAATGCGTAGATATCAGATAGAACACCAATGGCGAAGG
dde372be88a23b352c706b977cf40cb6	250	TACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTAAGTTAAGTCTGATGTGAAAGCCCCGGGCTCAACCTGGGAATGGCATTGGAAACTGGCTTGCTAGAGTGTGGCAGAGGGGGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGAGATGCGGAGGAACACCGATGGCGAAGG
94288680a53f2237eacf8ebab2d81bb2	250	TACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGCCCGATAAGTCAGCGGTGAAATCTCCCGGCTCAACCGGGAAATGGCCATTGATACTGTCGGGCTTGAATTATCAGGAAGCAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGATATTACATGGAATACCAATTGCGAAGG
11ba73c9478a99aef2f0dc33e75ebf93	250	TACGTAGGGGGCAAACGTTGTCCGGATTTACTGGGTGTAAAGGGTGCTCAGGCGGTTTTGTAAGTCAGAAGTGAAATCCTAGAGCTTAACTCTGGAACTGCTTTTGATACTGCAAGGCTTGAATGTGGAAGAGGAGGATGGAATTTCTGGTGTAGCAGTGAAATGCGTAGATATCAGAAAGAACACCAGTGGCGAAGG

- Looking at the sequence length statistics, we see that deblur did indeed **not** trim out any base pairs at the 5' end like we wanted
- We copy-pasted a sequence into a new word document to make sure that the following primer sequences were not included in the final sequence (via "find"):
    - **Illumina 5' adapter:** AATGATACGGCGACCACCGAGATCTACACGCT
    - **Forward primer pad:** TATGGTAATT
    - **515F forward primer (Parada):** GTGYCAGCMGCCGCGGTAA → Klaatu *parada* nikto

7.) Visualize and summarize the table from the deblur step:

Note: How to format the metadata file to a .tsv:

- Open a new Google Sheets spreadsheet
- Copy-paste all content from your master mapping file (your original excel sheet) into the Google Sheet
- Check it again with the Keemei extension
- File → download → as .tsv (tab separated values) → save to your QIIME folder

qiime feature-table summarize --i-table Turnbull-table-deblur.qza --o-visualization Turnbull-table-deblur.qzv --m-sample-metadata-file Metadata_Mapping_Turnbull_2019.tsv
Saved Visualization to: Turnbull-table-deblur.qzv

Summarized deblur-table qzv in qiime2 view:

Overview | Interactive Sample Detail | Feature Detail

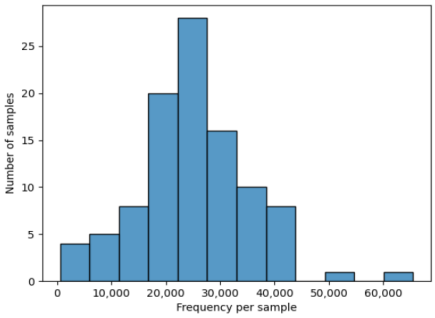
Table summary

Metric	Sample
Number of samples	101
Number of features	3,906
Total frequency	2,535,174

Frequency per sample

	Frequency
Minimum frequency	572.0
1st quartile	19,089.0
Median frequency	24,486.0
3rd quartile	31,613.0
Maximum frequency	65,471.0
Mean frequency	25,100.73267326733

Frequency per sample detail (csv | html)



- How many samples are in the dataset? → **101 samples**
- How many features (bacterial "species") are in the dataset? → **3,906**
- What is the total frequency (total number of DNA sequences in the dataset)? → **2,535,174 total sequences in the dataset**
- What is the frequency per sample? → **[mean] 25,100 per individual frog**
- What is the mean frequency per feature [scroll down!] (the mean number of sequences assigned to a feature, a.k.a., bacterial "species")? → **649 per bacterial "species"**

8.) Assign taxonomy to your sequences with Silva database:

For the sequence reference database:

Michael S Robeson II, Devon R O'Rourke, Benjamin D Kaehler, Michal Ziemski, Matthew R Dillon, Jeffrey T Foster, Nicholas A Bokulich. RESCRIPt: Reproducible sequence taxonomy reference database management for the masses. bioRxiv 2020.10.05.326504; doi: https://doi.org/10.1101/2020.10.05.326504

For SILVA in general:

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucl. Acids Res. 41: D590 – D560

For the taxonomic framework:

Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glöckner FO (2014) The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucl. Acids Res. 42: D643 – D648

Dr. Walke assigned taxonomy to my samples by running Shelby's classifier on her matching version of QIIME (my version was too recent to match). I filtered the mitochondria and chloroplasts from my table and converted it to a qzv.

The code Dr. Walke used to assign taxonomy:
(qiime2-2020.11) BIOL108946MP:Dana QIIME 13Mar22 jwalke$ qiime feature-classifier classify-sklearn --i-classifier /Users/jwalke/Desktop/Dana\ QIIME\ 13Mar22/ShelbyFettig_Silva_99_138.1_qiime_classifer.qza --i-reads /Users/jwalke/Desktop/Dana\ QIIME\ 13Mar22/TurnbullFrogs2019_deblur_seq.qza --o-classification TurnbullFrogs2019_taxonomy.qza
Saved FeatureData[Taxonomy] to: TurnbullFrogs2019_taxonomy.qza

9.) Visualize the taxonomy of your sequences:

qiime metadata tabulate --m-input-file TurnbullFrogs2019_taxonomy.qza --o-visualization TurnbullFrogs2019_taxonomy.qzv
Saved Visualization to: TurnbullFrogs2019_taxonomy.qzv

Taxonomy visualization in QIIME2 View: very useful file to refer to!

Feature ID #q2:types	Taxon categorical	Confidence categorical
00092942f7d559700e85f305fa5b522f	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prolixibacteraceae; g__WCHB1-32	0.9979145597239717
000e38f477632fa6299901badf2e0f06	d__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus	0.9999882155386602
001491973eeef44559f205335ceff4cb	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__NS9_marine_group; g__NS9_marine_group; s__uncultured_Bacteroidetes	0.9033906124760703
0048aeaec1b0a22fd3eb7333b2e3d8f5	d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Alkanindiges	0.7602771440973116
004cbc95e6564a64a0975801957ff6c9	d__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Polymorphobacter; s__Polymorphobacter_sp.	0.8715744760011738
0051287464c1d10fbe98c4e8d4e8ae7e	d__Bacteria; p__Bdellovibrionota; c__Bdellovibrionia; o__Bdellovibrionales; f__Bdellovibrionaceae; g__Bdellovibrio	0.999854747167069
0060e72c3bf48d3a722f1114e7190330	d__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Novosphingobium	0.8562866444582092
006932cfb328133269fc5de842e4507b	d__Bacteria; p__Planctomycetota; c__Planctomycetes; o__Isosphaerales; f__Isosphaeraceae; g__uncultured	0.960736285654819
00751fa634535475b17f4deb25546884	d__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Xanthobacteraceae; g__Tardiphaga	0.7667339248307755
007cbfb51ced95d24993b44a7141a508	d__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Pseudorhodobacter	0.7616651298237704
00e0eea60270dca1c743746655b66c98	d__Bacteria; p__Patescibacteria; c__Parcubacteria; o__Candidatus_Nomurabacteria; f__Candidatus_Nomurabacteria; g__Candidatus_Nomurabacteria; s__uncultured_bacterium	0.734150759745486

10.) Filter mitochondria and chloroplasts out of the table:

qiime taxa filter-seqs --i-sequences TurnbullFrogs2019_deblur_seq.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Mitochondria --o-filtered-sequences Turnbull-filtered-seqs-new.qza
Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Chloroplast --o-filtered-sequences Turnbull-filtered-seqs-new.qza
Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Unassigned --o-filtered-sequences Turnbull-filtered-seqs-new.qza
Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza

    - TurnbullFrogs201_taxonomy.qzv. 3,906 entries vs 3,693 sequences in the new one.

- Use the search bar at the top of the page to search for anything you need to remove. From a quick glance through this table, we found:
    - Pseudoalteromonas (2)
        - qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Pseudoalteromonas --o-filtered-sequences Turnbull-filtered-seqs-new.qza
          Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
    - Vibrio (125)? Well-known contaminant of DNA extraction kits. Generally kit contamination is even more pronounced in cases where the amount of host bacteria are low (as in our case with the bat skin bacteria).
        - qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Vibrio --o-filtered-sequences Turnbull-filtered-seqs-new.qza
          Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
    - Halomonas (9)
        - qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Halomonas --o-filtered-sequences Turnbull-filtered-seqs-new.qza
          Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
    - Idiomarina (7)
        - qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Idiomarina --o-filtered-sequences Turnbull-filtered-seqs-new.qza
          Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
    - Marinobacter (1)
        - qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Marinobacter --o-filtered-sequences Turnbull-filtered-seqs-new.qza
          Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
    - Marinomonas (0)
        - Still ran this code though I didn't find any in the taxa table.

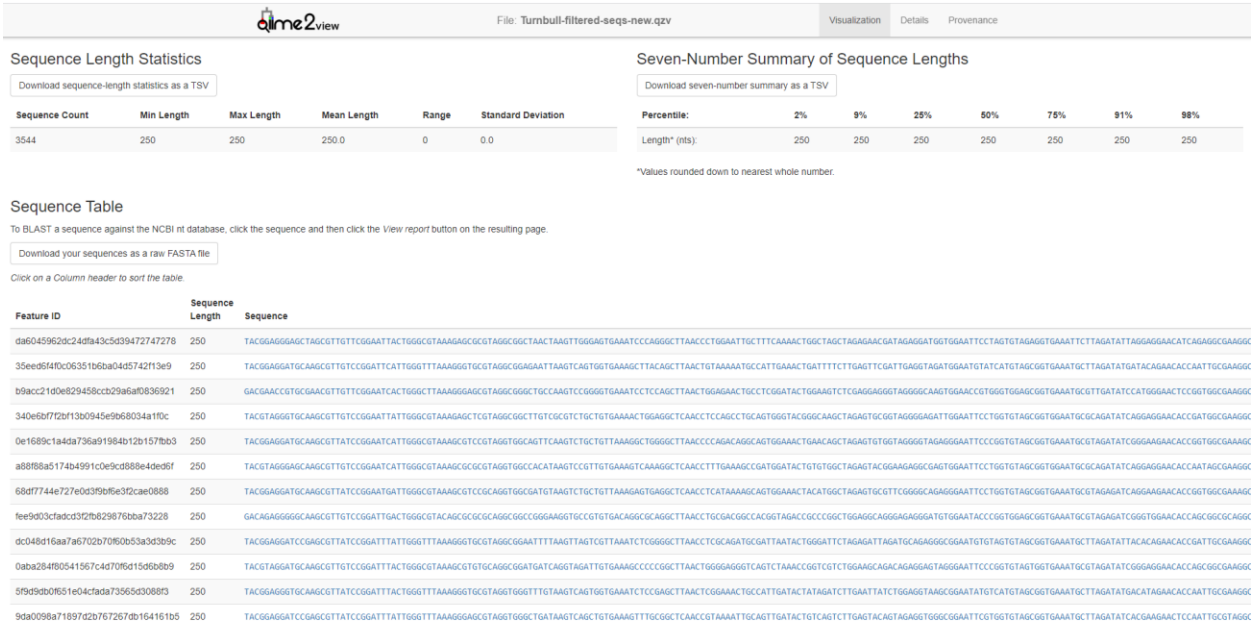
- qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Marinomonas --o-filtered-sequences Turnbull-filtered-seqs-new.qza
  Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza

- Salinisphaera (4)
  - qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Salinisphaera --o-filtered-sequences Turnbull-filtered-seqs-new.qza
    Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza
- Salinarimonas (0)
  - Still ran this code even though none were found searching the Taxonomy table
  - qiime taxa filter-seqs --i-sequences Turnbull-filtered-table-new.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Salinarimonas --o-filtered-sequences Turnbull-filtered-seqs-new.qza
    Saved FeatureData[Sequence] to: Turnbull-filtered-seqs-new.qza

11.) Check that the mitochondria and chloroplasts were filtered out from the table:

The Codes I used to filter out Mitochondrion, Chloroplasts, and unassigned sequences:
***For future reference, everything can be filtered at the same time by just adding a comma between the things being filtered. Example code below. See example code after the codes that I ran.**

qiime feature-table tabulate-seqs --i-data Turnbull-filtered-seqs-new.qza --o-visualization Turnbull-filtered-table-new.qzv
Saved Visualization to: Turnbull-filtered-seqs-new.qzv



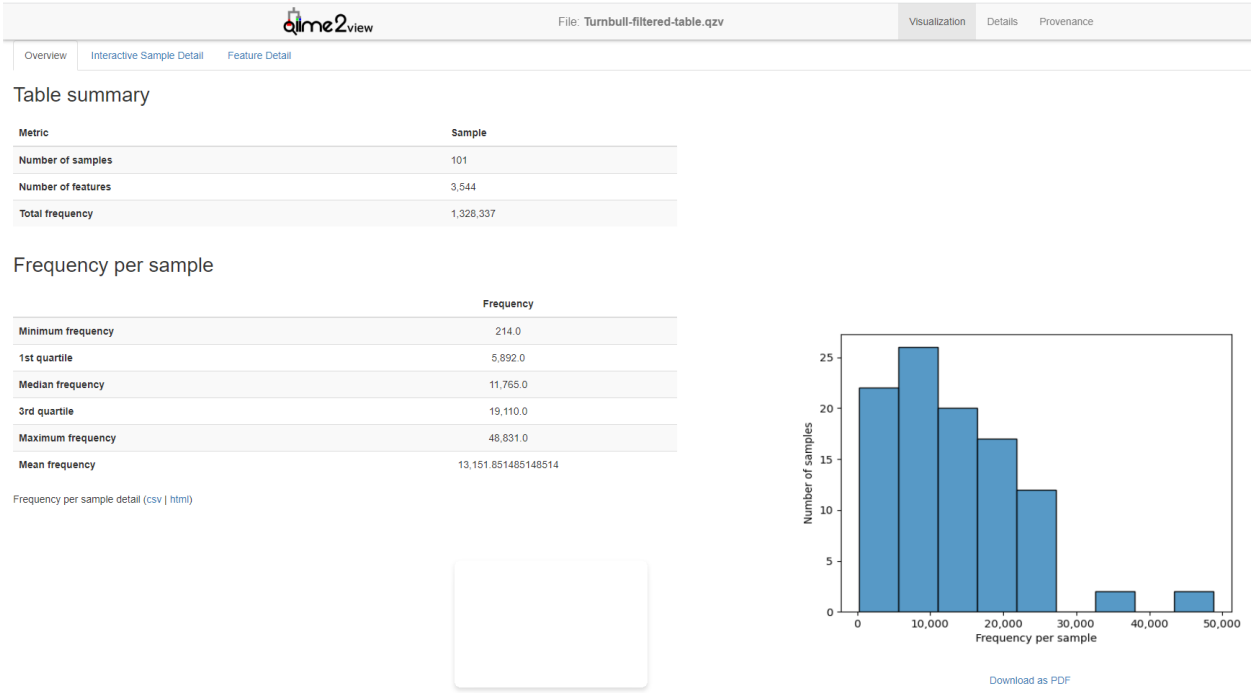
The Sequence count went from 3,906 to 3692 (after filtering out Mitochondria, Chloroplasts and unassigned) then to 3,544 after all of the filtering was completed. This confirmed that the sequences were filtered.

Next, the taxonomy table needs to be filtered

The Code I used to filter the taxonomy table:
qiime taxa filter-table --i-table TurnbullFrogs2019-deblur-table.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-exclude Mitochondria,Chloroplast,Unassigned,Pseudoalteromonas,Vibrio,Halomonas,Idiomarina,Marinobacter,Marinomonas,Salinisphaera,Salinarimonas --o-filtered-table Turnbull-filtered-table.qza
Saved FeatureTable[Frequency] to: Turnbull-filtered-table.qza

The code I used to visualize the filtered table:

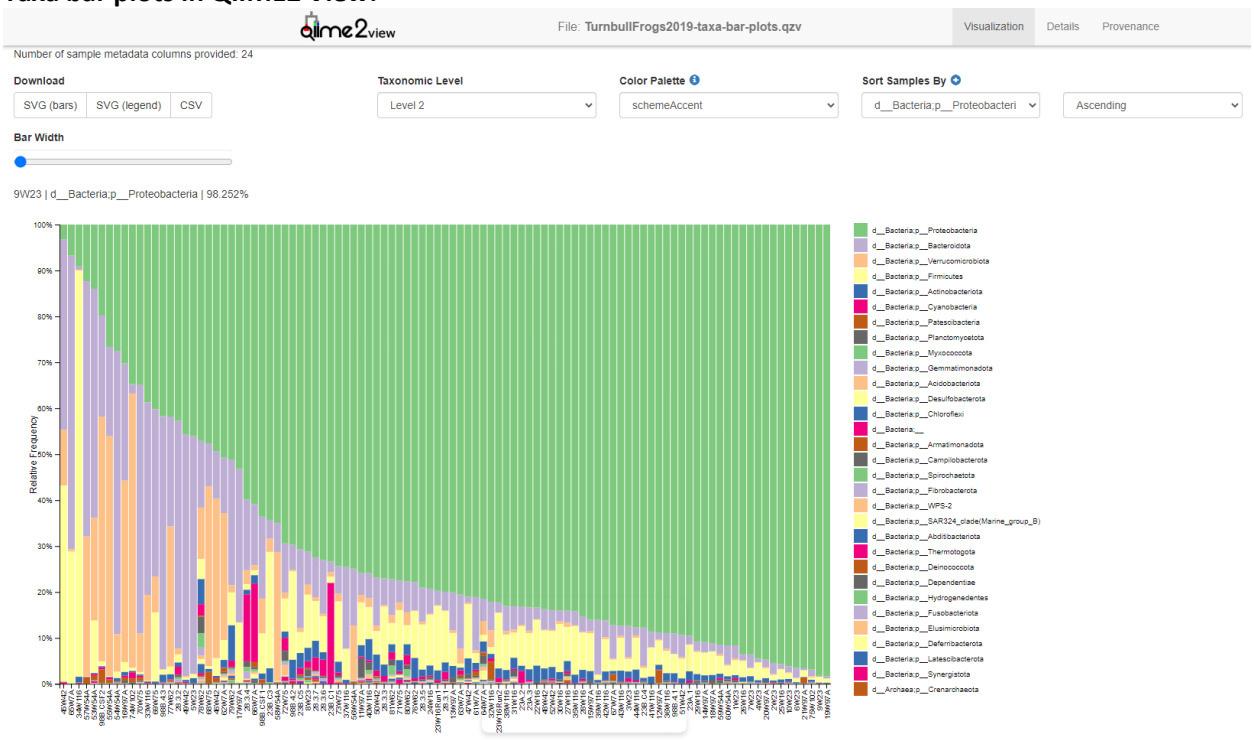
qiime feature-table summarize --i-table Turnbull-filtered-table.qza --o-visualization Turnbull-filtered-table.qzv --m-sample-metadata-file Metadata_Mapping_Turnbull_2019.tsv
Saved Visualization to: Turnbull-filtered-table.qzv



The number of features now match the number of sequences that were filtered. From 3,906 to 3,544.

<u>7.) Visualize the microbiome using taxa bar plots:</u>
qiime taxa barplot --i-table Turnbull-filtered-table.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization TurnbullFrogs2019-taxa-bar-plots.qzv
Saved Visualization to: TurnbullFrogs2019-taxa-bar-plots.qzv

Taxa bar plots in QIIME2 View:



12.) Generate a tree for phylogenetic diversity analysis and rarefy sequence data

qiime phylogeny align-to-tree-mafft-fasttree --i-sequences Turnbull-filtered-seqs-new.qza --o-alignment Turnbull-seqs_aligned.qza --o-masked-alignment Turnbull-seqs_masked-aligned.qza --o-tree Turnbull-seqs_unrooted-tree.qza --o-rooted-tree Turnbull-seqs_rooted-tree.qza
Saved FeatureData[AlignedSequence] to: Turnbull-seqs_aligned.qza
Saved FeatureData[AlignedSequence] to: Turnbull-seqs_masked-aligned.qza
Saved Phylogeny[Unrooted] to: Turnbull-seqs_unrooted-tree.qza
Saved Phylogeny[Rooted] to: Turnbull-seqs_rooted-tree.qza

13.) Alpha rarefaction plotting (Plots were rarefied at 3000 and 5000 to see if I would miss out on statistical significance rarefying at 300 vs 5000)

qiime diversity core-metrics-phylogenetic --i-phylogeny Turnbull-seqs_rooted-tree.qza --i-table Turnbull-filtered-table.qza --p-sampling-depth 3000 --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-rarefied-table Turnbull-rarefied-table-3000-new.qza --output-dir Turnbull2019-3000-new-core-metrics-results
Saved FeatureTable[Frequency] to: Turnbull-rarefied-table-3000-new.qza

qiime diversity core-metrics-phylogenetic --i-phylogeny Turnbull-seqs_rooted-tree.qza --i-table Turnbull-filtered-table.qza --p-sampling-depth 5000 --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-rarefied-table Turnbull-rarefied-table-5000-new.qza --output-dir Turnbull2019-5000-new-core-metrics-results
Saved FeatureTable[Frequency] to: Turnbull-rarefied-table-5000-new.qza

14.) Calculate alpha diversity statistics with nonparametric Kruskal-Wallis tests:

Shannon Diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-3000-new-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-results-shannon-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-results-shannon-significance.qzv

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-5000-new-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-results-shannon-significance-5000.qzv
Saved Visualization to: Turnbull2019-core-metrics-results-shannon-significance-5000.qzv

Observed OTU's:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-3000-new-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-results-observed-features-vector-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-results-observed-features-vector-significance.qzv

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-5000-new-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-5000-results-observed-features-vector-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-5000-results-observed-features-vector-significance.qzv

Faith's Phylogenetic Diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-3000-new-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-3000-results-faith-pd-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-3000-results-faith-pd-significance.qzv

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-5000-new-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-5000-results-faith-pd-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-5000-results-faith-pd-significance.qzv

Evenness:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-3000-new-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-core-metrics-3000-results-evenness-significance.qzv
Saved Visualization to: Turnbull-core-metrics-3000-results-evenness-significance.qzv

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-5000-new-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-core-metrics-5000-results-evenness-significance.qzv
Saved Visualization to: Turnbull-core-metrics-5000-results-evenness-significance.qzv

15.) Calculate beta diversity statistics using nonparametric PERMANOVAs:

Bray-Curtis Dissimilarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-3000-new-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-3000-resutls-Bray-Curtis-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-3000-resutls-Bray-Curtis-zoospore-significance.qzv

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-5000-new-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-5000-resutls-Bray-Curtis-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-5000-resutls-Bray-Curtis-zoospore-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-3000-new-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-3000-resutls-jaccard-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-3000-resutls-jaccard-zoospore-significance.qzv

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-5000-new-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-5000-resutls-jaccard-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-5000-resutls-jaccard-zoospore-significance.qzv

Unweighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-3000-new-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-3000-resutls-unweighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-3000-resutls-unweighted-UniFrac-zoospore-significance.qzv

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-5000-new-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file

Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-5000-resutls-unweighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-5000-resutls-unweighted-UniFrac-zoospore-significance.qzv

Weighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-3000-new-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-3000-resutls-weighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-3000-resutls-weighted-UniFrac-zoospore-significance.qzv

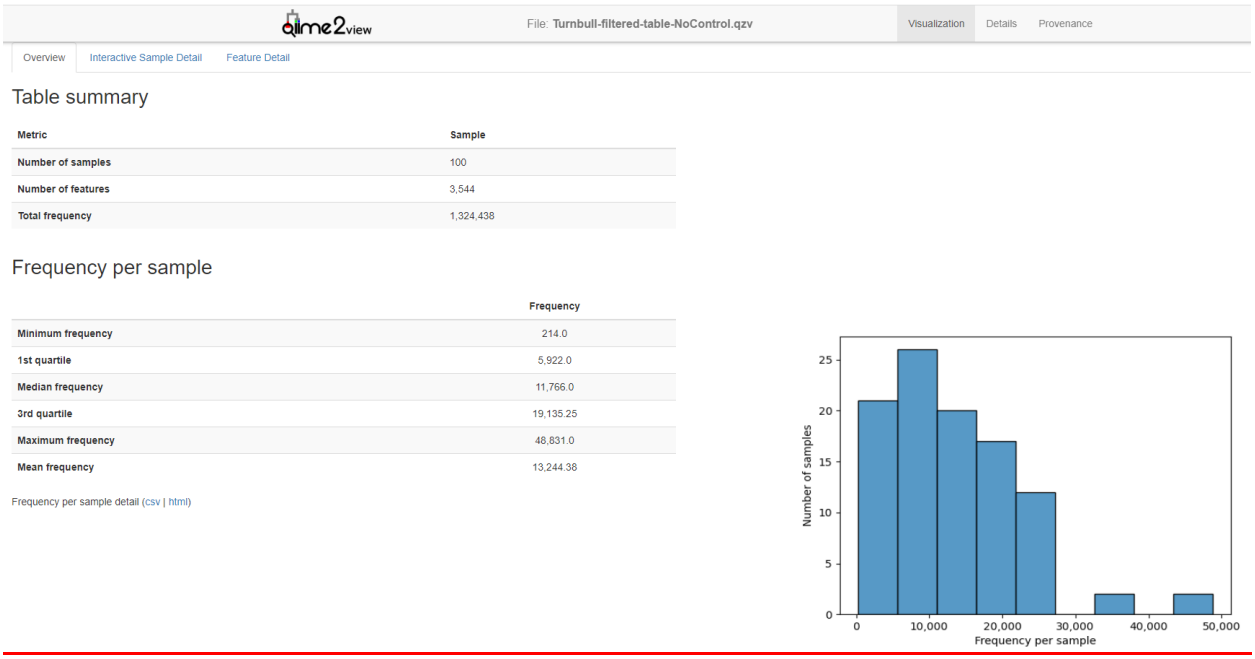
qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-5000-new-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-sore-metrics-5000-resutls-weighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-sore-metrics-5000-resutls-weighted-UniFrac-zoospore-significance.qzv

Since rarefying at 3,000 sequences per sample and 5,000 sequences per sample were practically the same with initial statistical results for the alpha and beta diversity plots, we decided to keep more samples and rarefy at 3,000. We also visualized the weighted UniFrac emperor plot to see if the control sample was the same, and it was (see below).

16. Filter out control sample

qiime feature-table filter-samples --i-table Turnbull-filtered-table.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --p-where "[SampleID]='23W16Run2'" --p-exclude-ids --o-filtered-table Trunbull-filtered-table-NoControl.qza
Saved FeatureTable[Frequency] to: Trunbull-filtered-table-NoControl.qza

Visualize the filtered table.
qiime feature-table summarize --i-table Turnbull-filtered-table-NoControl.qza --m-sample-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-filtered-table-NoControl.qzv
Saved Visualization to: Turnbull-filtered-table-NoControl.qzv



- How many samples are in the dataset? → **100 samples**
- How many features (bacterial "species") are in the dataset? → **3,544**
- What is the total frequency (total number of DNA sequences in the dataset)? → **1,324,438 total sequences in the dataset**
- What is the frequency per sample? → **[mean] 13,244.38 per individual frog**
- What is the mean frequency per feature [scroll down!] (the mean number of sequences assigned to a feature, a.k.a., bacterial "species")? → **383.7 per bacterial "species"**

17.) Calculating alpha and beta diversity of the filtered samples without the control and rarefied at 3000:

qiime diversity core-metrics-phylogenetic --i-phylogeny Turnbull-seqs_rooted-tree.qza --i-table Turnbull-filtered-table-NoControl.qza --p-sampling-depth 3000 --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-rarefied-table Turnbull-rarefied-table-real-3000.qza --output-dir Turnbull2019-real-3000-core-metrics-results
Saved FeatureTable[Frequency] to: Turnbull-rarefied-table-real-3000.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-real-3000-core-metrics-results/faith_pd_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-real-3000-core-metrics-results/observed_features_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-real-3000-core-metrics-results/shannon_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-real-3000-core-metrics-results/evenness_vector.qza
Saved DistanceMatrix to: Turnbull2019-real-3000-core-metrics-results/unweighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-real-3000-core-metrics-results/weighted_UniFrac_distance_matrix.qza

Saved DistanceMatrix to: Turnbull2019-real-3000-core-metrics-results/jaccard_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-real-3000-core-metrics-results/bray_curtis_distance_matrix.qza
Saved PCoAResults to: Turnbull2019-real-3000-core-metrics-results/unweighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-real-3000-core-metrics-results/weighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-real-3000-core-metrics-results/jaccard_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-real-3000-core-metrics-results/bray_curtis_pcoa_results.qza
Saved Visualization to: Turnbull2019-real-3000-core-metrics-results/unweighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-real-3000-core-metrics-results/weighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-real-3000-core-metrics-results/jaccard_emperor.qzv
Saved Visualization to: Turnbull2019-real-3000-core-metrics-results/bray_curtis_emperor.qzv

18.) Calculate alpha diversity statistics with nonparametric Kruskal-Wallis tests:

Shannon diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-real-results-shannon-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-real-results-shannon-significance.qzv

Observed OTU's:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-real-results-observed-features-vector-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-real-results-observed-features-vector-significance.qzv

Faith's Phylogenetic Diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-real-3000-results-faith-pd-significance.qzv
Saved Visualization to: Turnbull2019-core-metrics-real-3000-results-faith-pd-significance.qzv

Evenness:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-core-metrics-real-3000-results-evenness-significance.qzv

Saved Visualization to: Turnbull-core-metrics-real-3000-results-evenness-significance.qzv

18.) Alpha diversity with Spearman's Rank Correlation

Shannon diversity spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-real-3000-results-shannon-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-core-metrics-real-3000-results-shannon-correlation-spearman.qzv

Phylogenetic diversity spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-real-3000-results-faith-pd-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-core-metrics-real-3000-results-faith-pd-correlation-spearman.qzv

Evenness spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-real-3000-results-evenness-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-core-metrics-real-3000-results-evenness-correlation-spearman.qzv

Observed features spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-real-3000-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-core-metrics-real-3000-results-observed-features-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-core-metrics-real-3000-results-observed-features-correlation-spearman.qzv

19.) Calculate beta diversity statistics using nonparametric PERMANOVAs:

Bd PRESENCE/ABSENCE

Bray-Curtis Dissimilarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-core-metrics-real-3000-results-Bray-Curtis-zoospore-significance.qzv --p-pairwise

Saved Visualization to: Turnbull-core-metrics-real-3000-resutls-Bray-Curtis-zoospore-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-core-metrics-real-3000-resutls-jaccard-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-3000-resutls-jaccard-zoospore-significance.qzv

Unweighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-core-metrics-real-3000-resutls-unweighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-3000-resutls-unweighted-UniFrac-zoospore-significance.qzv

Weighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull-core-metrics-real-3000-resutls-weighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-real-3000-resutls-weighted-UniFrac-zoospore-significance.qzv

SITE

Bray-Curtis Dissimilarity: Site
qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull-core-metrics-real-3000-results-Bray-Curtis-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-real-3000-resutls-Bray-Curtis-site-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull-core-metrics-real-3000-resutls-jaccard-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-3000-resutls-jaccard-site-significance.qzv

Unweighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull-core-metrics-real-3000-resutls-unweighted-UniFrac-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-3000-resutls-unweighted-UniFrac-site-significance.qzv

Weighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull-core-metrics-real-3000-resutls-weighted-UniFrac-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-real-3000-resutls-weighted-UniFrac-site-significance.qzv

SPECIES

Bray-Curtis Dissimilarity: Species
qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Species --o-visualization Turnbull-core-metrics-real-3000-results-Bray-Curtis-species-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-real-3000-resutls-Bray-Curtis-species-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Species --o-visualization Turnbull-core-metrics-real-3000-resutls-jaccard-species-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-3000-resutls-jaccard-species-significance.qzv

Unweighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Species --o-visualization Turnbull-core-metrics-real-3000-resutls-unweighted-UniFrac-species-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-3000-resutls-unweighted-UniFrac-species-significance.qzv

Weighted UniFrac Distances:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Species --o-visualization Turnbull-core-metrics-real-3000-resutls-weighted-UniFrac-species-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-core-metrics-real-3000-resutls-weighted-UniFrac-species-significance.qzv

20.) Calculate beta diversity statistics using Mantel tests:

Bd PRESENCE/ABSENCE

Bray-Curtis Dissimilarity:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull-core-metrics-real-3000-results-bray_curtis-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull-core-metrics-real-3000-results-bray_curtis-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull-core-metrics-real-3000-results-bray_curtis-zoospores-correlation.qza
Saved Visualization to: Turnbull-core-metrics-real-3000-results-bray_curtis-zoospores-correlation.qzv

Jaccard Similarity:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull-core-metrics-real-3000-results-jaccard-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull-core-metrics-real-3000-results-jaccard-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull-core-metrics-real-3000-results-jaccard-zoospores-correlation.qza
Saved Visualization to: Turnbull-core-metrics-real-3000-results-jaccard-zoospores-correlation.qzv

Unweighted UniFrac Distances:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull-core-metrics-real-3000-results-unweighted-UniFrac-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull-core-metrics-real-3000-results-unweighted-UniFrac-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull-core-metrics-real-3000-results-unweighted-UniFrac-zoospores-correlation.qza

Saved Visualization to: Turnbull-core-metrics-real-3000-results-unweighted-UniFrac-zoospores-correlation.qzv

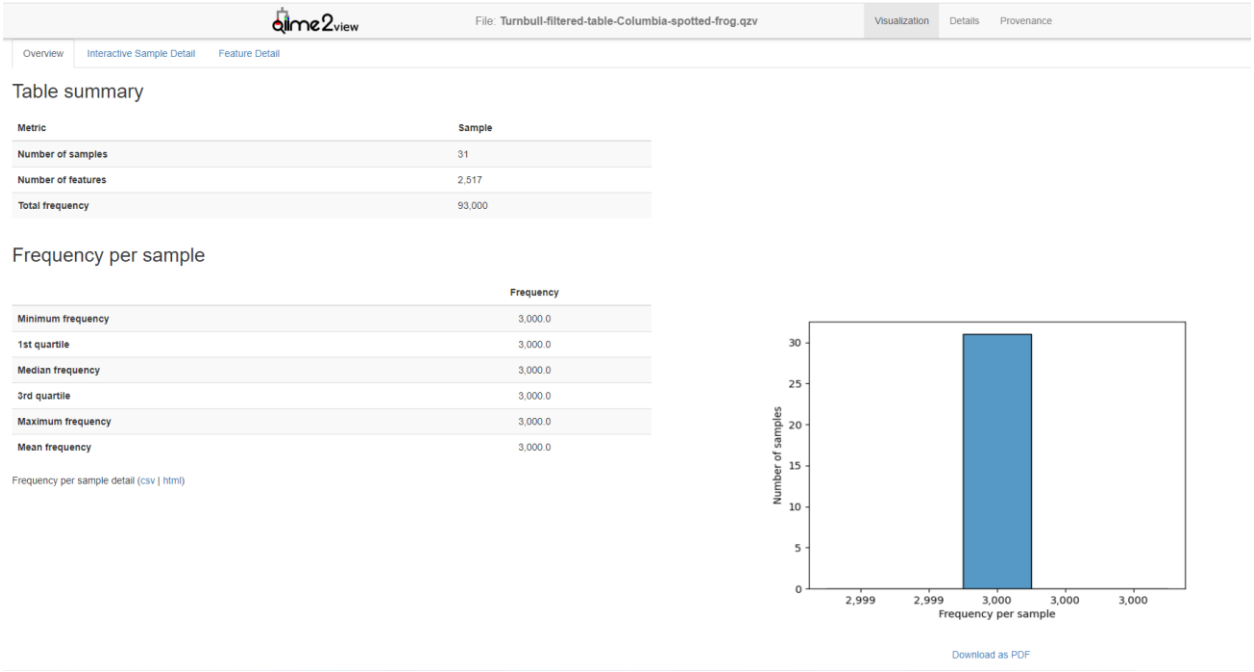
Weighted UniFrac Distances:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-real-3000-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull-core-metrics-real-3000-results-weighted-UniFrac-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull-core-metrics-real-3000-results-weighted-UniFrac-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull-core-metrics-real-3000-results-weighted-UniFrac-zoospores-correlation.qza
Saved Visualization to: Turnbull-core-metrics-real-3000-results-weighted-UniFrac-zoospores-correlation.qzv

21.) Evaluate CSF by itself (Bd presence/absence and zoospore equivalence)
Code I used to get just the Columbia Spotted Frog

qiime feature-table filter-samples --i-table Turnbull-rarefied-table-real-3000.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --p-where "[Species]='Columbia spotted frog'" --o-filtered-table Turnbull-filtered-table-Columbia-spotted-frog.qza
Saved FeatureTable[Frequency] to: Turnbull-filtered-table-Columbia-spotted-frog.qza

Code I used to visualize the filtered table.
qiime feature-table summarize --i-table Turnbull-filtered-table-Columbia-spotted-frog.qza --m-sample-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-filtered-table-Columbia-spotted-frog.qzv
Saved Visualization to: Turnbull-filtered-table-Columbia-spotted-frog.qzv



22.) The code I used to calculate alpha and beta diversity CSF:

qiime diversity core-metrics-phylogenetic --i-phylogeny Turnbull-seqs_rooted-tree.qza --i-table Turnbull-filtered-table-Columbia-spotted-frog.qza --p-sampling-depth 3000 --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-rarefied-table Turnbull-CSF-table.qza --output-dir Turnbull2019-CSF-core-metrics-results

Saved FeatureTable[Frequency] to: Turnbull-CSF-table.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-core-metrics-results/faith_pd_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-core-metrics-results/observed_features_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-core-metrics-results/shannon_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-core-metrics-results/evenness_vector.qza
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results/unweighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results/weighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results/jaccard_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results/bray_curtis_distance_matrix.qza
Saved PCoAResults to: Turnbull2019-CSF-core-metrics-results/unweighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-CSF-core-metrics-results/weighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-CSF-core-metrics-results/jaccard_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-CSF-core-metrics-results/bray_curtis_pcoa_results.qza

Saved Visualization to: Turnbull2019-CSF-core-metrics-results/unweighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results/weighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results/jaccard_emperor.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results/bray_curtis_emperor.qzv

22.) Alpha Diversity for CSF using KW
Shannon diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-shannon-significance.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-shannon-significance.qzv

Observed OTU's:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-observed-features-vector-significance.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-observed-features-vector-significance.qzv

Faith's Phylogenetic Diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-faith-pd-significance.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-faith-pd-significance.qzv

Evenness:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-core-metricsresults/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-evenness-significance.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-evenness-significance.qzv

23.) Beta diversity PERMANOVA CSF:

Bray-Curtis Dissimilarity: Bd Presence/absence

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-CSF-core-metrics-results-Bray-Curtis-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-CSF-core-metrics-resutls-Bray-Curtis-zoospore-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-CSF-core-metrics-resutls-jaccard-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-CSF-core-metrics-jaccard-zoospore-significance.qzv

Unweighted UniFrac:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-CSF-core-metrics-resutls-unweighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-CSF-core-metrics-resutls-unweighted-UniFrac-zoospore-significance.qzv

Weighted UniFrac:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-CSF-core-metrics-resutls-weighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-CSF-core-metrics-resutls-weighted-UniFrac-zoospore-significance.qzv

24.) Alpha diversity CSF with Spearman's Rank Correlation

Shannon diversity spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-CSF-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-shannon-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-shannon-correlation-spearman.qzv

Faith's phylogenetic diversity spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-CSF-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-faith-pd-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-faith-pd-correlation-spearman.qzv

Evenness spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-CSF-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-evenness-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-evenness-correlation-spearman.qzv

Observed features spearman's rank

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-CSF-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-core-metrics-results-observed-features-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-observed-features-correlation-spearman.qzv

25.) Calculate beta diversity CSF statistics using Mantel tests:

Bray-Curtis Dissimilarity:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-CSF-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-CSF-core-metrics-results-bray_curtis-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-CSF-core-metrics-results-bray_curtis-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results-bray_curtis-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-bray_curtis-zoospores-correlation.qzv

Jaccard Similarity:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-CSF-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-CSF-core-metrics-results-jaccard-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-CSF-core-metrics-results-jaccard-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results-jaccard-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-jaccard-zoospores-correlation.qzv

Unweighted UniFrac Distances:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-CSF-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-CSF-core-metrics-results-unweighted-

UniFrac-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-CSF-core-metrics-results-unweighted-UniFrac-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results-unweighted-UniFrac-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-unweighted-UniFrac-zoospores-correlation.qzv

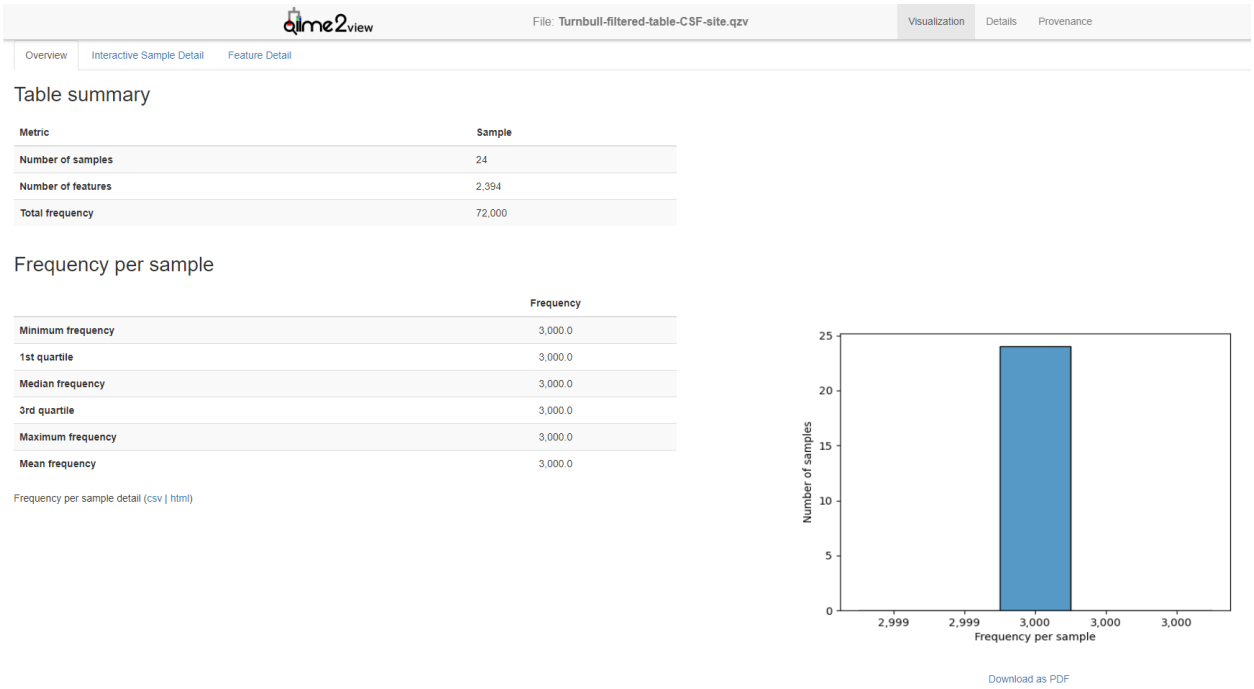
Weighted UniFrac Distances:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-CSF-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-CSF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-CSF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-CSF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-CSF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qzv

26.) Filter out sites with less than 3 frogs (CSF):
Code I used to get just the >3 Sites CSF
qiime feature-table filter-samples --i-table Turnbull-filtered-table-Columbia-spotted-frog.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --p-where "[Site] IN ('116','54A','7A','75','62')" --o-filtered-table Turnbull-filtered-table-CSF-site.qza
Saved FeatureTable[Frequency] to: Turnbull-filtered-table-CSF-site3.qza

27.) Visualize filtration

qiime feature-table summarize --i-table Turnbull-filtered-table-CSF-site.qza --m-sample-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-filtered-table-CSF-site.qzv
Saved Visualization to: Turnbull-filtered-table-CSF-site3.qzv



28.) Code I use to calculate Alpha and Beta Diversity for CSF Site

qiime diversity core-metrics-phylogenetic --i-phylogeny Turnbull-seqs_rooted-tree.qza --i-table Turnbull-filtered-table-CSF-site.qza --p-sampling-depth 3000 --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-rarefied-table Turnbull-table-CSF-site.qza --output-dir Turnbull2019-CSF-site-core-metrics-results

Saved FeatureTable[Frequency] to: Turnbull-table-CSF-site.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-site-core-metrics-results/faith_pd_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-site-core-metrics-results/observed_features_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-site-core-metrics-results/shannon_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-CSF-site-core-metrics-results/evenness_vector.qza
Saved DistanceMatrix to: Turnbull2019-CSF-site-core-metrics-results/unweighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-CSF-site-core-metrics-results/weighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-CSF-site-core-metrics-results/jaccard_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-CSF-site-core-metrics-results/bray_curtis_distance_matrix.qza
Saved PCoAResults to: Turnbull2019-CSF-site-core-metrics-results/unweighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-CSF-site-core-metrics-results/weighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-CSF-site-core-metrics-results/jaccard_pcoa_results.qza

Saved PCoAResults to: Turnbull2019-CSF-site-core-metrics-results/bray_curtis_pcoa_results.qza
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results/unweighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results/weighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results/jaccard_emperor.qzv
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results/bray_curtis_emperor.qzv

29.) The code I used to visualize Alpha Diversity KW for CSF Site:
Shannon diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-site-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-site-core-metrics-results-shannon-significance.qzv
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results-shannon-significance.qzv

Observed OTU's:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-site-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-site-core-metrics-results-observed-features-vector-significance.qzv
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results-observed-features-vector-significance.qzv

Faith's Phylogenetic Diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-site-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-site-core-metrics-results-faith-pd-significance.qzv
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results-faith-pd-significance.qzv

Evenness:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-CSF-site-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-CSF-site-core-metrics-results-evenness-significance.qzv
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-results-evenness-significance.qzv

30.) Beta Diversity CSF Site:

Bray-Curtis Dissimilarity: Bd Presence/absence

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-site-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-CSF-site-core-metrics-results-Bray-Curtis-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-CSF-site-core-metrics-resutls-Bray-Curtis-site-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-site-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-CSF-site-core-metrics-resutls-jaccard-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-jaccard-site-significance.qzv

Unweighted UniFrac:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-site-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-CSF-core-metrics-resutls-unweighted-UniFrac-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-resutls-unweighted-UniFrac-site-significance.qzv

Weighted UniFrac:

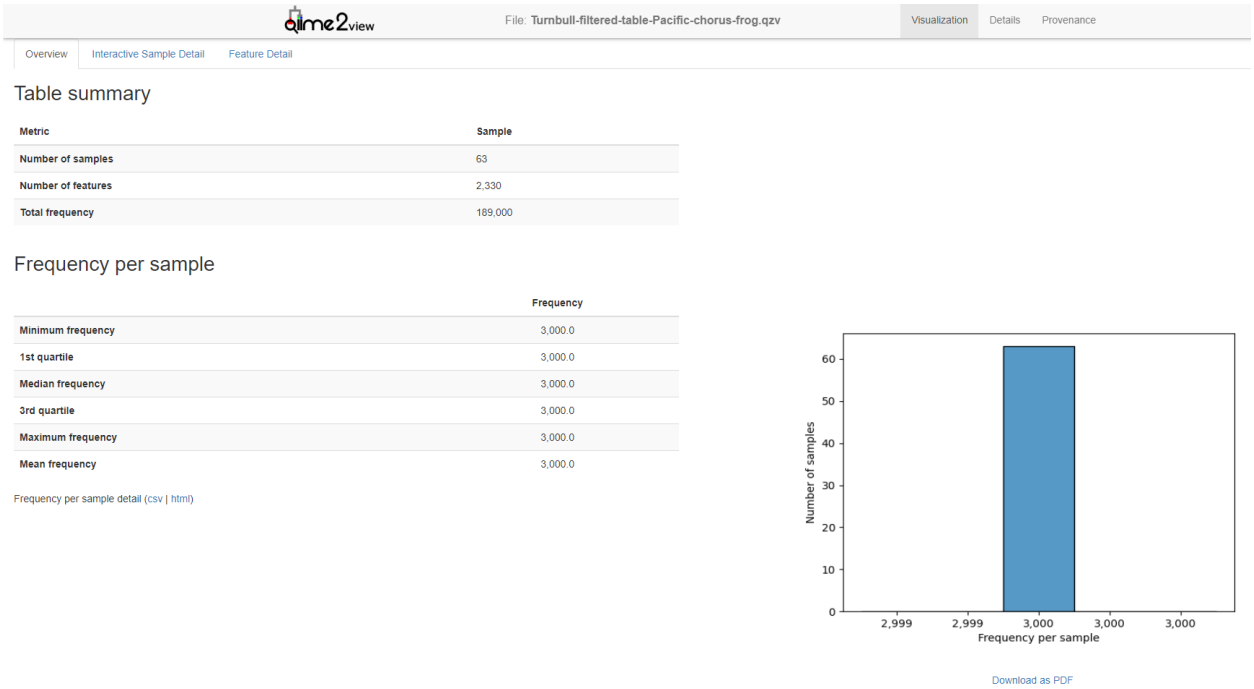
qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-CSF-site-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-CSF-site-core-metrics-resutls-weighted-UniFrac-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-CSF-site-core-metrics-resutls-weighted-UniFrac-site-significance.qzv

31.) Evaluate PCF by itself (Bd presence/absence and zoospore equivalence)

Code I used to get just the PCF by itself
qiime feature-table filter-samples --i-table Turnbull-rarefied-table-real-3000.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --p-where "[Species]='Pacific chorus frog'" --o-filtered-table Trunbull-filtered-table-Pacific-chorus-frog.qza
Saved FeatureTable[Frequency] to: Trunbull-filtered-table-Pacific-chorus-frog.qza

32.) Visualize the filtered table

qiime feature-table summarize --i-table Turnbull-filtered-table-Pacific-chorus-frog.qza --m-sample-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-filtered-table-Pacific-chorus-frog.qzv
Saved Visualization to: Turnbull-filtered-table-Pacific-chorus-frog.qzv



33.) Calculate Alpha/Beta Diversity for PCF:

qiime diversity core-metrics-phylogenetic --i-phylogeny Turnbull-seqs_rooted-tree.qza --i-table Turnbull-filtered-table-Pacific-chorus-frog.qza --p-sampling-depth 3000 --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-rarefied-table Turnbull-PCF-table.qza --output-dir Turnbull2019-PCF-core-metrics-results
Saved FeatureTable[Frequency] to: Turnbull-PCF-table.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-core-metrics-results/faith_pd_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-core-metrics-results/observed_features_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-core-metrics-results/shannon_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-core-metrics-results/evenness_vector.qza
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results/unweighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results/weighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results/jaccard_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results/bray_curtis_distance_matrix.qza
Saved PCoAResults to: Turnbull2019-PCF-core-metrics-results/unweighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-PCF-core-metrics-results/weighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-PCF-core-metrics-results/jaccard_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-PCF-core-metrics-results/bray_curtis_pcoa_results.qza

Saved Visualization to: Turnbull2019-PCF-core-metrics-results/unweighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results/weighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results/jaccard_emperor.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results/bray_curtis_emperor.qzv

34.) Alpha diversity for PCF using KW:

Shannon diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-shannon-significance.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-shannon-significance.qzv

Observed OTU's:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-observed-features-vector-significance.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-observed-features-vector-significance.qzv

Faith's Phylogenetic Diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-faith-pd-significance.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-faith-pd-significance.qzv

Evenness:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-evenness-significance.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-evenness-significance.qzv

35.) Beta diversity PERMANOVA PCF:

Bray-Curtis Dissimilarity: Bd Presence/absence

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-PCF-core-metrics-results-Bray-Curtis-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull-PCF-core-metrics-resutls-Bray-Curtis-zoospore-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-PCF-core-metrics-resutls-jaccard-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-PCF-core-metrics-jaccard-zoospore-significance.qzv

Unweighted UniFrac:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-PCF-core-metrics-resutls-unweighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-PCF-core-metrics-resutls-unweighted-UniFrac-zoospore-significance.qzv

Weighted UniFrac:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column BdPresence --o-visualization Turnbull2019-PCF-core-metrics-resutls-weighted-UniFrac-zoospore-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-PCF-core-metrics-resutls-weighted-UniFrac-zoospore-significance.qzv

36.) Alpha diversity PCF with Spearman's Rank Correlation

Shannon diversity spearman's rank:

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-shannon-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-shannon-correlation-spearman.qzv

Faith's phylogenetic diversity spearman's rank:

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-faith-pd-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-faith-pd-correlation-spearman.qzv

Evenness spearman's rank:

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-evenness-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-evenness-correlation-spearman.qzv

Observed features spearman's rank:

qiime diversity alpha-correlation --i-alpha-diversity Turnbull2019-PCF-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-core-metrics-results-observed-features-correlation-spearman.qzv
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-observed-features-correlation-spearman.qzv

37.) Calculate beta diversity PCF statistics using Mantel tests:

Bray-Curtis Dissimilarity:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-PCF-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-PCF-core-metrics-results-bray_curtis-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-PCF-core-metrics-results-bray_curtis-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results-bray_curtis-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-bray_curtis-zoospores-correlation.qzv

Jaccard Similarity:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-PCF-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-PCF-core-metrics-results-jaccard-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-PCF-core-metrics-results-jaccard-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results-jaccard-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-jaccard-zoospores-correlation.qzv

Unweighted UniFrac Distances:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-PCF-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-PCF-core-metrics-results-unweighted-

UniFrac-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-PCF-core-metrics-results-unweighted-UniFrac-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results-unweighted-UniFrac-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-unweighted-UniFrac-zoospores-correlation.qzv

Weighted UniFrac Distances:

qiime diversity beta-correlation --i-distance-matrix Turnbull2019-PCF-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column ZoosporeEquivalents --p-intersect-ids --o-metadata-distance-matrix Turnbull2019-PCF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qza --o-mantel-scatter-visualization Turnbull2019-PCF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qzv
Saved DistanceMatrix to: Turnbull2019-PCF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qza
Saved Visualization to: Turnbull2019-PCF-core-metrics-results-weighted-UniFrac-zoospores-correlation.qzv

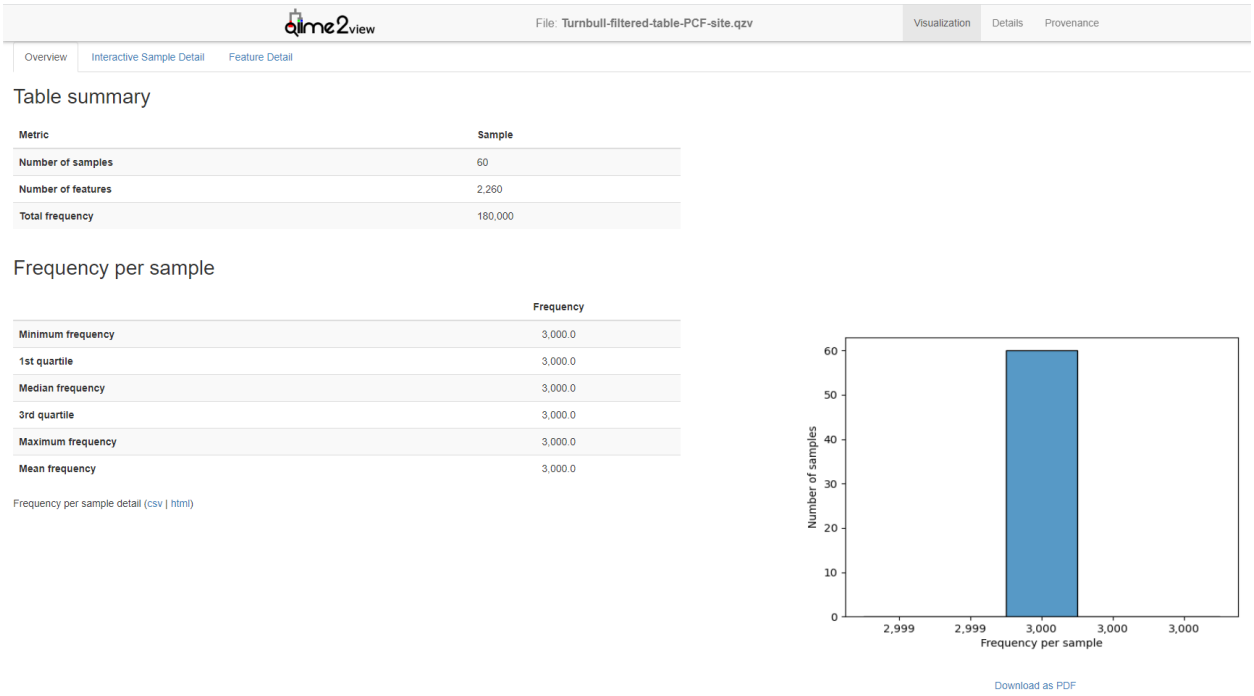
38.) Separate out PCF Site >3 frogs

Code I used to separate out sites

qiime feature-table filter-samples --i-table Turnbull-filtered-table-Pacific-chorus-frog.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --p-where "[Site] IN ('23','97A','16','116','42','75','23A','23B','28','98B')" --o-filtered-table Turnbull-filtered-table-PCF-site.qza
Saved FeatureTable[Frequency] to: Turnbull-filtered-table-PCF-site.qza

39.) Visualize sites

qiime feature-table summarize --i-table Turnbull-filtered-table-PCF-site.qza --m-sample-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull-filtered-table-PCF-site.qzv
Saved Visualization to: Turnbull-filtered-table-PCF-site.qzv



40.) Alpha and Beta diversity for PCF Site:

qiime diversity core-metrics-phylogenetic --i-phylogeny Turnbull-seqs_rooted-tree.qza --i-table Turnbull-filtered-table-PCF-site.qza --p-sampling-depth 3000 --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-rarefied-table Turnbull-table-PCF-site.qza --output-dir Turnbull2019-PCF-site-core-metrics-results

Saved FeatureTable[Frequency] to: Turnbull-table-PCF-site.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-site-core-metrics-results/faith_pd_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-site-core-metrics-results/observed_features_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-site-core-metrics-results/shannon_vector.qza
Saved SampleData[AlphaDiversity] to: Turnbull2019-PCF-site-core-metrics-results/evenness_vector.qza
Saved DistanceMatrix to: Turnbull2019-PCF-site-core-metrics-results/unweighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-PCF-site-core-metrics-results/weighted_UniFrac_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-PCF-site-core-metrics-results/jaccard_distance_matrix.qza
Saved DistanceMatrix to: Turnbull2019-PCF-site-core-metrics-results/bray_curtis_distance_matrix.qza
Saved PCoAResults to: Turnbull2019-PCF-site-core-metrics-results/unweighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-PCF-site-core-metrics-results/weighted_UniFrac_pcoa_results.qza
Saved PCoAResults to: Turnbull2019-PCF-site-core-metrics-results/jaccard_pcoa_results.qza

Saved PCoAResults to: Turnbull2019-PCF-site-core-metrics-results/bray_curtis_pcoa_results.qza
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results/unweighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results/weighted_UniFrac_emperor.qzv
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results/jaccard_emperor.qzv
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results/bray_curtis_emperor.qzv

41.) Evaluate Alpha KW Diversity for PCF Site:

Shannon diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-site-core-metrics-results/shannon_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-site-core-metrics-results-shannon-significance.qzv
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results-shannon-significance.qzv

Observed OTU's:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-site-core-metrics-results/observed_features_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-site-core-metrics-results-observed-features-vector-significance.qzv
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results-observed-features-vector-significance.qzv

Faith's Phylogenetic Diversity:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-site-core-metrics-results/faith_pd_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-site-core-metrics-results-faith-pd-significance.qzv
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results-faith-pd-significance.qzv

Evenness:

qiime diversity alpha-group-significance --i-alpha-diversity Turnbull2019-PCF-site-core-metrics-results/evenness_vector.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --o-visualization Turnbull2019-PCF-site-core-metrics-results-evenness-significance.qzv
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-results-evenness-significance.qzv

42.) Beta Diversity PCF Site:

Bray-Curtis Dissimilarity: Site

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-site-core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-PCF-site-core-metrics-results-Bray-Curtis-site-significance.qzv --p-pairwise

Saved Visualization to: Turnbull-PCF-site-core-metrics-resutls-Bray-Curtis-site-significance.qzv

Jaccard Similarity:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-site-core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-PCF-site-core-metrics-resutls-jaccard-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-jaccard-site-significance.qzv

Unweighted UniFrac:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-site-core-metrics-results/unweighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-PCF-core-metrics-resutls-unweighted-UniFrac-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-resutls-unweighted-UniFrac-site-significance.qzv

Weighted UniFrac:

qiime diversity beta-group-significance --i-distance-matrix Turnbull2019-PCF-site-core-metrics-results/weighted_UniFrac_distance_matrix.qza --m-metadata-file Metadata_Mapping_Turnbull_2019.tsv --m-metadata-column Site --o-visualization Turnbull2019-PCF-site-core-metrics-resutls-weighted-UniFrac-site-significance.qzv --p-pairwise
Saved Visualization to: Turnbull2019-PCF-site-core-metrics-resutls-weighted-UniFrac-site-significance.qzv

LEfSe was not working correctly in QIIIME, so I used Indicator Species Analysis in RStudio. First, I had to convert my feature table to a CSV file in QIIME

43.) Code I used to calculate relative frequency for a collapsed table

qiime taxa collapse --i-table Turnbull-rarefied-table-real-3000.qza --i-taxonomy TurnbullFrogs2019_taxonomy.qza --p-level 6 --o-collapsed-table Turnbull2019-indicator-species-analysis6.qza
Saved FeatureTable[Frequency] to: Turnbull2019-indicator-species-analysis6.qza

qiime feature-table relative-frequency --i-table Turnbull2019-indicator-species-analysis6.qza --o-relative-frequency-table Turnbull2019-relative-frequency-table.qza
Saved FeatureTable[RelativeFrequency] to: Turnbull2019-relative-frequency-table.qza

44.) Export biome file from QIIME:

qiime tools export --input-path Turnbull2019-indicator-species-analysis6.qza --output-path Turnbull2019-indicator-species-analysis6-files
Exported Turnbull2019-indicator-species-analysis6.qza as BIOMV210DirFmt to directory Turnbull2019-indicator-species-analysis6-files

```
biom convert --input-fp feature-table6.biom --output-fp Turnbull2019-indicator-species-analysis6-feature-table6.txt --header-key "taxonomy" --to-tsv
```

QIIME2 TABLE OUTPUTS

1. Outputs from rarefaction steps showing the differences between rarefying at 3,000 sequences per sample vs 5,000 sequences per sample.

Alpha KW 3000				
Statistic	**Site**	**Species**	**Date Collected**	**Bd Presence**
Shannon p-value	0.007	0.07	0.007	0.11
Shannon H	28.59	3.07	26.95	2.51
Faith's p-value	0.00089	0.08	0.0005	0.04
Faith's H	34.8	2.96	34.7	4.1
Evenness p-value	0.02	0.013	0.027	0.21
Evenness H	25	6.13	23.01	1.508
Observed features p-value	0.0009	0.21	0.000359888	0.09
Observed features H	34.79	1.51	35.7	2.7

Alpha KW 5000				
Statistic	**Site**	**Species**	**Date Collected**	**Bd Presence**
Shannon p-value	0.008	0.2	0.01	0.2
Shannon H	28.38	1.56	25.73	1.4
Faith's p-value	0.001	0.19	0.0005	0.059
Faith's H	34.5	1.68	34.63	3.558
Evenness p-value	0.01	0.07	0.02	0.33
Evenness H	25.9	3.24	22.89	0.94
Observed features p-value	0.001	0.2	0.0005	0.167
Observed features H	33	1.53	34.59	1.9

Beta PERMANOVA 3000	
Statistic	**Species**
Bray-Curtis p-value	0.138
Bray-Curtis pseudo-F	1.5
Jaccard p-value	0.002
Jaccard pseudo-F	1.48
Weighted UniFrac p-value	0.028
Weighted UniFrac pseudo-F	2.704
Unweighted UniFrac p-value	0.004
Unweighted UniFrac pseudo-F	2.13

Beta PERMANOVA 5000	
Statistic	**Species**
Bray-Curtis p-value	0.09
Bray-Curtis pseudo-F	1.67
Jaccard p-value	0.002
Jaccard pseudo-F	1.56
Weighted UniFrac p-value	0.041
Weighted UniFrac pseudo-F	2.5
Unweighted UniFrac p-value	0.005
Unweighted UniFrac pseudo-F	2.05

2. Alpha diversity (Kruskal-Wallis) for the whole dataset and the two frog species

Alpha KW 3000

Statistic	Site	Species	Date Collected	Bd Presence
Shannon p-value	0.0056	0.095	0.00635	0.116
Shannon H	29.48	2.78	27.59	2.47
Faith's p-value	0.00111	0.163	0.000769	0.057
Faith's H	34.23	1.95	33.64	3.61
Evenness p-value	0.0236	0.0153	0.0295	0.222
Evenness H	24.92	5.88	22.8	1.49
Observed features p-value	0.00094	0.265	0.000396	0.122
Observed features H	34.72	1.24	35.46	2.39

Alpha KW CSF

Statistic	Site	Date Collected	Bd Presence
Shannon p-value	0.059	0.059	0.29
Shannon H	14.97	14.97	1.12
Faith's p-value	0.0188	0.0188	0.1763
Faith's H	18.24	18.24	1.83
Evenness p-value	0.0726	0.0726	0.291
Evenness H	14.37	14.37	1.12
Observed features p-value	0.0189	0.0189	0.281
Observed features H	18.32	18.32	1.16

Alpha KW PCF

Statistic	Site	Date Collected	Bd Presence
Shannon p-value	0.0207	0.0252	0.0552
Shannon H	22.5	20.46	3.67
Faith's p-value	0.529	0.0435	0.481
Faith's H	19.48	18.75	0.497
Evenness p-value	0.0443	0.0575	0.0454
Evenness H	10.08	17.85	4
Observed features p-value	0.108	0.0567	0.642
Observed features H	17	17.9	0.216

Alpha KW CSF Site (sites with less than 3 frogs were removed)

Statistic	Site
Shannon p-value	0.0282
Shannon H	10.86
Faith's p-value	0.0136
Faith's H	12.56
Evenness p-value	0.0329
Evenness H	10.5
Observed features p-value	0.0161
Observed features H	12.17

Alpha KW PCF Site (sites with less than 3 frogs were removed)

Statistic	Site
Shannon p-value	0.0128
Shannon H	10.97
Faith's p-value	0.0274
Faith's H	18.75
Evenness p-value	0.0297
Evenness H	18.51
Observed features p-value	0.06
Observed features H	16.35

3. Alpha diversity Spearman's rank correlation for the whole dataset and both frog species.

Alpha Spearman's Rank 3000

Statistic	Zoospore Equivalence
Shannon p-value	0.102
Shannon H	-0.017
Faith's p-value	0.0352
Faith's H	-0.218
Evenness p-value	0.138
Evenness H	-0.154
Observed features p-value	0.128
Observed features H	-0.158

Alpha Spearman's Rank CSF

Statistic	Zoospore Equivalence
Shannon p-value	0.127
Shannon H	-0.28
Faith's p-value	0.054
Faith's H	-0.349
Evenness p-value	0.164
Evenness H	-0.257
Observed features p-value	0.0884
Observed features H	-0.311

Alpha Spearman's Rank PCF

Statistic	Zoospore Equivalence
Shannon p-value	0.0007
Shannon H	-0.415
Faith's p-value	0.301
Faith's H	-0.132
Evenness p-value	0
Evenness H	-0.4901
Observed features p-value	0.635
Observed features H	-0.061

4. Beta diversity PERMANOVA for the whole dataset and both frog species.

Beta PERMANOVA 3000

Statistic	Site	Species	Bd Presence
Bray-Curtis p-value	0.001	0.001	0.13
Bray-Curtis pseudo-F	2.04	17.13	1.5
Jaccard p-value	0.001	0.001	0.003
Jaccard pseudo-F	1.59	3.15	1.5
Weighted UniFrac p-value	0.004	0.001	0.03
Weighted UniFrac pseudo-F	1.89	19.07	2.63
Unweighted UniFrac p-value	0.001	0.001	0.005
Unweighted UniFrac pseudo-F	1.87	5.45	2.11

Beta PERMANOVA CSF

Statistic	Bd Presence
Bray-Curtis p-value	0.909
Bray-Curtis pseudo-F	0.566
Jaccard p-value	0.447
Jaccard pseudo-F	0.996
Weighted UniFrac p-value	0.744
Weighted UniFrac pseudo-F	0.527
Unweighted UniFrac p-value	0.325
Unweighted UniFrac pseudo-F	1.06

Beta PERMANOVA PCF

Statistic	Bd Presence
Bray-Curtis p-value	0.003
Bray-Curtis pseudo-F	3.97
Jaccard p-value	0.205
Jaccard pseudo-F	1.07
Weighted UniFrac p-value	0.021
Weighted UniFrac pseudo-F	3.67
Unweighted UniFrac p-value	0.179
Unweighted UniFrac pseudo-F	1.16

Beta PERMANOVA CSF Site (Sites with less than 3 frogs were removed)

Statistic	Site

Bray-Curtis p-value	0.047
Bray-Curtis pseudo-F	1.46
Jaccard p-value	0.001
Jaccard pseudo-F	1.43
Weighted UniFrac p-value	0.174
Weighted UniFrac pseudo-F	1.36
Unweighted UniFrac p-value	0.007
Unweighted UniFrac pseudo-F	1.53

Beta PERMANOVA PCF Site (Sites with less than 3 frogs were removed)

Statistic	Site
Bray-Curtis p-value	0.002
Bray-Curtis pseudo-F	2.37
Jaccard p-value	0.001
Jaccard pseudo-F	1.46
Weighted UniFrac p-value	0.002
Weighted UniFrac pseudo-F	2.45
Unweighted UniFrac p-value	0.001
Unweighted UniFrac pseudo-F	1.7

5. Beta diversity Mantel for the whole dataset and both frog species.

Beta Mantel 3000

Statistic	Zoospore Equivalence
Bray-Curtis p-value	0.0949
Bray-Curtis Spearman's rho	0.0044
Jaccard p-value	0.112
Jaccard Spearman's rho	-0.0965
Weighted UniFrac p-value	0.914
Weighted UniFrac Spearman's rho	-0.0063
Unweighted UniFrac p-value	0.04
Unweighted UniFrac Spearman's rho	-0.12

Beta Mantel CSF	
Statistic	**Zoospore Equivalence**

Bray-Curtis p-value	0.689
Bray-Curtis Spearman's rho	-0.0464
Jaccard p-value	0.873
Jaccard Spearman's rho	-0.0141
Weighted UniFrac p-value	0.931
Weighted UniFrac Spearman's rho	-0.0086
Unweighted UniFrac p-value	0.788
Unweighted UniFrac Spearman's rho	0.0226

Beta Mantel PCF

Statistic	**Zoospore Equivalence**
Bray-Curtis p-value	0.001
Bray-Curtis Spearman's rho	0.332
Jaccard p-value	0.083
Jaccard Spearman's rho	0.115
Weighted UniFrac p-value	0.001
Weighted UniFrac Spearman's rho	0.32
Unweighted UniFrac p-value	0.846
Unweighted UniFrac Spearman's rho	-0.0138

RStudio Steps for Inhibition Data

1.) Clear out RStudio and load in dataset

```
rm(list=ls())
Turnbull=read.csv("InhibitionData.csv",header=TRUE)
names(Turnbull)
```

2.) Hartigan's diptest to examine the distribution of isolate inhibition

```
library(diptest)
hist(Turnbull$Inhibition)
library(ggplot2)
dip.test(Turnbull$Inhibition,simulate.p.value = FALSE,B=2000)
dip.test(Turnbull$Inhibition)
```

3.) Visualize the distribution using the EM algorithm in the mixtools package

```
library(mixtools)
test=normalmixEM(Turnbull$Inhibition)
plot(test)
plot(test,density=TRUE)
```

4.) Calculate the cutoff to classify Inhibition categories

```
test[c("lambda", "mu", "sigma")]
cutoff=test$mu[2]-1.96*test$sigma[2]
cutoff
```

5.) Create cumulative frequency graph

```
library(ggplot2)
ggplot(Turnbull,aes(y=Inhibition,x=1-..x..))+stat_ecdf()+theme_classic()+
geom_vline(xintercept=0.224638,linetype='dotted')+geom_vline(xintercept=0.855072,lin
etype='dotted')+labs(x="Proportion of Bacteria",y="Inhibition Score")
length(which(Turnbull$Inhibition>0))/length(Turnbull$Inhibition)
```

6.) Go through each 96-well plate to calculate significant difference between the sample's growth rate and the positive control growth rate. Note: any sample

significantly higher is significantly facilitative and anything significantly lower is significantly inhibitory of *B.*

```
BdGrowthPlate1=read.csv("BdGrowthRatePlate1.csv",header=TRUE)

names(BdGrowthPlate1)

#for (i in 1:29) BdGrowthPlate2$Isolate[3*(i-1)+2]=BdGrowthPlate2$Isolate[3*(i-1)+1]

#for (i in 1:29) BdGrowthPlate2$Isolate[3*(i-1)+3]=BdGrowthPlate2$Isolate[3*(i-1)+1]

#Use T-test

BdGrowthPlate1$Isolate=as.factor(BdGrowthPlate1$Isolate)

class(BdGrowthPlate1$Isolate)

p.values=NULL

for (i in 1:27) p.values=c(p.values,
t.test(BdGrowthPlate1$GrowthRate[which(BdGrowthPlate1$Isolate==levels(BdGrowthP
late1$Isolate)[i])],
BdGrowthPlate1$GrowthRate[which(BdGrowthPlate1$Isolate=="Positive")],
alt="two.sided", conf.int=T, paired=F)$p.values)

newalpha=0.05/27

which(p.values<newalpha)

levels(BdGrowthPlate1$Isolate)[which(p.values<newalpha)]

#Use Mann-Whitney U-test to classify Bd-inhibition ability

BdGrowthPlate1$Isolate=as.factor(BdGrowthPlate1$Isolate)

BdGrowthPlate1$GrowthRate=as.numeric(BdGrowthPlate1$GrowthRate)

p.values=NULL

for (i in 1:27) p.values=c(p.values,
wilcox.test(BdGrowthPlate1$GrowthRate[which(BdGrowthPlate1$Isolate==levels(BdGr
owthPlate1$Isolate)[i])],
BdGrowthPlate1$GrowthRate[which(BdGrowthPlate1$Isolate=="Positive")],
alt="two.sided", conf.int=T, paired=F)$p.value)
```

RStudio Comparison of *Bd* infection by Location

1. Import Data

```
CSF=read.csv("BdInfection_Site_CSF.csv",header=TRUE)
PCF=read.csv("BDInfection_Site_PCF.csv",header=TRUE)
```

2. Make Zoospore Equivalents into numeric values

```
CSF$ZE=as.numeric(CSF$ZoosporeEquivalents)
PCF$ZEPCF=as.numeric(PCF$ZoosporeEquivalents)
```

3. Use the Shapiro test to test for normal distribution (for both frog species)

```
shapiro.test(CSF$ZE)
shapiro.test(PCF$ZEPCF)
```

4. Use the Levene's Test for equal variance (for both frog species)

```
library(car)
CSF$site.f=as.factor(CSF$Site)
leveneTest(ZE~site.f,CSF)
PCF$site.fPCF=as.factor(PCF$Site)
leveneTest(ZEPCF~site.fPCF,PCF)
```

5. Use the Kruskal-Wallis test to see if wetland site is a significant predictor for zoospore equivalents (for both frog species)

```
kruskal.test(ZE~site.f,data=CSF)
kruskal.test(ZEPCF~site.fPCF,data=PCF)
```

6. Site was found to be a significant predictor for zoospore equivalents for the PCF species, so the Dunn test was used as a post-hoc comparison using *Bd* infection intensity as the response variable.

```
library(dunn.test)
dunn.test(PCF$ZEPCF,PCF$site.fPCF,method = "bonferroni")
```

RStudio Relative Abundance Differences (Frog Species)

```
rm(list=ls())

Abundance = read.csv("Relative Abundance Species.csv", header=T)

library(ggplot2)

ggplot(Abundance,aes(x=Species,y=Sum))+geom_point()

ggplot(Abundance,aes(x=Species,y=Sum))+stat_summary(fun="mean",
geom="point")+stat_summary(fun= "mean", fun.min=function(x) mean(x)-
sd(x),fun.max=function(x) mean(x)+sd(x), geom="errorbar")+ theme_classic()

wilcox.test(Sum~Species,data=Abundance)

summary(Abundance$Sum)

tapply(Abundance$Sum,Abundance$Species,summary)

tapply(Abundance$Sum,Abundance$Species,sd)


Abundance2 = read.csv("Rel-abundance-sum-species2.csv", header=F)

Species=Abundance2[1,3:102]

Sum=Abundance2[29,3:102]

Abundance=rbind(Species=Species,Sum=Sum)

Abundance=as.data.frame(t(Abundance))

Abundance$Sum=as.numeric(Abundance$Sum)


ggplot(Abundance,aes(x=Species,y=Sum))+geom_point()

ggplot(Abundance,aes(x=Species,y=Sum))+stat_summary(fun="mean",
geom="point")+stat_summary(fun= "mean", fun.min=function(x) mean(x)-
sd(x),fun.max=function(x) mean(x)+sd(x), geom="errorbar")+ theme_classic()

wilcox.test(Sum~Species,data=Abundance)

summary(Abundance$Sum)

tapply(Abundance$Sum,Abundance$Species,summary)

tapply(Abundance$Sum,Abundance$Species,sd)


Species=Abundance2[1,3:102]
```

```
Sum=Abundance2[3,3:102]

Abundance=rbind(Species=Species,Sum=Sum)

Abundance=as.data.frame(t(Abundance))

Abundance$Sum=as.numeric(Abundance$Sum)


ggplot(Abundance,aes(x=Species,y=Sum))+geom_point()

ggplot(Abundance,aes(x=Species,y=Sum))+stat_summary(fun="mean",
geom="point")+stat_summary(fun= "mean", fun.min=function(x) mean(x)-
sd(x),fun.max=function(x) mean(x)+sd(x), geom="errorbar")+ theme_classic()

wilcox.test(Sum~Species,data=Abundance)

summary(Abundance$Sum)

tapply(Abundance$Sum,Abundance$Species,summary)

tapply(Abundance$Sum,Abundance$Species,sd)
```

RStudio Indicator Species Analysis

```
rm(list=ls())

#LeFSe Analysis
frog = read.csv("Turnbull-Indicator-species-analysis.csv", header=T)
metadata= read.csv ("Metadata_Mapping_Turnbull_2019.csv", header=T)

species=unlist(lapply(2:96,function(x)
metadata$Species[which(metadata$SampleID==substring(names(frog)[x],2,nchar(names
(frog)[x]))])))

#Trim extra taxonomy column from data table
frog = frog[,-96]
row.names(frog)=frog[,1]
frog=frog[,-1]
#removes 1st row

for (i in 1:94) frog[,i]=as.numeric(frog[,i])
#convert that one column


#Transpose dataset
frog=t(frog)


#load in the indicspecies package
library(indicspecies)
indval=multipatt(frog,species)
summary(indval)
#Trying to figure out which groups of bacteria are associated with each group, which
bacteria associated with which group
#mixes up group randomly, compares randomly to actual, which is not like the random
#only 5 spp that are associated with EP/non-EP group
#permutational test, larger = better?
#124 species specific to CSF and 11 species specific to PCF


#Site CSF
frog1=frog[which(species=="Columbia spotted frog"),]
site=unlist(lapply(1:31,function(x)
metadata$Site[which(metadata$SampleID==substring(row.names(frog1)[x],2,nchar(row.
names(frog1)[x]))])))
indval=multipatt(frog1,site)
summary(indval)
table(site)
```

```
#Site PCF
frog2=frog[which(species=="Pacific chorus frog"),]
site2=unlist(lapply(1:63,function(x)
metadata$Site[which(metadata$SampleID==substring(row.names(frog2)[x],2,nchar(row.
names(frog2)[x]))))]))
indval=multipatt(frog2,site2)
summary(indval)
table(site)

#Bd pres/abs CSF
Bd1=unlist(lapply(1:31,function(x)
metadata$BdPresence[which(metadata$SampleID==substring(row.names(frog1)[x],2,nc
har(row.names(frog1)[x]))))]))
indval=multipatt(frog1,Bd1)
summary(indval)


#Bd pres/abs PCF
Bd2=unlist(lapply(1:63,function(x)
metadata$BdPresence[which(metadata$SampleID==substring(row.names(frog2)[x],2,nc
har(row.names(frog2)[x]))))]))
indval=multipatt(frog2,Bd2)
summary(indval)

rm(list=ls())

#Indicator Species ASV
frog = read.csv("Turnbull-filtered-table-lefse.csv", header=T)
metadata= read.csv ("Metadata_Mapping_Turnbull_2019.csv", header=T)

species=unlist(lapply(2:101,function(x)
metadata$Species[which(metadata$SampleID==substring(names(frog)[x],2,nchar(names
(frog)[x]))))]))

#Trim extra taxonomy column from data table
frog = frog[,-102]
row.names(frog)=frog[,1]
frog=frog[,-1]
#removes 1st row

for (i in 1:100) frog[,i]=as.numeric(frog[,i])
#convert that one column


#Transpose dataset
```

```
frog=t(frog)

ASVSpecies = read.csv("ASVSpecies.csv", header=T)
#load in the indicspecies package
library(indicspecies)
indval=multipatt(frog,species)
summary(indval)
table(species)
indval$sign
antiBd_ASVspecies=indval$sign[unlist(lapply(1:22,function(x)
which(row.names(indval$sign)==ASVSpecies$X.ASV.ID[x]))),]
#Trying to figure out which groups of bacteria are associated with each group, which
bacteria associated with which group
#mixes up group randomly, compares randomly to actual, which is not like the random
#only 5 spp that are associated with EP/non-EP group
#permutational test, larger = better?
#124 species specific to CSF and 11 species specific to PCF


#Site CSF
frog1=frog[which(species=="Columbia spotted frog"),]
site=unlist(lapply(1:31,function(x)
metadata$Site[which(metadata$SampleID==substring(row.names(frog1)[x],2,nchar(row.
names(frog1)[x])))]))
indval=multipatt(frog1,site)
summary(indval)
table(site)
indval$sign
antiBd_ASVSite1=indval$sign[unlist(lapply(1:22,function(x)
which(row.names(indval$sign)==ASVSpecies$X.ASV.ID[x]))),]

#Site PCF
frog2=frog[which(species=="Pacific chorus frog"),]
site2=unlist(lapply(1:69,function(x)
metadata$Site[which(metadata$SampleID==substring(row.names(frog2)[x],2,nchar(row.
names(frog2)[x])))]))
indval=multipatt(frog2,site2)
summary(indval)
table(site2)
indval$sign
antiBd_ASVSite2=indval$sign[unlist(lapply(1:22,function(x)
which(row.names(indval$sign)==ASVSpecies$X.ASV.ID[x]))),]


#Bd pres/abs CSF
```

```
Bd1=unlist(lapply(1:31,function(x)
metadata$BdPresence[which(metadata$SampleID==substring(row.names(frog1)[x],2,nc
har(row.names(frog1)[x])))]))
indval=multipatt(frog1,Bd1)
summary(indval)
indval$sign
antiBd_ASVbd1=indval$sign[unlist(lapply(1:22,function(x)
which(row.names(indval$sign)==ASVSpecies$X.ASV.ID[x]))),]


#Bd pres/abs PCF
Bd2=unlist(lapply(1:69,function(x)
metadata$BdPresence[which(metadata$SampleID==substring(row.names(frog2)[x],2,nc
har(row.names(frog2)[x])))]))
indval=multipatt(frog2,Bd2)
summary(indval)
antiBd_ASVbd2=indval$sign[unlist(lapply(1:22,function(x)
which(row.names(indval$sign)==ASVSpecies$X.ASV.ID[x]))),]
```

INDICATOR SPECIES R MARKDOWN

KD

5/23/2022

```
rm(list=ls())

#LeFSe Analysis
frog = read.csv("Turnbull-Indicator-species-analysis.csv", header=T)
metadata= read.csv ("Metadata_Mapping_Turnbull_2019.csv", header=T)

species=unlist(lapply(2:96,function(x) metadata$Species[which(metadata$SampleID==s
ubstring(names(frog)[x],2,nchar(names(frog)[x])))]))

#Trim extra taxonomy column from data table
frog = frog[,-96]
row.names(frog)=frog[,1]
frog=frog[,-1]
#removes 1st row

for (i in 1:94) frog[,i]=as.numeric(frog[,i])
#convert that one column


#Transpose dataset
frog=t(frog)


#load in the indicspecies package
library(indicspecies)

## Warning: package 'indicspecies' was built under R version 4.1.3

## Loading required package: permute

## Warning: package 'permute' was built under R version 4.1.2

indval=multipatt(frog,species)
summary(indval)

##
##  Multilevel pattern analysis
##  ---------------------------
##
##  Association function: IndVal.g
##  Significance level (alpha): 0.05
##
##  Total number of species: 787
```

```
##  Selected number of species: 134
##  Number of species associated to 1 group: 134
##
##  List of species associated to each combination:
##
##  Group Columbia spotted frog  #sps.  122
##
p.value
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Verrucomicrobiales;__;
__                                                  0.005
## d__Bacteria;p__Patescibacteria;c__Gracilibacteria;o__Gracilibacteria;f__Gracilibacte
ria;g__Gracilibacteria                              0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rho
docyclaceae;__                                      0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;__
0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__Lentimicrobia
ceae;g__Lentimicrobiaceae                           0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Tannerellaceae;g__
Parabacteroides                                     0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Me
thylomonadaceae;g__Crenothrix                       0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g_
_Bacteroides                                        0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;__;__
0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prolixibacteraceae;
g__WCHB1-32                                         0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__
Rikenella                                           0.005
## d__Bacteria;p__Verrucomicrobiota;c__Kiritimatiellae;o__WCHB1-41;f__WCHB1-4
1;g__WCHB1-41                                       0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__
Alistipes                                           0.005
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Verrucomicrobiales;f__
Akkermansiaceae;g__Akkermansia                      0.005
## d__Bacteria;p__Desulfobacterota;c__Desulfuromonadia;o__Geobacterales;f__Geobac
teraceae;g__Geobacter                               0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Marinifilaceae;g__
Odoribacter                                         0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Nitr
osomonadaceae;g__Ellin6067                          0.005
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Pedosphaerales;f__Ped
osphaeraceae;g__uncultured                          0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rho
docyclaceae;g__Dechloromonas                        0.010
```

```
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Segetibacter 0.050
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Tannerellaceae;__
0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;__
0.005
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__uncult
ured;g__uncultured 0.005
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinck
iaceae;g__Rhodoblastus 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;__
0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Me
thylomonadaceae;g__Methylobacter 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;__;__;__
0.005
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelotrichaceae;g
__Faecalitalea 0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Chrom
atiaceae;g__Thiodictyon 0.015
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales;f__A
naerovoracaceae;__ 0.005
## d__Bacteria;p__Actinobacteriota;c__Thermoleophilia;o__Gaiellales;f__uncultured;g_
_uncultured 0.005
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Intrasporan
giaceae;__ 0.025
## d__Bacteria;p__Fibrobacterota;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacterac
eae;g__uncultured 0.010
## d__Bacteria;p__Acidobacteriota;c__Acidobacteriae;o__Acidobacteriae;f__Acidobact
eriae;g__Paludibaculum 0.010
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g_
_Angelakisella 0.005
## d__Bacteria;p__Verrucomicrobiota;c__Lentisphaeria;o__Victivallales;f__vadinBE97;
g__vadinBE97 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;__;__
0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__
Oscillibacter 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;__;__
0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales;f__A
naerovoracaceae;g__Anaerovorax 0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prolixibacteraceae;
g__BSV13 0.010
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Me
thylomonadaceae;g__Methylomonas 0.020
```

```
## d__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Caldilineales;f__Caldilineaceae;g__
uncultured                                                    0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clost
ridium_sensu_stricto_1                                        0.005
## d__Bacteria;p__Acidobacteriota;c__Vicinamibacteria;o__Vicinamibacterales;f__Vici
namibacteraceae;g__Vicinamibacteraceae                        0.020
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rho
docyclaceae;g__Ferribacterium                                 0.010
## d__Bacteria;p__WPS-2;c__WPS-2;o__WPS-2;f__WPS-2;g__WPS-2
0.030
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__SC-I
-84;g__SC-I-84                                                0.005
## d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Polyangiaceae;g__P
ajaroellobacter                                               0.040
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rho
docyclaceae;g__Candidatus_Accumulibacter                      0.010
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Steroidobacterales;f__St
eroidobacteraceae;g__uncultured                               0.020
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__
uncultured                                                    0.005
## d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Polyangiaceae;g__u
ncultured                                                     0.005
## d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;__;
__                                                            0.005
## d__Bacteria;p__Desulfobacterota;c__Desulfobulbia;o__Desulfobulbales;f__Desulfob
ulbaceae;g__Desulfobulbus                                     0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Thiotri
chaceae;g__Thiothrix                                          0.005
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Pedosphaerales;f__Ped
osphaeraceae;__                                               0.015
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__R
hodanobacteraceae;g__uncultured                               0.020
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidetes_vadi
nHA17;g__Bacteroidetes_vadinHA17                              0.050
## d__Bacteria;p__Actinobacteriota;c__Thermoleophilia;o__Solirubrobacterales;f__Soli
rubrobacteraceae;g__Conexibacter                              0.010
## d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Polyangiaceae;g__P
olyangium                                                     0.025
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Kineosporiales;f__Kineospori
aceae;__                                                      0.025
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiale
s_Incertae_Sedis;g__Alsobacter                                0.030
## d__Bacteria;p__Actinobacteriota;c__Thermoleophilia;o__Solirubrobacterales;f__67-1
4;g__67-14                                                    0.025
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;g__Mesorhizobium                                          0.030
```

```
## d__Bacteria;p__Campilobacterota;c__Campylobacteria;o__Campylobacterales;f__Sul
furimonadaceae;g__Sulfuricurvum 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospirales;g__H
ydrogenoanaerobacterium 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__
Anaerostignum 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Eubacteriales;f__Eubacteriaceae;g__Eu
bacterium 0.005
## d__Bacteria;p__Spirochaetota;c__Leptospirae;o__Leptospirales;f__Leptospiraceae;g_
_Leptospira 0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__
dgA-11_gut_group 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__I
ntestinimonas 0.015
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Paracaedibacterales;f__Pa
racaedibacteraceae;g__Candidatus_Paracaedibacter 0.030
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methyloli
gellaceae;g__uncultured 0.005
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelotrichaceae;g
__Erysipelotrichaceae 0.030
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__AKYH767;g_
_AKYH767 0.015
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Frankiales;f__Geodermatophi
laceae;g__Blastococcus 0.045
## d__Bacteria;p__Planctomycetota;c__Planctomycetes;o__Planctomycetales;f__Rubini
sphaeraceae;g__SH-PL14 0.030
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia_UCG-014;f__Clostridia_UC
G-014;g__Clostridia_UCG-014 0.015
## d__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolineaceae;
g__Leptolinea 0.015
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelotrichaceae;g
__[Anaerorhabdus]_furcosa_group 0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Microscillaceae;g_
_Ohtaekwangia 0.030
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Me
thylomonadaceae;g__Methylovulum 0.025
## d__Bacteria;p__Bacteroidota;c__SJA-28;o__SJA-28;f__SJA-28;g__SJA-28
0.040
## d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales;f__Eggerthel
laceae;g__Gordonibacter 0.015
## d__Bacteria;p__Verrucomicrobiota;c__Lentisphaeria;o__Victivallales;f__Victivallace
ae;g__Victivallaceae 0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Hyd
rogenophilaceae;g__uncultured 0.025
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__
Natranaerovirga 0.005
```

```
## d__Bacteria;p__Spirochaetota;c__Spirochaetia;o__Spirochaetales;f__Spirochaetaceae
;g__Spirochaeta_2 0.010
## d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__
Sporomusaceae;g__Anaerosinus 0.020
## d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Sandaracinaceae;g__
uncultured 0.010
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;g__Phyllobacterium 0.010
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellacea
e;g__Christensenellaceae_R-7_group 0.030
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Pedosphaerales;f__Ped
osphaeraceae;g__ADurb.Bin063-1 0.025
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prolixibacteraceae;
g__uncultured 0.025
## d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__
Selenomonadaceae;__ 0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales;f__A
naerovoracaceae;g__[Eubacterium]_brachy_group 0.005
## d__Bacteria;p__Myxococcota;c__Myxococcia;o__Myxococcales;f__Myxococcaceae;
__ 0.035
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelatoclostridiac
eae;g__Coprobacillus 0.010
## d__Bacteria;p__Chloroflexi;c__KD4-96;o__KD4-96;f__KD4-96;g__KD4-96
0.030
## d__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolineaceae;
g__uncultured 0.020
## d__Bacteria;p__Desulfobacterota;c__Desulfuromonadia;o__Geobacterales;f__Geobac
teraceae;g__Citrifermentans 0.040
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelotrichaceae;_
_ 0.025
## d__Bacteria;p__Acidobacteriota;c__Blastocatellia;o__Blastocatellales;f__Blastocatell
aceae;g__JGI_0001001-H03 0.040
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__X
anthomonadaceae;g__Pseudoxanthomonas 0.050
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g_
_Incertae_Sedis 0.040
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Dongiales;f__Dongiaceae;
g__Dongia 0.035
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__SB-5;g__SB-5
0.045
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Tistrellales;f__Geminicoc
caceae;__ 0.025
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia;f__Hungateiclostridiaceae;__
0.045
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__[Eubacterium]_copro
stanoligenes_group;g__[Eubacterium]_coprostanoligenes_group 0.030
```

```
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Azospirillales;f__Azospiri
llaceae;g__Azospirillum                                    0.025
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Flavobacteriace
ae;g__uncultured                                           0.030
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g_
_Ruminococcus                                              0.045
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Muribaculaceae;g_
_Muribaculaceae                                            0.035
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Microscillaceae;g_
_OLB12                                                     0.040
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Phormidia
ceae;g__Planktothrix_NIVA-CYA_15                           0.040
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobac
teraceae;g__Rhodopseudomonas                               0.030
## d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Polyangiaceae;__
0.030
## d__Bacteria;p__Acidobacteriota;c__Holophagae;o__Holophagales;f__Holophagaceae
;g__Holophagaceae                                          0.045
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Kaistiacea
e;g__Kaistia                                               0.050
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Elsterales;f__Elsteraceae;
g__uncultured                                              0.050
##  Group Pacific chorus frog  #sps.  12
##                                                                                p.value
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Propionibacteriales;f__Propio
nibacteriaceae;__           0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Fibrella                    0.015
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Nit
rincolaceae;g__Nitrincola     0.010
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__Sphingobacte
riaceae;g__Sphingobacterium   0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Rudanella                   0.005
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lac
tobacillus                  0.030
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;g__Nitratireductor        0.005
## d__Bacteria;p__Campilobacterota;c__Campylobacteria;o__Campylobacterales;f__Ar
cobacteraceae;g__Malaciobacter   0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Flavobacteriace
ae;g__Mesonia               0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__uncultured;g__uncultur
ed                          0.025
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Opitutales;f__Opitutace
ae;g__IMCC26134             0.030
```

```
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Niabella                0.035
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

#Trying to figure out which groups of bacteria are associated with each group, which ba
cteria associated with which group
#mixes up group randomly, compares randomly to actual, which is not like the random
#only 5 spp that are associated with EP/non-EP group
#permutational test, larger = better?
#124 species specific to CSF and 11 species specific to PCF


#Site CSF
frog1=frog[which(species=="Columbia spotted frog"),]
site=unlist(lapply(1:31,function(x) metadata$Site[which(metadata$SampleID==substrin
g(row.names(frog1)[x],2,nchar(row.names(frog1)[x])))]))
indval=multipatt(frog1,site)
summary(indval)

##
##  Multilevel pattern analysis
##  ---------------------------
##
##  Association function: IndVal.g
##  Significance level (alpha): 0.05
##
##  Total number of species: 787
##  Selected number of species: 48
##  Number of species associated to 1 group: 23
##  Number of species associated to 2 groups: 9
##  Number of species associated to 3 groups: 7
##  Number of species associated to 4 groups: 5
##  Number of species associated to 5 groups: 2
##  Number of species associated to 6 groups: 1
##  Number of species associated to 7 groups: 1
##  Number of species associated to 8 groups: 0
##
##  List of species associated to each combination:
##
##  Group 98B  #sps.  2
##                                                                        p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Orbales;f__Orbaceae;g_
_Gilliamella             0.005
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;g__Nitratireductor   0.045
```

```
##  Group W42  #sps.  4
##                                                                                                    p.value
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptococcales;f__Peptococcaceae;g__un
cultured                    0.015
## d__Bacteria;p__Verrucomicrobiota;c__Lentisphaeria;o__Victivallales;f__vadinBE97;
g__vadinBE97                  0.010
## d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__
Sporomusaceae;g__Anaerosinus   0.010
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__
Anaerostignum                 0.045

##
##  Group W62  #sps.  5
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Segetibacter             0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Flavisolibacter           0.045
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocace
ae;g__Trichormus_HINDAK_2001-4   0.050
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;__                          0.045
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__NS9_marine_gr
oup;g__NS9_marine_group         0.030

##  Group W75  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Azospirillales;f__Azospiri
llaceae;g__Azospirillum   0.005

##  Group W7A  #sps.  3
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Thiotri
chaceae;g__Thiothrix   0.025
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__PeM15;f__PeM15;g__PeM15
0.035
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Chrom
atiaceae;g__Thiodictyon   0.030

##  Group W97A  #sps.  8
p.value
## d__Bacteria;p__Firmicutes;c__Desulfitobacteriia;o__Desulfitobacteriales;f__Desulfit
obacteriaceae;g__Desulfosporosinus   0.035
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Pseudonocardiales;f__Pseudo
nocardiaceae;g__Crossiella             0.035
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
```

```
Rudanella                                   0.005
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clost
ridium_sensu_stricto_9              0.025
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Phormidia
ceae;g__Planktothrix_NIVA-CYA_15         0.020
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__X
anthomonadaceae;g__Pseudoxanthomonas         0.010
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Streptomycetales;f__Strepto
mycetaceae;g__Streptomyces              0.040
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulo
bacteraceae;g__uncultured               0.050

##  Group 98B+W62  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Bur
kholderiaceae;g__Limnobacter   0.005

##  Group 98B+W97A  #sps.  2
p.value
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lac
tobacillus              0.04
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;g__Pseudochrobactrum    0.04

##  Group W102+W62  #sps.  1
p.value
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocace
ae;__   0.005

##  Group W62+W7A  #sps.  3
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__NS11-12_mar
ine_group;g__NS11-12_marine_group    0.04
## d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Phaselicystidaceae;g
__Phaselicystis                 0.02
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinck
iaceae;g__Rhodoblastus          0.05

##  Group W62+W97A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alt
eromonadaceae;g__Rheinheimera   0.045

##  Group W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia;f__Hungateiclostridiaceae;g_
```

```
_uncultured    0.05

##  Group 98B+W62+W7A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__X
anthomonadaceae;g__Arenimonas    0.03

##  Group 98B+W62+W97A  #sps.  2
p.value
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocace
ae;g__Cuspidothrix_LMECYA_163   0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Flavobacteriace
ae;g__Myroides                0.025

##  Group W54A+W62+W7A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Oxal
obacteraceae;g__Massilia   0.045

##  Group W62+W75+W7A  #sps.  2
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;__;__
0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__uncultured;g__u
ncultured   0.035

##  Group W62+W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Ferruginibacter   0.025

##  Group 98B+W62+W75+W97A  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Weeksellaceae;g
__Elizabethkingia    0.02

##  Group 98B+W62+W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Propionibacteriales;f__Propio
nibacteriaceae;__    0.035

##  Group W102+W62+W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sp
hingomonadaceae;g__Porphyrobacter   0.015
```

```
##  Group W116+W62+W75+W97A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinck
iaceae;__   0.035

##  Group W62+W75+W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Com
amonadaceae;g__Hydrogenophaga   0.025

##  Group W102+W116+W62+W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinck
iaceae;g__alphaI_cluster   0.035

##  Group W102+W62+W75+W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Verrucomicrobiales;f__
Verrucomicrobiaceae;g__uncultured   0.045

##  Group W102+W116+W54A+W62+W75+W7A  #sps.  1
p.value
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Microbacte
riaceae;__    0.04

##  Group 98B+W102+W116+W62+W75+W7A+W97A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;__    0.05

## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

table(site)

## site
##  98B W102 W116  W42 W54A  W62  W75  W7A W97A
##    2    2    3    2    6    5    3    7    1

#Site PCF
frog2=frog[which(species=="Pacific chorus frog"),]
site2=unlist(lapply(1:63,function(x) metadata$Site[which(metadata$SampleID==substri
ng(row.names(frog2)[x],2,nchar(row.names(frog2)[x])))]))
indval=multipatt(frog2,site2)
summary(indval)

##
##  Multilevel pattern analysis
```

```
##  -------------------------
##
##  Association function: IndVal.g
##  Significance level (alpha): 0.05
##
##  Total number of species: 787
##  Selected number of species: 73
##  Number of species associated to 1 group: 44
##  Number of species associated to 2 groups: 6
##  Number of species associated to 3 groups: 7
##  Number of species associated to 4 groups: 5
##  Number of species associated to 5 groups: 4
##  Number of species associated to 6 groups: 2
##  Number of species associated to 7 groups: 0
##  Number of species associated to 8 groups: 1
##  Number of species associated to 9 groups: 2
##  Number of species associated to 10 groups: 1
##  Number of species associated to 11 groups: 1
##
##  List of species associated to each combination:
##
##  Group 23B  #sps.  4
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;__;__
0.025
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Bur
kholderiaceae;g__Lautropia      0.030
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rho
docyclaceae;g__Dechloromonas    0.015
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhod
ospirillaceae;g__uncultured   0.050

##  Group W42  #sps.  1
p.value
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Catellicoccaceae;g__Cat
ellicoccus   0.045

##  Group W54A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Com
amonadaceae;g__Polaromonas    0.04

##  Group W62  #sps.  23
p.value
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Micrococc
aceae;g__Glutamicibacter                    0.020
```

```
## d__Bacteria;p__Latescibacterota;c__Latescibacterota;o__Latescibacterota;f__Latesci
bacterota;g__Latescibacterota 0.020
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;g__Hoeflea 0.020
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Nitr
osomonadaceae;g__MND1 0.020
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__X
anthomonadaceae;g__Lysobacter 0.020
## d__Bacteria;p__Actinobacteriota;c__Thermoleophilia;o__Solirubrobacterales;f__Soli
rubrobacteraceae;g__Solirubrobacter 0.010
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Frankiales;f__Geodermatophi
laceae;g__Modestobacter 0.010
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Weeksellaceae;_
_ 0.010
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacterales;f__Yer
siniaceae;__ 0.020
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Jeotgalib
acillus 0.030
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Frankiales;__;__
0.010
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Pseudanabaenales;f__Pseudana
baenaceae;g__Pseudanabaena_PCC-7403 0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Microscillaceae;__
0.035
## d__Bacteria;p__Actinobacteriota;c__Acidimicrobiia;o__Microtrichales;f__Iamiaceae;
g__Iamia 0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Fluviimonas 0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Dinghuibacter 0.020
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocace
ae;g__Calothrix_PCC-6303 0.050
## d__Bacteria;p__Chloroflexi;c__TK10;o__TK10;f__TK10;g__TK10
0.030
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Micrococc
aceae;g__Micrococcus 0.045
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Gammaproteobacteria_I
ncertae_Sedis;f__Unknown_Family;g__Acidibacter 0.030
## d__Bacteria;p__Acidobacteriota;c__Acidobacteriae;o__Acidobacteriales;f__Acidobac
teriaceae_(Subgroup_1);g__Bryocella 0.050
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Corynebacteriales;f__Coryne
bacteriaceae;g__Corynebacterium 0.030
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Frankiales;f__Frankiaceae;g_
_Jatrophihabitans 0.050

##  Group W75  #sps.  15
```

```
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Stappiacea
e;__                                0.005
## d__Bacteria;p__Acidobacteriota;c__Acidobacteriae;o__Acidobacteriales;f__Acidobac
teriaceae_(Subgroup_1);g__Granulicella   0.025
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinck
iaceae;g__FukuN57                   0.035
## d__Bacteria;p__Chloroflexi;c__Chloroflexia;o__Chloroflexales;f__Roseiflexaceae;g_
_uncultured                         0.030
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Cyclobacteriaceae;
g__Algoriphagus                     0.030
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Crocinitomicace
ae;g__Wandonia                      0.035
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Chit
inibacteraceae;g__Chitinibacter          0.030
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g_
_Subdoligranulum                    0.030
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__R
hodanobacteraceae;g__Aquimonas           0.045
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Hyph
omonadaceae;g__UKL13-1              0.030
## d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Polyangiaceae;g__P
olyangium                           0.050
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomic
robiaceae;g__Hyphomicrobium              0.025
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Me
thylomonadaceae;__                  0.045
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocace
ae;g__Anabaena_PCC-7122             0.050
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Azospirillales;f__Azospiri
llaceae;g__Niveispirillum            0.045

##  Group 23A+W54A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Chrom
atiaceae;g__Thiodictyon   0.025

##  Group 23A+W62  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sp
hingomonadaceae;g__Sandaracinobacter   0.045

##  Group 23B+28  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;__;__   0.04
```

```
##  Group 23B+W62  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Terrimonas   0.035

##  Group 23B+W75  #sps.  2
p.value
## d__Bacteria;__;__;__;__;__                                          0.040
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Lacihabitans   0.015

##  Group 23A+23B+28  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Runella    0.02

##  Group 23A+23B+W75  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__uncultured;g__u
ncultured    0.01

##  Group 23A+W54A+W62  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__Sphingobacte
riaceae;g__Sphingobacterium   0.035

##  Group 23B+W42+W75  #sps.  2
p.value
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__uncultured;f__unculture
d;g__uncultured    0.01
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;__;__
0.04

##  Group 23B+W54A+W75  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Crocinitomicace
ae;g__Fluviicola    0.01

##  Group W42+W62+W75  #sps.  1
p.value
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Pseudanabaenales;f__Pseudana
baenaceae;g__Pseudanabaena_PCC-7429   0.005

##  Group 23A+23B+28+98B  #sps.  2
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Orbales;f__Orbaceae;g_
```

```
_Gilliamella                0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Neis
seriaceae;g__Snodgrassella   0.035

##  Group 23B+28+98B+W75  #sps.  1
p.value
## d__Bacteria;p__Gemmatimonadota;c__Gemmatimonadetes;o__Gemmatimonadales;f
__Gemmatimonadaceae;g__Gemmatimonas    0.01

##  Group 23B+28+W23+W54A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiac
eae;g__Aureimonas    0.03

##  Group 23B+28+W62+W75  #sps.  1
p.value
## d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Verrucomicrobiales;f__
Rubritaleaceae;g__Luteolibacter   0.045

##  Group 23A+23B+28+98B+W62  #sps.  1
p.value
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocace
ae;g__Aphanizomenon_NIES81    0.01

##  Group 23A+23B+28+98B+W75  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Saprospiraceae;
g__uncultured   0.005

##  Group 23B+28+98B+W23+W54A  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Fibrella    0.02

##  Group 23B+28+W42+W54A+W75  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Arcicella   0.005

##  Group 23A+23B+28+98B+W23+W54A  #sps.  1
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__
Rudanella   0.005

##  Group 23A+23B+28+98B+W42+W75  #sps.  1
##                                                                  p.value
```

```
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium   0.015

##  Group 23B+28+W116+W16+W23+W54A+W75+W97A  #sps.  1
p.value
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Microbacteriaceae;__    0.01

##  Group 23A+23B+28+98B+W116+W42+W54A+W62+W75  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Methylophilaceae;__    0.01

##  Group 98B+W116+W16+W23+W42+W54A+W62+W75+W97A  #sps.  1
p.value
## d__Bacteria;p__Firmicutes;c__Bacilli;o__Staphylococcales;f__Staphylococcaceae;g__Staphylococcus   0.005

##  Group 23A+23B+28+98B+W16+W23+W42+W54A+W62+W75  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Devosiaceae;g__Devosia    0.01

##  Group 23A+23B+28+98B+W116+W16+W42+W54A+W62+W75+W97A  #sps.  1
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae;g__Aeromonas    0.05

## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

table(site)

## site
##  98B W102 W116  W42 W54A  W62  W75  W7A W97A
##    2    2    3    2    6    5    3    7    1

#Bd pres/abs CSF
Bd1=unlist(lapply(1:31,function(x) metadata$BdPresence[which(metadata$SampleID==substring(row.names(frog1)[x],2,nchar(row.names(frog1)[x])))]))
indval=multipatt(frog1,Bd1)
summary(indval)

##
##  Multilevel pattern analysis
##  ---------------------------
##
##  Association function: IndVal.g
```

```
##  Significance level (alpha): 0.05
##
##  Total number of species: 787
##  Selected number of species: 23
##  Number of species associated to 1 group: 23
##
##  List of species associated to each combination:
##
##  Group Negative  #sps.  23
p.value
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Hymenobacteracea
e;g__Hymenobacter                    0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__
Rikenella                          0.040
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Ferruginibacter                0.050
## d__Bacteria;p__Desulfobacterota;c__Desulfuromonadia;o__Geobacterales;f__Geobac
teraceae;g__Geobacter                0.050
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;__                               0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__env.OPS_17;
g__env.OPS_17                      0.025
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;__;__
0.045
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;__;__;__
0.015
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__uncult
ured;g__uncultured                0.020
## d__Bacteria;p__Actinobacteriota;c__Thermoleophilia;o__Solirubrobacterales;f__Soli
rubrobacteraceae;g__Conexibacter   0.020
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Microscillaceae;__
0.035
## d__Bacteria;p__Chloroflexi;c__Ktedonobacteria;o__C0119;f__C0119;g__C0119
0.015
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sp
hingomonadaceae;g__Sphingobium        0.015
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagacea
e;g__Edaphobaculum                0.015
## d__Bacteria;p__Chloroflexi;__;__;__;__
0.015
## d__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;__;
__                                 0.045
## d__Bacteria;p__Myxococcota;c__Myxococcia;o__Myxococcales;f__Myxococcaceae;
g__P3OB-42                         0.040
## d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g_
_Incertae_Sedis                    0.050
```

```
## d__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolineaceae;
g__uncultured                    0.045
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Frankiales;f__Geodermatophi
laceae;g__Blastococcus           0.050
## d__Bacteria;p__Acidobacteriota;c__Vicinamibacteria;o__Subgroup_17;f__Subgroup
_17;g__Subgroup_17               0.045
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobac
teraceae;g__Bradyrhizobium       0.045
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocace
ae;g__Trichormus_HINDAK_2001-4   0.045

## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

#Bd pres/abs PCF
Bd2=unlist(lapply(1:63,function(x) metadata$BdPresence[which(metadata$SampleID==
substring(row.names(frog2)[x],2,nchar(row.names(frog2)[x])))]))
indval=multipatt(frog2,Bd2)
summary(indval)

##
##  Multilevel pattern analysis
##  ---------------------------
##
##  Association function: IndVal.g
##  Significance level (alpha): 0.05
##
##  Total number of species: 787
##  Selected number of species: 32
##  Number of species associated to 1 group: 32
##
##  List of species associated to each combination:
##
##  Group Negative  #sps.  32
p.value
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacterales;f__Yer
siniaceae;__                     0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__Sphingobacte
riaceae;g__Pedobacter            0.050
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Gammaproteobacteria_I
ncertae_Sedis;f__Unknown_Family;g__Acidibacter   0.005
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadace
ae;g__Porphyromonas              0.040
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sp
hingomonadaceae;g__Sphingobium   0.020
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Com
amonadaceae;g__Aquabacterium     0.010
```

```
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiales_Incertae_Sedis;g__uncultured 0.035
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocaceae;g__Nostoc_PCC-73102 0.030
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__NS11-12_marine_group;g__NS11-12_marine_group 0.045
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Corynebacteriales;f__Mycobacteriaceae;g__Mycobacterium 0.025
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocaceae;g__Aphanizomenon_NIES81 0.050
## d__Bacteria;p__Armatimonadota;c__Armatimonadia;o__Armatimonadales;f__Armatimonadales;g__Armatimonadales 0.025
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Propionibacteriales;f__Nocardioidaceae;g__Nocardioides 0.050
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Cytophagales;f__Spirosomaceae;g__Spirosoma 0.045
## d__Bacteria;p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocaceae;g__Scytonema_UTEX_2349 0.005
## d__Bacteria;p__Armatimonadota;c__Fimbriimonadia;o__Fimbriimonadales;f__Fimbriimonadaceae;g__Fimbriimonadaceae 0.005
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Streptomycetales;f__Streptomycetaceae;g__Streptomyces 0.010
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Micrococcaceae;g__Micrococcus 0.005
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__R7C24;f__R7C24;g__R7C24 0.010
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Intrasporangiaceae;g__Aquipuribacter 0.010
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagaceae;g__Haoranjiania 0.035
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__A0839;g__A0839 0.045
## d__Bacteria;p__Planctomycetota;c__Planctomycetes;o__Isosphaerales;f__Isosphaeraceae;g__uncultured 0.045
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Alcaligenes 0.040
## d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Rikenella 0.035
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales;f__Micrococcaceae;g__Glutamicibacter 0.050
## d__Bacteria;p__Latescibacterota;c__Latescibacterota;o__Latescibacterota;f__Latescibacterota;g__Latescibacterota 0.050
## d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Hoeflea 0.050
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Nitrosomonadaceae;g__MND1 0.050
```

```
## d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__X
anthomonadaceae;g__Lysobacter                    0.050
## d__Bacteria;p__Actinobacteriota;c__Thermoleophilia;o__Solirubrobacterales;f__Soli
rubrobacteraceae;g__Solirubrobacter          0.050
## d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Frankiales;f__Geodermatophi
laceae;g__Modestobacter                      0.050
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

VITA

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Degrees Awarded: Bachelor of Science in General Biology minor in Biomedical Science, 2019, Liberty University

Honors and Awards: Graduate Service Appointment, Biology Department, 2020-2022, Eastern Washington University

Biology Department Mini Research Grant, Eastern Washington University Biology Department, Winter Quarters 2021 & 2022

Sigma Xi Research Grant, Sigma Xi, Scientific Research Honor Society, Grants in Aid of Research

Dean's Honors List, Liberty University School of Health Sciences, Spring Semesters 2017 & 2019

Liberty Champion Award, Liberty University, Fall Semester 2017 through Fall Semester 2019

Elizabeth High School Alumni Scholarship, Elizabeth High School Alumni Association, Spring Semester 2016

Research Experience: Graduate Research Project, Eastern Washington University, Walke Lab Fall Quarter 2020 to Spring Quarter 2021, Honeybee Gut Microbiome,

Undergraduate Research Project, Liberty University, Becker Lab, Spring Semester 2019 to Fall Semester 2019, Newt skin microbiome and *Batrachochytrium dendrobatidis.*

Work Experience: Graduate Teaching Assistant, Eastern Washington University Biology Department, 2020-2022

Veterinary Assistant, Blackhawk Veterinary Hospital, Cheney, WA, July 2021-present

Veterinary Assistant, VCA Animal Clinic of Parker, Parker, CO, January 2020-August 2020

Undergraduate Teaching Assistant, Liberty University, Lynchburg, VA, January 2019-December 2019

Conference Presentations: Dodd, K., Walke, J. (2022, July). *The interactions of Batrachochytrium dendrobatidis infection levels and frog cutaneous microbiomes in Cheney, WA, USA.* Joint Meeting of Ichthyologists and Herpetologists, Spokane, WA

Dodd, K., Walke, J. (2022, Aug.). *The in vitro and in vivo interactions of amphibian skin microbiomes and Batrachochytrium*

dendrobatidis. The first Global Amphibian and Reptile Disease Conference Knoxville, TN.

Duggins, M., Dodd, K., Becker, M. (2019 April). *Prevalence and infection intensity of fungal pathogen Batrachochytrium dendrobatidis in the red spotted eastern newt (Notophtalmus viridescens) in relation to seasonal change.* Liberty University Research Week, Lynchburg, VA.