16S rRNA SEQUENCE ANALYSIS OF SILVER CARP MICROBIAL COMMUNITY IN eDNA WATER SAMPLES

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Environmental Engineering in Civil Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

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ABSTRACT

A 14-day study was conducted in a closed system taking triplicate environmental DNA (eDNA) water samples from 150 L tanks containing set numbers of invasive Silver Carp. Analysis eDNA samples typically involves only targeting the species of interest. However, eDNA detection alone cannot undeniably confirm the presence of the species of interest and a second line of evidence would be beneficial in making this assay more robust. A specific gut microbe within the phylum *Fusobacteria* has been identified as unique to fish species and Silver Carp specifically. The ability to detect these species alongside the Silver Carp DNA would establish two lines of evidence to determine the presence of a live fish in an area of interest. A better understanding of the composition of microbial biome in the gut as it is represented in the natural environment and a tool to track its presence was needed to effectively complete this work as well as an understand of how Silver Carp biomass affects these results. The study was scheduled over the course of several days and different numbers of Silver Carp present to elucidate the impact of time on the microbe of interest. For this, DNA was extracted and analyzed through 16S rRNA sequencing on the Illumina MiSeq platform yielding 25 million reads and over 16,000 OTUs after initial clean up. Following analysis, there were multiple microbial groups, in particular 127 OTUs within the *Fusobacteria* phylum previously determined to be specialized to the gut of Silver Carp that were present in eDNA samples throughout the length of study and this was most significantly present in study tanks with the greatest amount of Silver Carp. This work confirms the potential of implementing a second target for eDNA sampling of Silver Carp through the assistance of the gut microbiome composition.

ACKNOWLEDGEMENTS

This work could not have been undertaken without the help from a plethora of individuals. Thanks to Dr. Ye Lin for his knowledge on this particular topic, as well as advice and figure help for this document. Additionally, I would like to thank the Liu Lab Group for their support and advice with my thesis research. Special thanks to the Upper Midwest Environmental Sciences Center for the years of training and guidance, particularly Dr. Jon Amberg and my mentor, S. Grace McCalla. Finally, I would like to thank all the women in science I have had to the privilege to work with over the last several years. This work was funded through USGS funds.

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CHAPTER 1: INTRODUCTION

1.1 Invasion of Bigheaded Carp in the Midwest Region

Efforts to control the spread of Bigheaded Carps and eradicate established populations in the Upper Midwest Region continue to be a high priority for multiple agencies in the United States (Asian Carp Regional Coordinating Committee, 2016). Bigheaded Carps began an invasion of US waters during the 1970s and have continued to spread to new rivers and tributaries (Koel et al., 2000).

Bigheaded carp are within the genus *Hypophthalmichthys* and include both the Bighead Carp (*Hypophthalmichthys nobilis*) and Silver Carp (*Hypophthalmichthys molitrix*) (Kolar et al., 2005). Other carps of invasive concern include the Black Carp (*Mylopharyngodon piceus*) and the Grass Carp (*Ctenopharyngodon idella*) (Chapman et al., 2013; Nico, 2005). The focus for our study here will be with Silver Carp (*Hypophthalmichthys molitrix*).

Invasion of these species can have several implications. The manner in which these fish species can behave, specifically that of Silver Carp being observed jumping out of the water at the approach of motor boats, can have a detrimental effect on water recreation (Kolar et al., 2005; Ready et al., 2018). Additionally, Silver Carps disrupt established ecological systems in the waterways they invade (Pendleton et al., 2017). While Silver Carp are planktivorous and only compete directly with other planktivores, this can additionally have radiating effects felt across the aquatic food web (DeBoer et al., 2018). There is significant concern for the movement of these carps up the Mississippi River, through its tributaries, and into many of the lakes and additional water bodies that are connected (DeGrandchamp et al., 2011; Kolar et al., 2005). This includes the Laurentian Great Lakes, a concern for both the United States and Canada (Lauber

et al., 2016). Multiple agencies across the state and federal government levels have joined forces to research, combat, and prevent further and more detrimental expansion of the Silver Carp populations (Asian Carp Regional Coordinating Committee, 2016).

1.2 eDNA as a Tool for Monitoring and Detecting

There are multiple methods currently undertaken to deter this expansion. These can include: monitoring, detection, capture, and the use of physical barriers. Some facilities have a bounty on particular invasive carp species. This gives an incentive for anglers to capture and remove these invasive species from the waterway by providing a financial award for each documented capture (Asian Carp Regional Coordinating Committee, 2016). Encouraged and increased fishing has the potential to help decrease the spread of Silver Carp as well as handicap the present populations (Tsehaye et al., 2013). Some individuals are proponents for encouraging the consumption of invasive carp. Although Silver Carp themselves are edible, most people have an aversion to consuming this particular species due to their negative association with invasive species (Varble and Secchi, 2013). Other agencies are actively researching effective physical barriers that would prevent the movement of Silver Carp into unestablished waters. These include the use of complex sound waves and carbon dioxide applied at the invasion front (Cupp et al., 2017; Murchy K. A. et al., 2017; Vetter et al., 2017). The final method mentioned, monitoring, is significant to the work described in this paper.

One of the key components to minimizing the risk of population expansion is early detection (Vander Zanden et al., 2010). Traditional monitoring of invasive fishes has been conducted through capture and observation, but Silver Carps typically avoid standard capture

techniques making these methods unreliable (Jerde et al., 2011). The use of molecular tools like environmental DNA (eDNA) have become more common in monitoring programs (Thomsen and Willerslev, 2015). eDNA was first incepted for biodiversity assessment and as a monitoring tool for aquatic invasive species in 2008 (Ficetola et al., 2008). Since this time, it has been used across a variety of aquatic systems, both marine and freshwater environments, and a variety of species from fish, reptiles, mammals, and birds (Hunter et al., 2015; Hunter et al., 2018; Larson et al., 2017; Ushio et al., 2018). The U.S. Fish and Wildlife Service has taken advantage of this method and currently has an eDNA program to monitor Silver Carps throughout the Great Lakes Basin. Typically, eDNA monitoring relies on the shedding of DNA from the species of interest through feces, slime, and excretion into the water (Barnes et al., 2014; Rees et al., 2014). Water samples are collected, and the DNA extracted is then amplified qualitatively or quantitatively through PCR or next-generation sequencing.

1.3 Molecular Methods and eDNA

Three different possible methods of PCR with eDNA samples are generally undertaken based up the end goal of the researcher (Nathan et al., 2014). All three rely on species-specific DNA primers to detect the species of interest and amplification of DNA present. Conventional PCR takes amplified DNA and observes it through staining and gel electrophoresis. This process is more qualitative, but some quantitative data can be amassed through numbers of replicates run or by undertaking a fish count survey to relate to the water samples taken (Jerde et al., 2011). Quantitative or real-time PCR uses fluorescent probes to measure copies of DNA during the amplification process. Along with basic quantification for each sample, some researchers

have also applied these data to inform on biodiversity and biomass (Lodge et al., 2012; Takahara et al., 2012). Digital PCR is a recently developed tool that has also been applied to eDNA samples. This method also uses fluorescence as an indicator but has been shown to have better detections than Real-Time PCR particularly with field samples and with the presence of inhibitors, both of which are high concerns for eDNA samples (Doi et al., 2015). Silver Carp DNA, such as we used in our study, can be targeted through species-specific primers and a PCR method mentioned above or by comparison to a validated Silver Carp's DNA sequence following next-generation sequencing (Farrington et al., 2015).

Next-generation sequencing will analyze extracted DNA and provide the DNA sequence data. Through bioinformatics analysis, this sequence can be matched to a validated sequence in a compiled database and the species related to the DNA extracted can be confirmed. This eliminates the need for species specific primer development. Through all of these methods, researchers can begin to answer questions concerning eDNA samples. A DNA sample positively determined to be Silver Carp DNA can suggest to researchers that the sampling location is inhabited by Silver Carp. Where the DNA is present, then the assumption is made that the organism is present as well. In this way, the detection of DNA in environmental samples can be a powerful tool.

1.4 Concerns with eDNA

However even with such molecular tools available, eDNA detection alone cannot undeniably confirm the presence of Silver Carp and other invasive species (Kelly, 2016). The United States Geological Survey (USGS) at the Upper Midwest Environmental Sciences Center

(UMESC) in La Crosse, Wisconsin demonstrated that DNA from Silver Carp could be detected from multiple different vectors including bird feces and carcasses (Merkes et al., 2014). These data suggest that an eDNA positive detection may not always indicate the presence of a live Silver Carp, resulting in a false positive (Goldberg et al., 2016; Song et al., 2017). All positive results noted by researchers must be thoroughly investigated before they can be documented as true positives that are indicative of a live fish or species of interest. Therefore, a second line of evidence would be beneficial in making this assay more robust.

Some eDNA monitoring efforts have created multiplexed assays. These assays use multiple primers sets to detect different sequences for a single target species or multiple targets in a single sample (Goldberg et al., 2016). To take advantage of this latter multiplexing method, there would need to be a second target that could be amplified in tandem with Silver Carp specific sequences. The University of Illinois at Urbana-Champaign (UIUC) has previously conducted research with microbial community analysis. In a comparison study with other fish species, researchers found distinct microbes within the phylum *Fusobacterium* in the intestinal tract of Silver Carp captured from rivers throughout the upper Midwest (Ye et al., 2014). The detection of this microbial DNA in together with Silver Carp DNA would minimize that risk of false positives and establish two lines of evidence to determine the presence of a live fish in an area of interest.

1.5 Understanding of Gut Microbe and Potential as Detection Target

The *Fusobacterium* species has been observed to be exclusive to the intestinal tract of Silver Carps in their native and invaded river habitats (Ye et al., 2014). In order to use this

advantageously with eDNA samples this microbe must also be released as well as persist in the water environment. If the microbe exists within the intestinal tract but is maintained, it will be undetectable in the water and have no use as a potential target for environmentally based water samples. However, if the microbe is released from the intestinal tract and exists in the water it could be a target for molecular tools. Molecular tools such as PCR primers and DNA sequencing could be successful at amplifying and detecting these species from environmental water samples. These tools would help provide additional evidence of a live fish present in a waterbody since the only source of the marker is a fish and not any of the known vectors/fomites (Ye et al., 2014). First the presence of this microbe needed to be affirmed through testing. This was studied in a controlled setting, in cultured, not captured, Silver Carps at UMESC.

1.6 Considerations for Continuation of Previous Research

As the initial research was conducted an UIUC samples were gathered concerning the gut microbiota exclusively in that of wild caught Silver Carp, this was held in consideration as the current study was conducted with Silver Carp raised in a controlled, captive setting (Ye et al., 2014). The differences between the two sources of fish could arise from diet, size of environment, or water temperatures (Piper et al., 1982). These factors could be negligible for the presence of the *Fusobacteria* group but could be confirmed by analyzing the fecal material of the fish in the current study. As with the initial study, fecal samples can be taken to determine if the same microbial environment is observed within the cultured Silver Carp. With

this confirmation, the tanks with cultured Silver carp can be used to collect water samples from to determine the potential of an eDNA primer set.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Period

Silver Carp were raised in a controlled, cultured setting to determine if the microbial environment is observed in water samples to determine the effectiveness and timeframe for eDNA monitoring. The experimentation period was divided into two sections: accumulation and decay. For the accumulation study, fish were held in a recirculating tank for 7 days with sampling each day before being removed for the decay study and sampling continuing for an additional 7 days (Figure 1).

Individually recirculating tanks were used for this study. Each of three tanks were stocked with 1 fish each, another three tanks were stocked with 5 fish, three tanks were stocked with 25 fish, and the last three tanks did not contain any fish and represented our control (Figure 1). Fish were obtained from the UMESC fish culture facility, which has extensive experience with the raising of these fish. Fish were only included if the mortality of the stock tank was less than 0.2% per day for 3 consecutive days before transport (UMESC SOP GEN 132). Fish were used regardless of gender or age, although smaller fish were specifically selected to prevent the loss of water quality as external recirculation would be stopped at the beginning of the experimental period. Fish were held in the test system for at least 6 days to allow for an acclimation of DNA shedding and to flush excess DNA shed during handling and transport (Klymus et al., 2015). The study was initiated by stopping water flow to all 12 tanks, minimizing the dilution of fecal microbes and allowing for them to accumulate within the system. Before each sampling fresh water (10% total volume) was added to each tank. This was done to help

maintain habitable water quality and oxygen levels for the duration of the accumulation study. These methods were adapted from static toxicity publications where a static system received a water renewal during the course of the study (Orvos et al., 2002; Tieman and Goodwin, 2001). After adding the fresh water, three 50 ml water samples were collected from each tank. Sampling, processing and analysis was repeated daily for 7 days. Due to a decreased circulation rate in tank 11 on the fourth day of the accumulation study, the dissolved oxygen levels were low enough such that an airstone was added to the system for the remainder of the seven days. The airstone had minimal special intrusion to the system but did provide an increased, not excessive, oxygen flow. At the conclusion of the accumulation study, 10 L of water was removed from each tank and placed into separate holding tanks. The actual presence of Silver Carp was no longer necessary to the study at this point in the process. Each day for the following 7 days, three 50 ml samples were collected from each holding tank. These water samples were processed and analyzed immediately after collection, identically to those of the accumulation study.

In addition to the samples taken during experimentation, seven Silver Carp fecal material samples were taken directly from the stock raising tanks prior to daily cleaning. Samples were chosen specifically for high quality and stored in RNAlater until extraction could commence.

2.2 DNA Extractions

DNA was extracted from samples immediately following collection. Samples were centrifuged at 5000 x g for 30 minutes separate water and water contents. The supernatant

was carefully poured off and removed, after which the resulting pellet was processed by DNA extraction. Extractions were performed in a room specific to this purpose and all surfaces were cleaned with 50% bleach before beginning. Extraction protocol and reagents were provided in the IBI gMAX Mini Genomic DNA Kit (IBI Scientific, Peosta, Iowa, USA). Deviations from the standard blood, tissue, and cultured cells protocol include: initial addition of GSB buffer (provided in kit) for transfer of pellet from original 50 mL tube to extraction tube. A negative control containing 100 µL of molecular grade water was extracted alongside each set of samples. Finals elution volumes were 100 µl. Extractions were stored at -20 °C until analysis by PCR.

2.3 PCR

Amplification of DNA for sequencing was accomplished through PCR. To allow for equal sequencing of each sample, samples were analyzed with a Nanodrop spectrophotometer and diluted with molecular grade water to a standard concentration of 15 ng before addition into the PCR reaction mix. Total volume of PCR reaction mix of 25 µl was used for each sample. This included: 1µl forward primer, 1 µl reverse primer, 12.5 µl 2x Taq and mastermix, 5 µl of diluted DNA, and 5.5 µl of molecular grade water. A unique combination of forward and reverse primers were used for each sample as primers also contained a distinctive barcode that would be used to differentiate the samples after sequencing was achieved. Primers targeted the V4 and V5 region of the 16s rRNA gene (Kozich et al., 2013). To avoid the risk of contamination, PCR was performed in a lab designated PCR hood and the hood space was thoroughly cleaned before beginning. Samples were amplified using a BioRad T100 thermocycler with the following

PCR program: 94°C for 3 m, then 25 cycles of 94 °C for 30s, 55 °C for 45s, and 72 °C for 1 m, with a final temp of 72 °C for 10 m. The resulting amplicon length was 400 base pairs (bp). After amplification by PCR, samples were run on 1% agarose gels with a 100 bp ladder to visualize success and strength of amplification. For each sample, gels were viewed with UV light and the amplicon region was excised from the gel, avoiding additionally cutting out any other nonspecific banding within the gel. The amplified DNA was extracted from the gel using a gel purification kit to remove impurities and undesirable product. After purification, samples were measured on the Qubit machine and samples were pooled and prepared for sequencing.

2.4 Sequencing and Sequence Processing

Sequencing was performed by the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. The pooled 2.87 ng/µl sample was submitted and run on the Illumina MiSeq v3 sequencing platform. Following the completion of sequencing, quality checks and development of contigs were performed initially through Mothur pipelines, followed by removal of added barcode sequences from the bacterial sequences, and finally QIIME pipelines for demultiplexing and quality checks (Caporaso et al., 2010; Schloss et al., 2009). Operational Taxonomic Units (OTU) picking was performed with QIIME and taxonomy assigned to OTUs using both Silva and Greengenes (at 97% species similarity) databases to ensure all potential *Fusobacteria* OTUs were included (DeSantis et al., 2006; Quast et al., 2013). These picked OTUs were then used for all downstream analysis performed by a variety of programs.

2.5 Analysis

Analysis was performed using the relative abundance of OTUs found by taking the amount observed for each OTU and the total number of sequences from the MiSeq platform. These values where then used with QIIME and R statistical programs to develop figures (Caporaso et al., 2010). Alpha-Diversity of all OTUs was analyzed through both Chao1 and Observed_OTUs methodology performed using a QIIME established workflow. QIIME was additionally used to develop taxonomy composition plots and perform Bray-Curtis statistical analysis and develop a Principal Coordinate Analysis plot (PCoA). R statistical program was used to develop the non-metric multidimensional scaling plots (NMDS), to develop figures based on the abundance data and finally to produce heatmaps to express relative abundance of OTUs. To secure a higher resolution of taxonomic assignment, all samples that had been compared to databases and assigned as phylum *Fusobacteria* were also compared to a phylogenetic tree constructed through the ARB Project from the previous Silver Carp gut microbiota study (Ludwig et al., 2004; Ye et al., 2014).

CHAPTER 3: RESULTS

3.1 Sequencing Output

Sequencing with the MiSeq platform returned just over 25 million total reads. After cleanup, filtering, building contigs through a Mothur pipeline followed by removal of barcodes, removal of chimeric sequences, and OTU picking through the QIIME workflow, this resulted in over 16,000 OTUs for further analysis (Caporaso et al., 2010; Schloss et al., 2009). All samples were visualized through a PCoA plot to observe similarity across samples (Figure 14).

OTUs were analyzed initially through alpha diversity. This was done through QIIME with both Chao1 (Figure 2) analysis for species richness and Observed OTUs (Figure 3) analysis for number of OTUs for each sample. Chao1 showed the richness or variety of species present in each sample, this was averaged across fish number (Control, 1 Fish, 5 Fish, and 25 Fish) and reported for each day of the study. Species richness both decreased by Day 14 of the study as well as was more similar across the different amounts of fish present (Figure 2). Observed OTUs gives the total OTU count for each sample. This was also averaged across fish number and reported for each day of the study as with chao1. Similar to the species richness analysis, the number of observed OTUs both decreased by Day 14 of the study as well as was more similar across the different amounts of fish present (Figure 3).

To ensure complete identification of the species of interest for this study both Greengenes and Silva databases (at 97% sequence similarity) were used in analysis (DeSantis et al., 2006; Quast et al., 2013). From this, a total of 127 OTUs were identified through both Greengenes and Silva database comparisons as *Fusobacteria*. This output was then used for downstream analysis as well as a basis for comparison.

3.2 Taxonomy Analysis of all Study Samples

QIIME analysis provided taxonomy bar graphs based on percentage the desired taxonomic level occupies the whole composition of OTUs. At the phylum level (Figure 4), the water samples were dominated by *Proteobacteria*. *Proteobacteria* comprised 81.78% of Day 1 samples, 87.37% of Day 3 samples, 52.37% of Day 7 samples, 62.70% for Day 9, and 49.65% for Day 14. The fecal samples were comprised of 55.97% from phylum *Proteobacteria*. One of the other major phylum represented was *Bacteriodetes*. *Bacteriodetes* comprised 3.56% of Day 1 samples, 5.93% of Day 3 samples, 17.57% of Day 7 samples, 21.66% for Day 9, and 26.46% for Day 14. The fecal samples were comprised of 2.50% from phylum *Bacteriodetes*. The final phylum of interest, *Fusobacteria* was significantly represented in the fecal samples at 40.36%. It was to a lesser degree, but present, within water samples. The phylum *Fusobacteria* comprised 1.4% of Day 1 samples, 0.4% of Day 3 samples, 0.4% of Day 7 samples, 0.3% of Day 9 samples, and finally 0% of Day 14 samples.

At the phylogenetic order level (Figure 5), water samples were composed significantly by *Burkholderiales* of class *Betaproteobactera* within phylum *Proteobacteria*. 22.08% of Day 1 samples were comprised by order *Burkholderiales*, 43.98% of Day 3 samples, 20.59% of Day 7 samples, 32.16% of Day 9 samples, and 17.18% of Day 14 samples. Fecal samples were only comprised 0.78% by order *Burkholderiales*. Fecal samples were more comprised by order *Aeromonadales* from class *Gammaproteobacteria* within phylum *Proteobacteria* at 39.45%. Order *Fusobacteriales* was still very present within the fecal samples at 40.36%. *Fusobacteriales* was also present within the water samples at 1.41% within Day 1 samples, 0.43% of Day 3

samples, 0.45% of Day 7 samples, 0.26% of Day 9 samples, and comprised 0% of Day 14 samples.

At the family level of phylogeny (Figure 6), one family groups from the phylum *Proteobacteria* is dominant across all the water samples and one is strongly present across some of the water samples. All of the water samples have a high percentage of OTUs from the family *Comamonadaceae*, with 14.25% in Day 1 samples, 37.51% in Day 3 samples, 18.0% in Day 7 samples, 30.56% in Day 9 samples, and 16.33% in Day 14 samples. *Comamonadaceae* was present at only 0.52% in the fecal samples. If family *Comamonadaceae* did not comprise the largest percentage within the water samples, then for some water samples the largest percentage was from family *Chromatiaceae*. This family was present at 27.05 % for Day 1 samples, 27.47% for Day 3 samples, 5.5% for Day 7 samples, 3.83% for Day 9 Samples, and 1.15% for Day 14 samples. This family group only comprised 0.96% for the fecal samples. For the family of peak interest*, Fusobacteriaceae* from the *Fusobacteria* phylum, this was only present at 40.33% within the fecal samples and in the water samples at 1.05% of Day 1 samples, 0.37% of Day 3 samples, 0.3% of Day 7 samples, 0.18% of Day 9 samples, and 0% of Day 14 samples.

3.3 Sample Similarity through Coordinate Plots

Non-metric multidimensional scaling (NMDS) plots used relative abundance of OTUs within samples to show relationships between the *Fusobacteria* community of water samples collected over 14 days (Figure 7 and 8). Following this analysis, one of the triplicate samples from each day 9 and 14 were established as outliers and removed from NMDS plot. Values on

the axis are negligible but spatial relationship between points of significance and should be used to study this figure. Points that are closer together on the plot are more similar than those located further apart. Groups of points represent higher similarity. Figure 7 shows the relationship between the fecal samples in comparison to all the water samples taken throughout the study. Close grouping of all fecal samples separate from water samples shows similarity between samples taken from the same source i.e., samples of water origin versus fecal origin. Significant points include the grouping of Day 1 and 3 samples at the beginning of study, grouping of Day 7 and 9 samples even though the sampling occurred with fish present for Day 7 and fish removed by Day 9. Day 9 and Day 14 samples were both taken during the degradation portion of the study. However, Day 9's samples similarity to Day 7 shows that Day 14 samples were more significantly impacted by degradation. *Fusobacteria* can still be observed in abundance on Day 9 regardless of the source being removed. Tanks with 25 fish samples are most similar to each other as the greatest number of *Fusobacteria* has accumulated in these samples regardless of day (Figure 8).

3.4 *Fusobacteria* OTU presence through ARB tree

All OTUs that were designated by taxonomy assignment within the phylum *Fusobacteria* were then compared to an ARB developed tree to pinpoint further taxonomic assignment to strain level (Figure 9-Provided by Ye Lin) (Ludwig et al., 2004). The Silva database was used to develop the ARB phylogenetic tree (Quast et al., 2013). With this tree, two groups within the phylum *Fusobacteria* were identified, *Cetobacterium* and Hados.Sed.Eubac.3. Within each of these, two sub-clusters were identified in each. Within *Cetobacterium* are the sub-cluster

groups Ceto-1 and Ceto-2. Within Hados.Sed.Eubac.3. are the sub-cluster groups Hados-1 and Hados-2. These relationships are expressed in Figure 9. This phylogenetic tree allowed for sequence data generated within this study to be applied to the tree based on sequence similarity and then assigned more precise taxonomy than was able to be elucidated through the QIIME pipeline. The publication associated with this particular ARB tree is currently in preparation. There were 127 *Fusobacteria* OTUs before additional analysis. Most of these species fell within the family *Fusobacteriaceae* (48 OTUs), however *Leptotrichia* (36 OTUs) falls within the family *Leptotrichiaceae*. After ARB analysis, these 127 OTUs were broken down as follows: 30 OTUs defined as Ceto-1, 11 OTUs Ceto-2, 6 OTUs *Fusobacterium*-1, 2 OTUs *Fusobacterium*-2, 20 OTUs Hados-1, 3 OTUs Hados-2, 2 OTUs *Leptotrichia*-1, 8 OTUs *Leptotrichia*-2, and 26 OTUs defined as *Leptotrichia*-3 species. A few OTUs were of unknown species even after ARB analysis or only a single OTU was defined to a specific species such as *Psychilyobacter* but these species were not of great significance to the completed study.

3.5 Relative Abundance of Select Species

A heat-map was developed for the detected 127 *Fusobacteria* OTUs determined through OTU picking. The significant OTUs were assigned to one of 9 taxonomic groups and the heat map was developed using relative abundance for OTUs in each sample taken across the study. The relative abundances were higher for some species within the fecal samples than the water samples, as could be seen through the taxonomy bar graphs (Figures 4-6). In general, fecal samples would be expected to have higher concentrations of fecal microbes as fecal microbes in water samples have been diluted when added to the environment. Of these 9

taxonomic groups, two are of specific interest because of their unique appearance in Silver Carp (Ye et al. unpublished). These are Ceto-1, Ceto-2 and Hados-1, Hados-2, sub-cluster groups within the *Fusobacteria* group (Ye et al., 2014; Ye et al. unpublished). Fecal samples 1-7 are shown to have very abundant amounts of both Ceto and Hados sub-clusters (Figure 10). As stated above, species abundant in the fecal samples were also present within the water samples taken during the experimental period, in lower abundance.

For both Ceto-1 and Ceto-2, sub-cluster groups the highest relative abundance was observed within fecal samples, specifically at a relative abundance of 0.0002787 Ceto-1 in Fecal Sample 3 and a relative abundance of 0.5412 Ceto-2 also in Fecal Sample 3. These species were still observed in the water samples, at a lower abundance. Ceto-1 was present in Day 3 sample from a tank with 25 fish at 8.23315×10^{-5} and Ceto2 was present in Day 7 sample from a tank with 25 fish at 0.0264.

For both Hados-1 and Hados-2 sub-cluster groups, the highest relative abundance was also observed within the fecal samples. For Hados-1 this was observed in Fecal Sample 6 at a relative abundance of 0.0008 and for Hados-2 this was observed in Fecal Sample 3 at a relative abundance of 0.025. For the water samples, Hados-1 was abundant in the late stages of the accumulation study on Day 7 sampled from a tank with 25 fish at a relative abundance of 7.3×10^{-6} and in the early stages of the decay study on Day 9 sampled from a tank with 25 fish at a relative abundance of 1.3×10^{-5}. Hados-2 was abundant alternatively in early and late stages of the accumulation study. In Day 1 sample from a tank with 1 fish Hados-2 had a relative abundance of 0.002 and in Day 7 sample from a tank with 25 fish this species had a relative abundance of 0.0011.

Leptotrichia was notably in low abundance in the fecal samples. The fecal sample with the highest relative abundance of *Leptotrichia* species was only at 0.0012 and was present within analyzed water samples. Two Day 1 water samples were abundant with *Leptotrichia* species. Day 1 sample from a tank with 5 fish had a relative abundance of 0.034 and a Day 1 sample from a tank with 25 fish with a relative abundance of 0.028. A Day 3 water sample was taken from a tank with 25 fish and had a relative abundance of 0.019. *Leptotrichia* was more abundant within the first part of the accumulation study.

Greatest abundance within water samples is observed in samples taken from the tanks housing 25 fish at the end of the accumulation study. Detections are significantly decreased after the fish were removed from the tanks during the decay study.

3.6 Accumulation and Degradation of *Cetobacterium* and *Leptotrichia*

The log transformed relative abundance was plotted for all *Fusobacteria* OTUs determined by UCLUST and BLAST analysis (Figure 11). This was analyzed across the days of the study and by the number of fish in each tank. To look closer at groups within phylum *Fusobacteria*, UCLUST aligned relative abundance of water samples were plotted by species of interest (Figures 12 and 13). Here, *Cetobacterium* represents the species in entirety and the sub-cluster groups of interest Ceto-1 and Ceto-2 are included with all other *Cetobacterium.* Ceto-1 and Ceto-2 were specifically observed to be unique to the gut in Silver Carp (Ye et al., unpublished). These microbes were then also observed in the surrounding water environment through our sampling. Additionally, the abundance of this microbe is different as the fish were in the system longer as well as different relative abundance observed dependent on how many

fish were in the system. A second microbe was observed to behave in a different manner. *Leptotrichia* is a Silver Carp skin-associated microbe. It is observed in the environmental water samples but is not observed within the fecal samples. The relative abundance of this microbe also changed as the study progressed, starting at an initially higher level and decreasing across the experimental period.

CHAPTER 4: DISCUSSION

4.1 Fecal Matter as Environmental Indicator

The study of fecal contamination of pollution has been used significantly by agencies as an indicator (Zhang et al., 2015). Often researchers use it as an indicator of polluted or unsafe water environments (O'Mullan et al., 2017). Thus, it is common for fecal material to be a large component of interest and detailed research, as it was in the precursor to this study (Ye et al. 2014). Other studies have gone to show that fecal material can be unique to the species or even the location can have an impact on the composition of the species of focus (Eichmiller et al., 2016; Roeselers et al., 2011; Sullam Karen E. et al., 2012). It was important for this work to establish that previous findings would apply to this work (Ye et al., 2014). This study has shown that for this particular unique species of *Fusobacteria* within Silver Carp fecal material was conserved based between two different environments that the fish originated, the wild-caught river environment in previous work and the captive raised Silver Carp from UMESC.

4.2 Targeted Molecular Approaches

In additional to using fecal material as a presence or absence indicator for water environments, multiple studies have molecularly analyzed this source for the potential of molecular tools usage (Kreader, 1995; Pegard et al., 2009). These tools are of particular use in helping distinguish one source from another (Bernhard and Field, 2000). Having a reliable method that can affirmatively pinpoint a source species is essential to eDNA monitoring efforts. Fecal DNA has been continually described as a strong contributor to the DNA found in eDNA samples (Klymus et al., 2015; Rees et al., 2014). However, the use of unique and particular

microbiome species as a potential detection point for eDNA samples had yet to be examined until this study.

4.3 Combining Microbiome Analysis with eDNA Monitoring

There have been numerous studies that have delved into and studied the gut microbiome of fishes, particularly those that have invaded waters, in the hopes that a better understanding will improve removal efforts (Eichmiller et al., 2016; Llewellyn et al., 2014). Molecular work has been done to detect the original of microbiome components (Wu et al., 2012). But our work here looked at the fate, rather than origin, of gut microbes, as present in the water environment. Research into invasive species microbiome, has often been undertaken tangentially with the study of native fishes' microbiomes (Ye et al., 2014). This was important to establish the species-specific nature that is particular to eDNA sampling. Understanding the gut microbiome before proceeding with the work done in this study and in future work was significant in understanding potential vectors for the *Fusobacteria* species.

4.4 *Leptotrichia* as a Possible New Target

The observance of *Leptotrichia* species essentially exclusively in water samples and not fecal samples in this study as well as its absence in previous work on Silver Carp fecal matter suggests this microbe is not associated with fecal material and potentially is related to skin/scale-needs additional studies focused on *Leptotrichia* (Ye et al., 2014). This species could have been particularly abundant at the initiation of the study due to the stress on the fish from the change in its environment, specifically in water flow (Klymus et al., 2015). As with fecal

material, skin and scale sloughing is considered a contributor to eDNA samples (Barnes and Turner, 2016; Thomsen and Willerslev, 2015). This could be further studied through swabbed samples of Silver Carp as well as compared to other fish species to see if this species could be another potential marker for Silver Carp eDNA samples.

4.5 Further Studies and Endeavors

Following this study, lab testing primer sets with specific *Fusobacteria* sequences, particularly within the sequences of Ceto-1 and Ceto-2 as well as Hados-1 and Hados-2 sub-cluster groups should begin. On the completion and success of this, the method should be applied and explored in natural waters systems. It is important to confirm that lab results will be mimicked in field-based samples. With field samples and particularly with eDNA samples, there is the high chance of inhibition within samples and steps will need to be taken to clean up these samples (McKee et al., 2015). Field tests could determine the persistence of the detectible DNA in the environment. This could help to associate detections with time of fish presence, within a small but crucial window of time, possibly for sightings in new locations or on the invasion front. Additionally, these primer sets would need to be tested with desired Silver Carp markers, to ensure these could indeed be used as a multiplexed assay to analyze the same water samples accurately (Goldberg et al., 2016).

Along with traditional PCR primer development, there is the potential to develop more advanced primers that would lead to great ease of use and faster detection. LAMP assays can be used to quickly analyze field samples will circumventing some of the standard steps of PCR and could be adapted for use in monitoring efforts (Fu et al., 2011; Gallas-Lindemann et al.,

2013). These assays can provide fast preliminary information about a system. This could be employed with both Silver Carp and *Fusobacteria* markers.

4.6 Conclusions

This study supports the viability of specific species of *Fusobacteria* to be used in environmental DNA monitoring. Species that were both present in the fecal samples and within the eDNA water samples. The use of markers designed to target *Fusobacteria* would help eliminate the false positives in eDNA monitoring programs. It was crucial in this work to build upon the knowledge of *Fusobacteria* present in the gut of Silver Carp and to determine if its fate was within the water system we developed at UMESC. Having study tanks containing different numbers of Silver Carp demonstrated that the number of fish present have an impact on the detection of *Fusobacteria*. This study showed that detections of *Fusobacteria* were strongest within study tanks with greatest amount of Silver Carp present. This should knowledge should be applied to eDNA monitoring sampling efforts. Positive detections with this marker in tandem with positive detections of Silver Carp will ascertain the presence of a Silver Carp with a stronger level of confidence than a detection of Silver Carp alone. As a whole, this will be supportive to the accuracy of multiple monitoring efforts conducted by a variety of both federal and state agencies and serve to enhance the improvement of our natural waters and ecosystems.

FIGURES

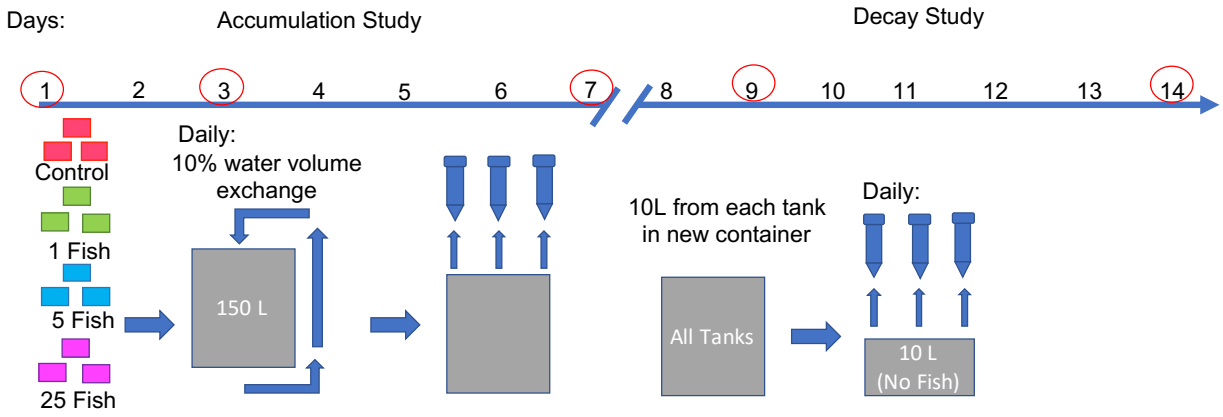

Figure 1. Schematic of sampling process for the accumulation study, days 1-7, and decay study, days 8-14. Circled days were sample days used within this analysis.

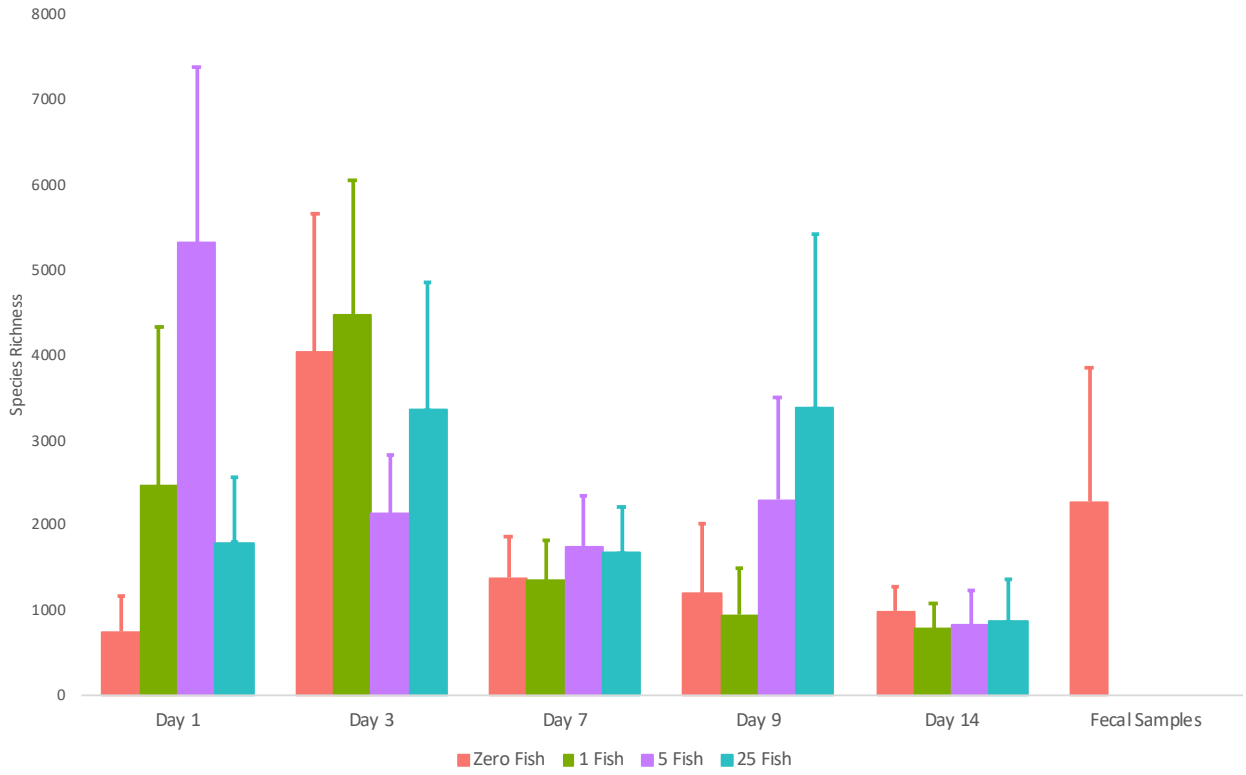

Figure 2. Alpha Diversity with chao1 for species richness each day of the study, with samples grouped and averaged base on the amount of fish present in the system of sampling.

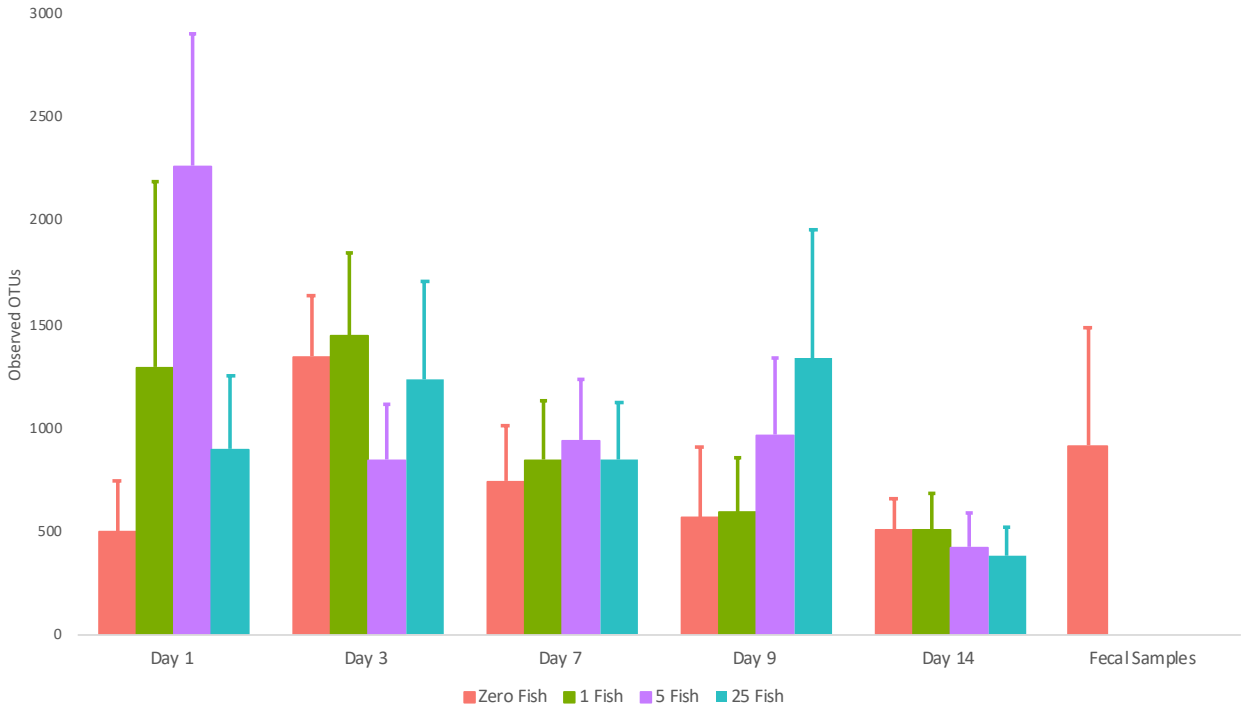

Figure 3. Alpha Diversity with observed OTUs analysis for the number of OTUs in each day of the study, with samples grouped and averaged base on the amount of fish present in the system of sampling.

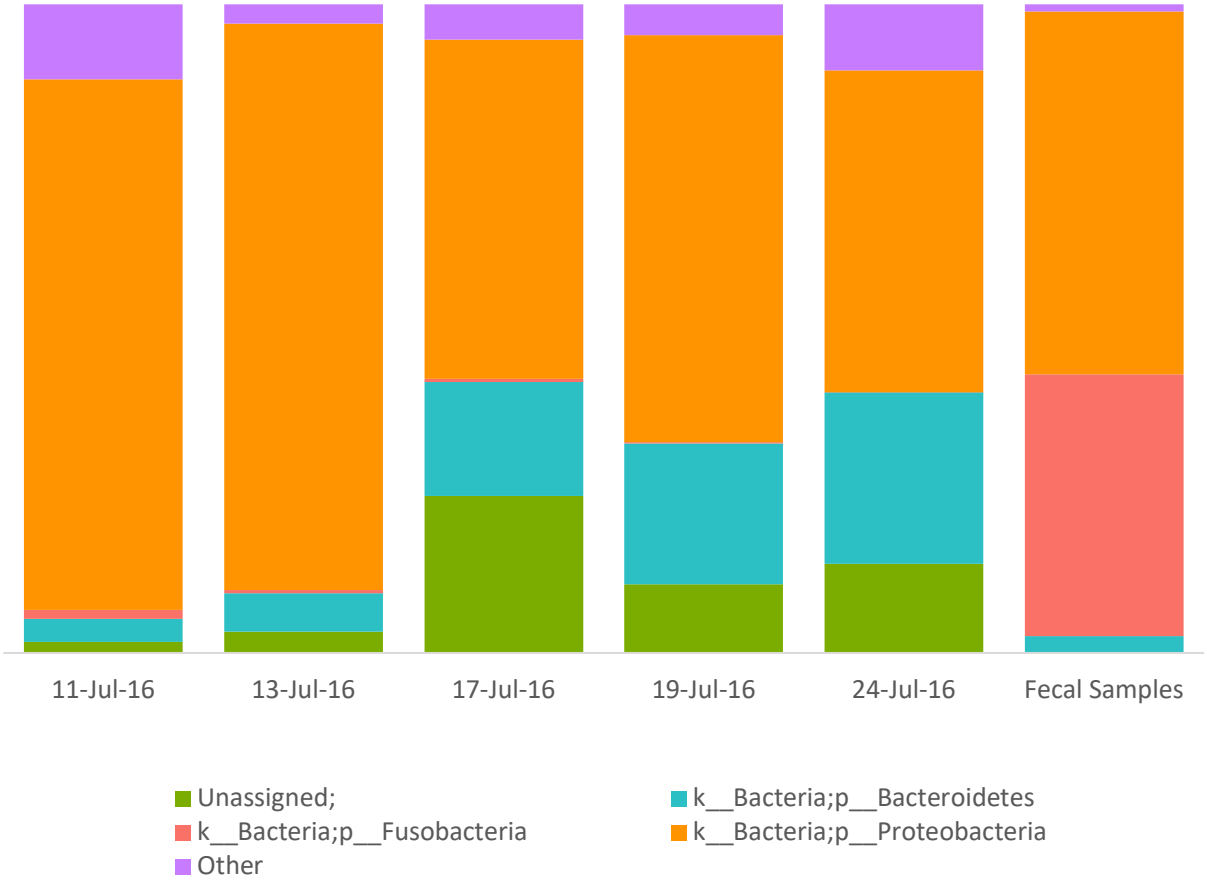

Figure 4. QIIME output generated bar graph of experimental days assigned by taxonomic phylum.

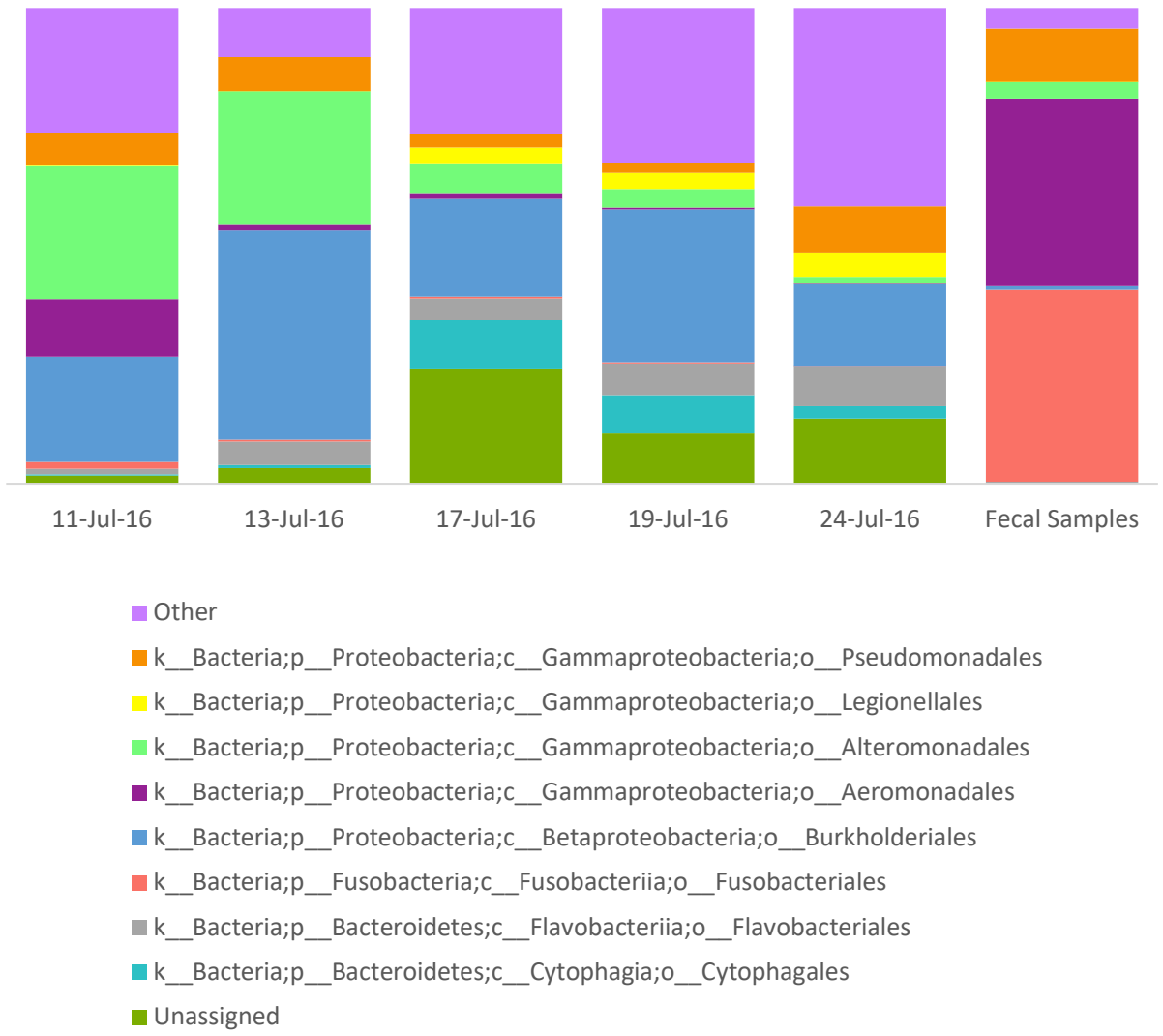

Figure 5. QIIME output generated bar graph of experimental days assigned by taxonomic order.

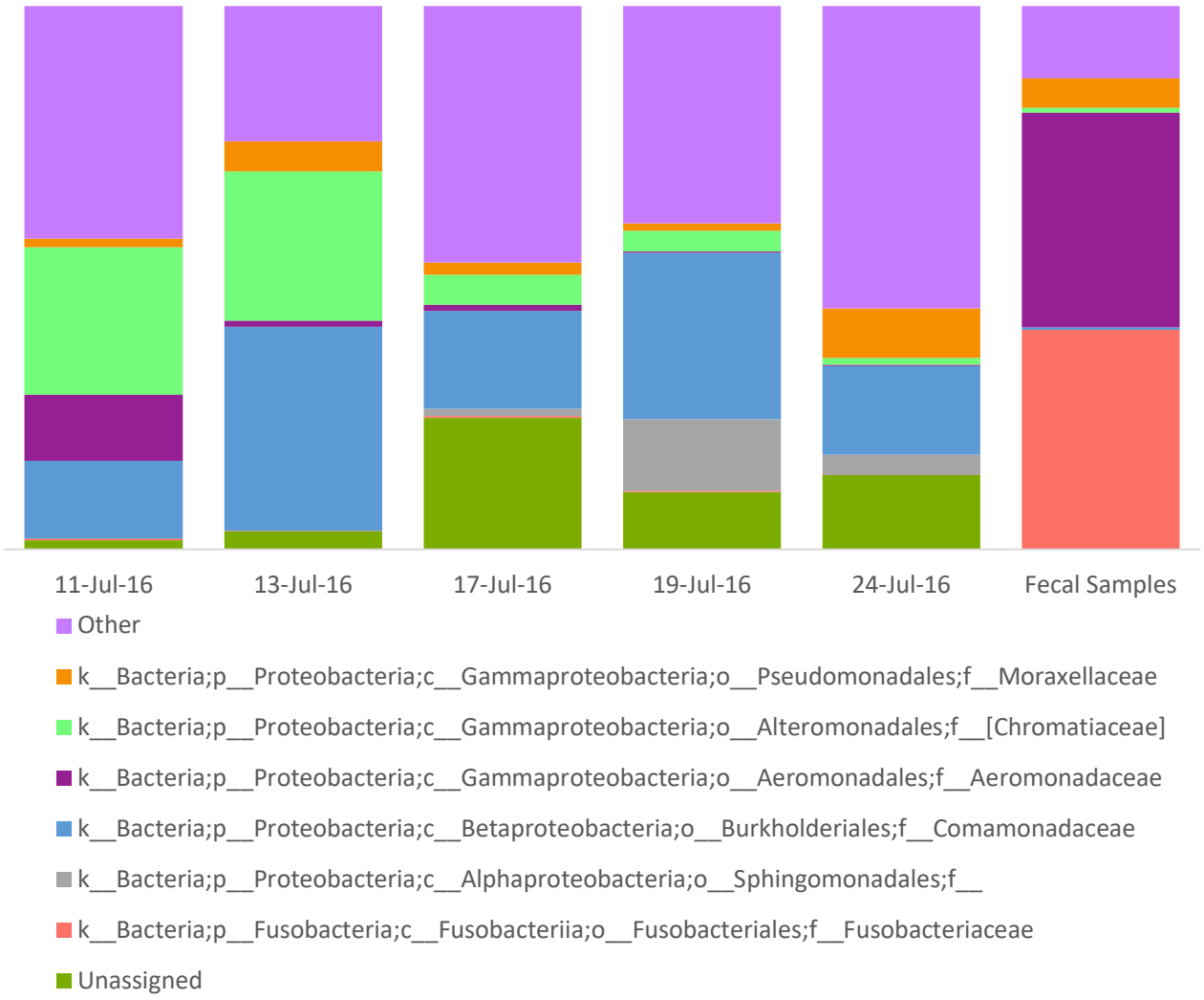

Figure 6. QIIME output generated bar graph of experimental days assigned by taxonomic family.

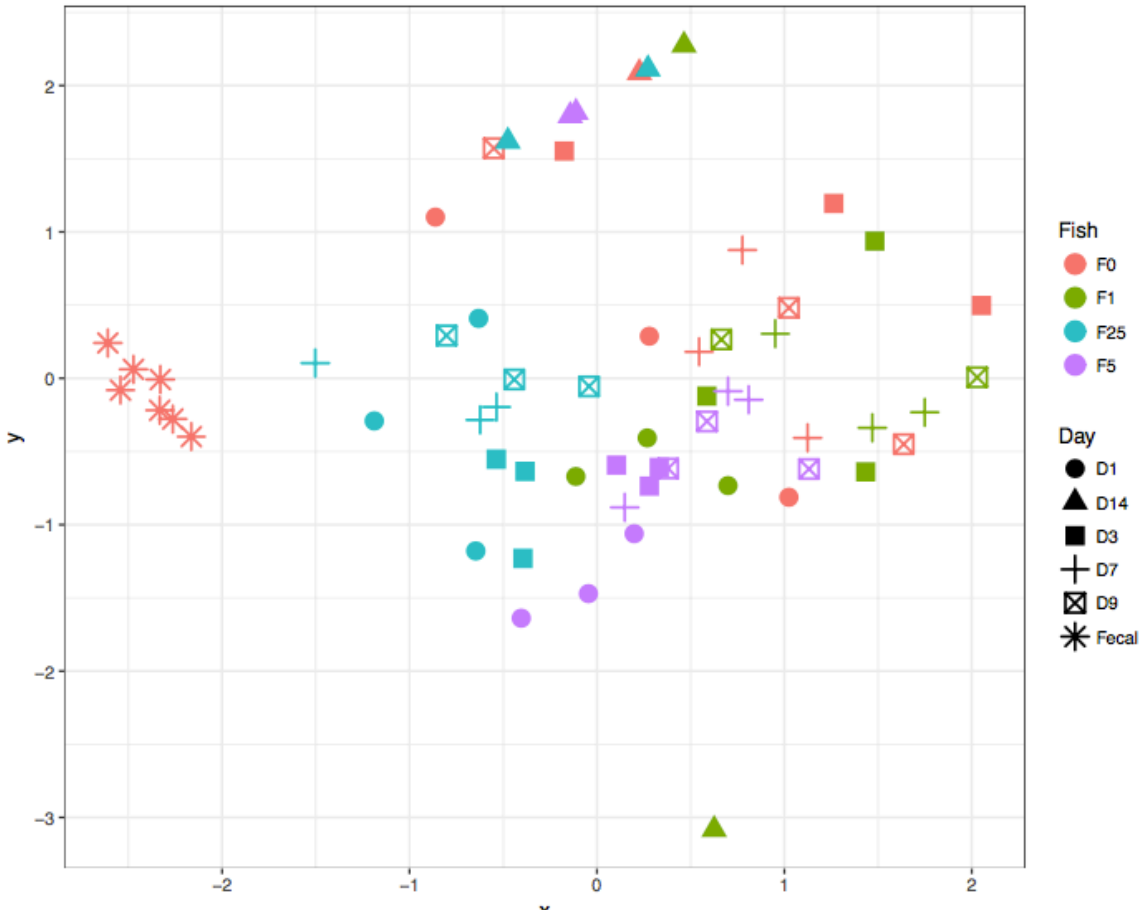

Figure 7. NMDS Plot for 127 *Fusobacteria* OTUs of fecal and water samples taken throughout the accumulation and decay study. Color of plotted point denotes number of fish in the tank system and the shape of the point represents the day the sample was taken. Fecal samples represented by a unique shape, but not unique color.

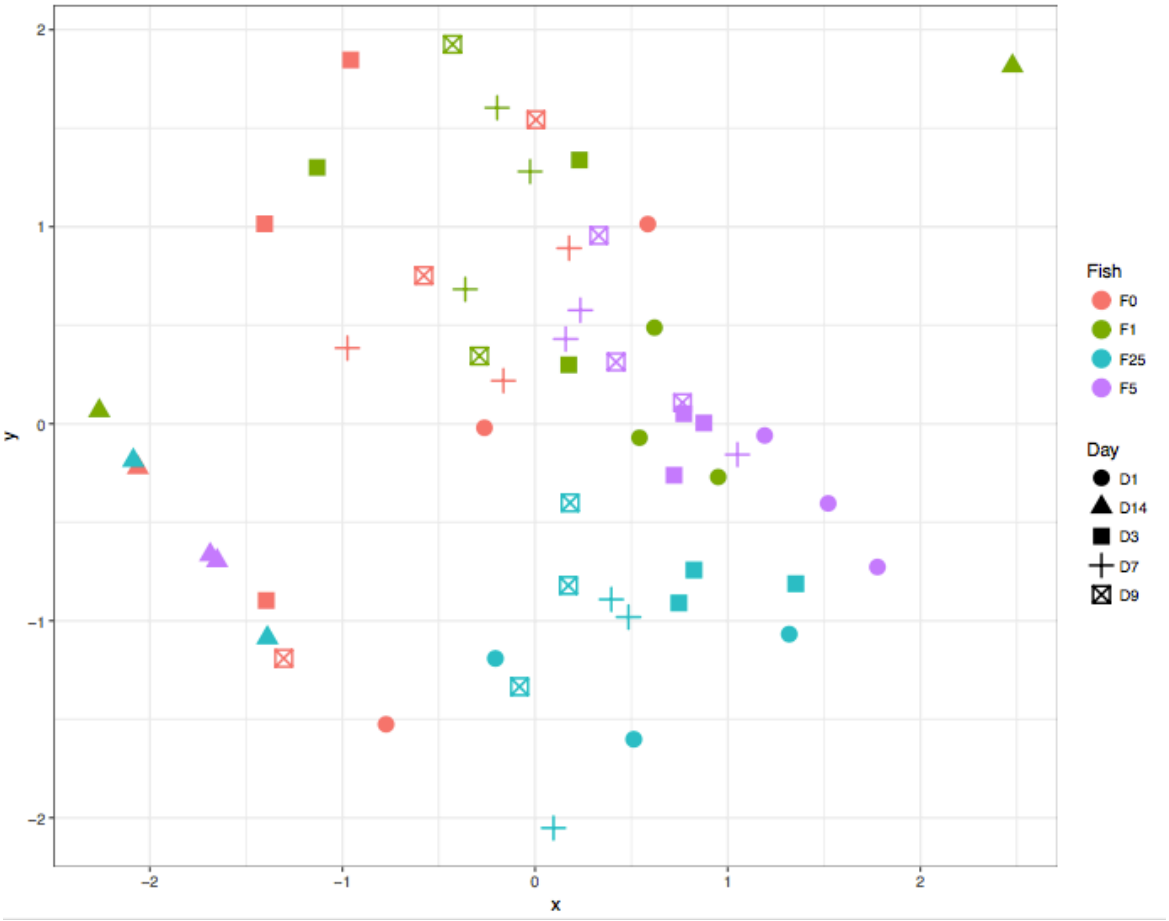

Figure 8. NMDS Plot for the 127 *Fusobacteria* OTUs of only water samples taken throughout the accumulation and decay study. Color of plotted point denotes number of fish in the tank system and the shape of the point represents the day the sample was taken.

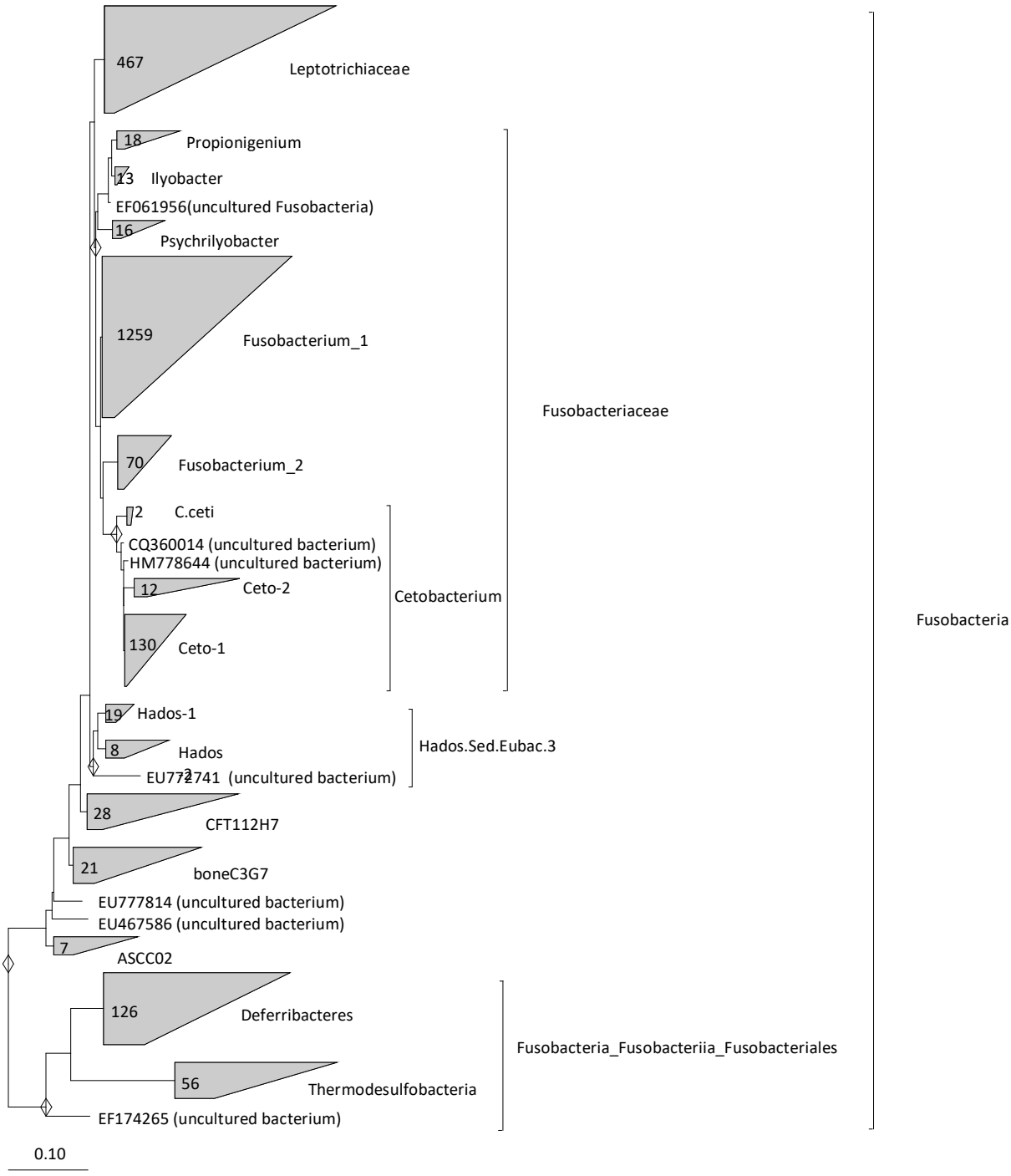

Figure 9. ARB based phylogenetic tree for phylum *Fusobacteria* developed and provided by Ye Lin.

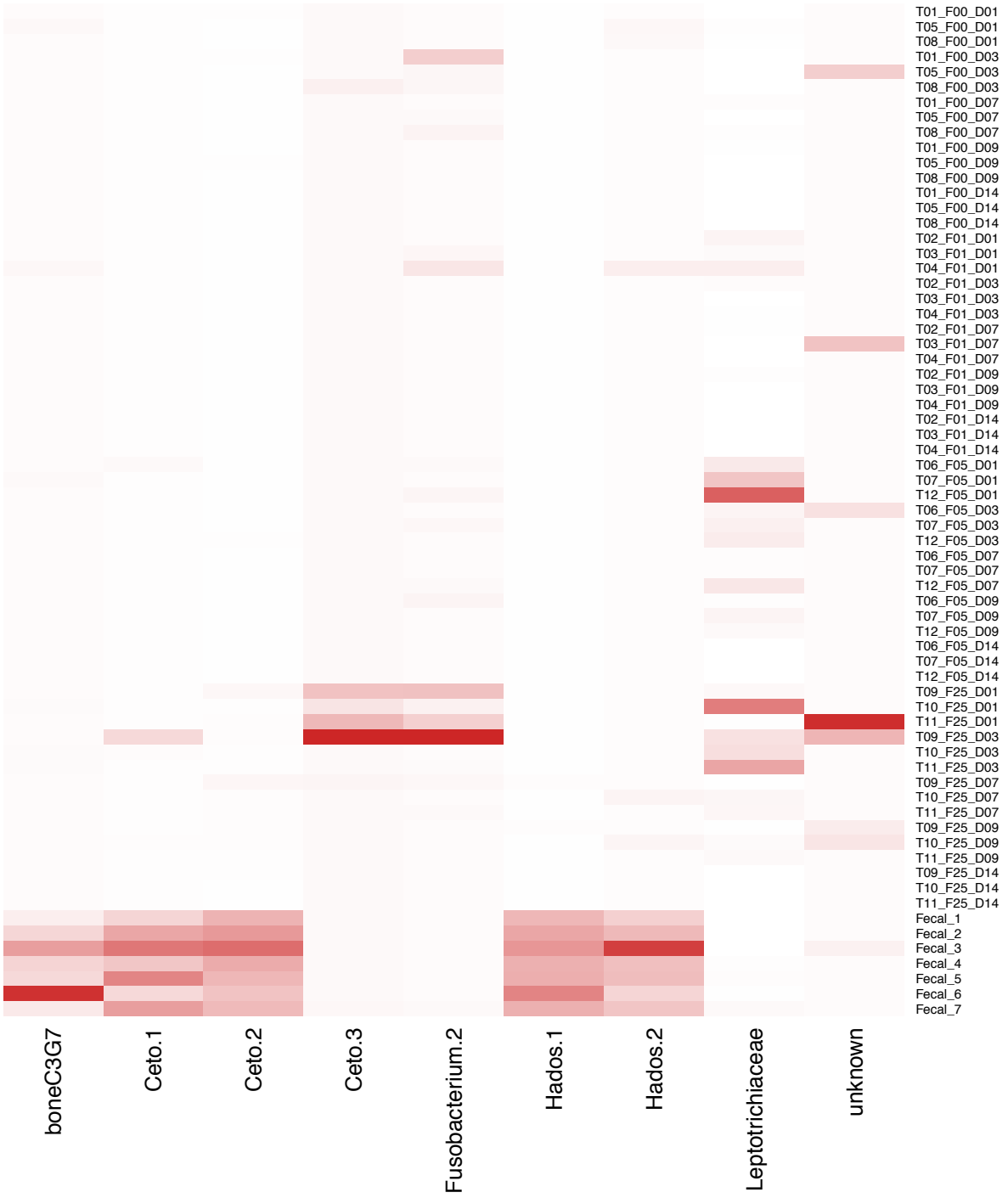

Figure 10. Heat map data based on relative abundance of *Fusobacteria* species along the x-axis. Color gradient represents abundance of species in comparison to the total.

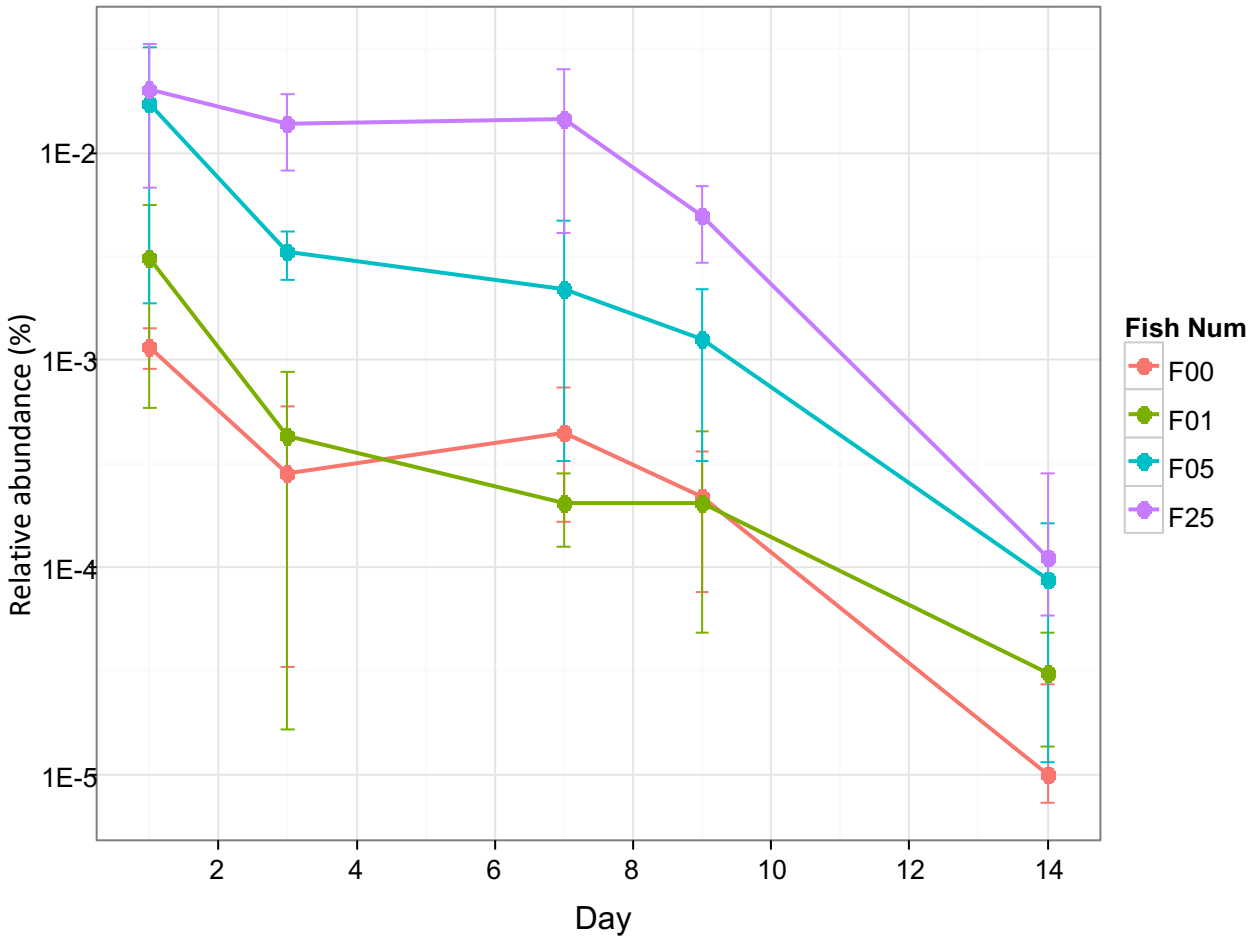

Figure 11. Changes of log transformed relative abundances of total *Fusobacteria* in water samples over time.

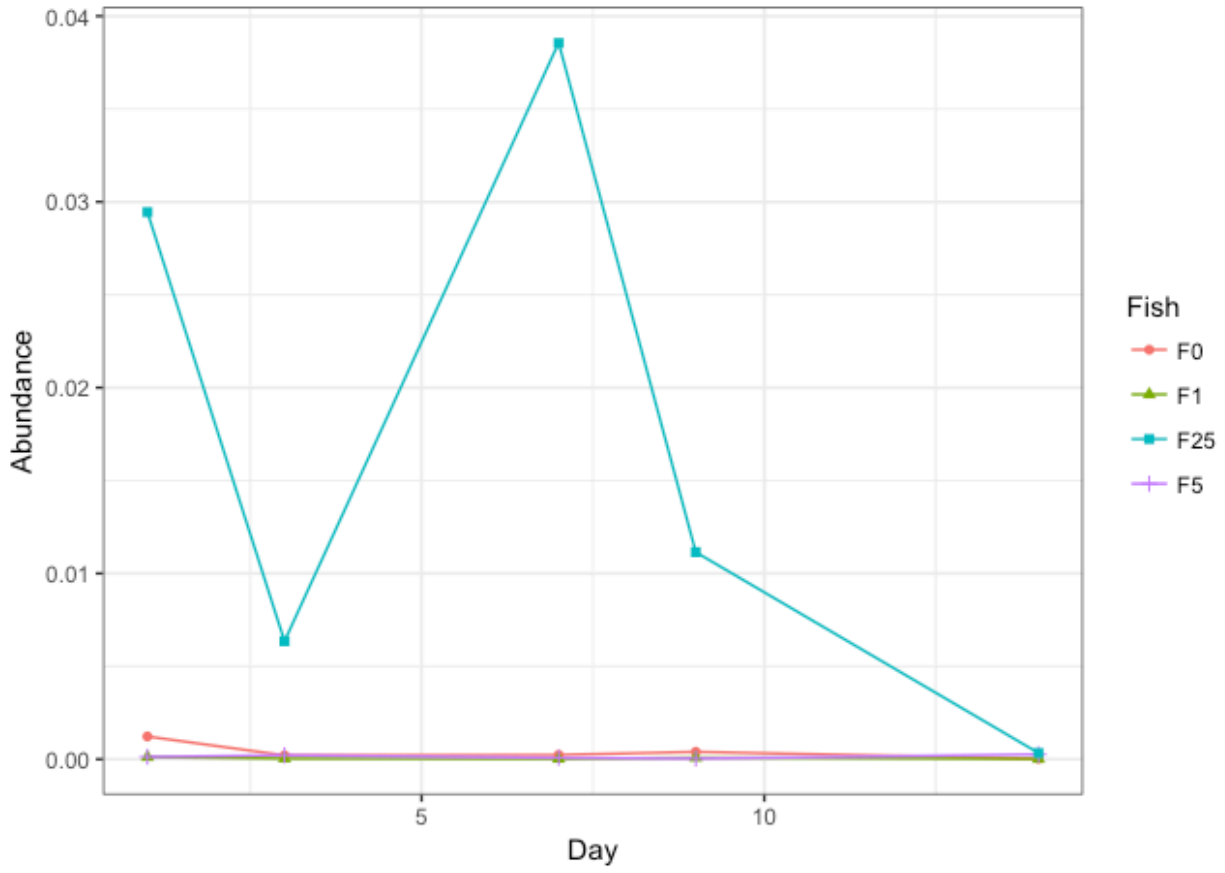

Figure 12. Changes of relative abundance of *Cetobacterium* species in water samples of time.

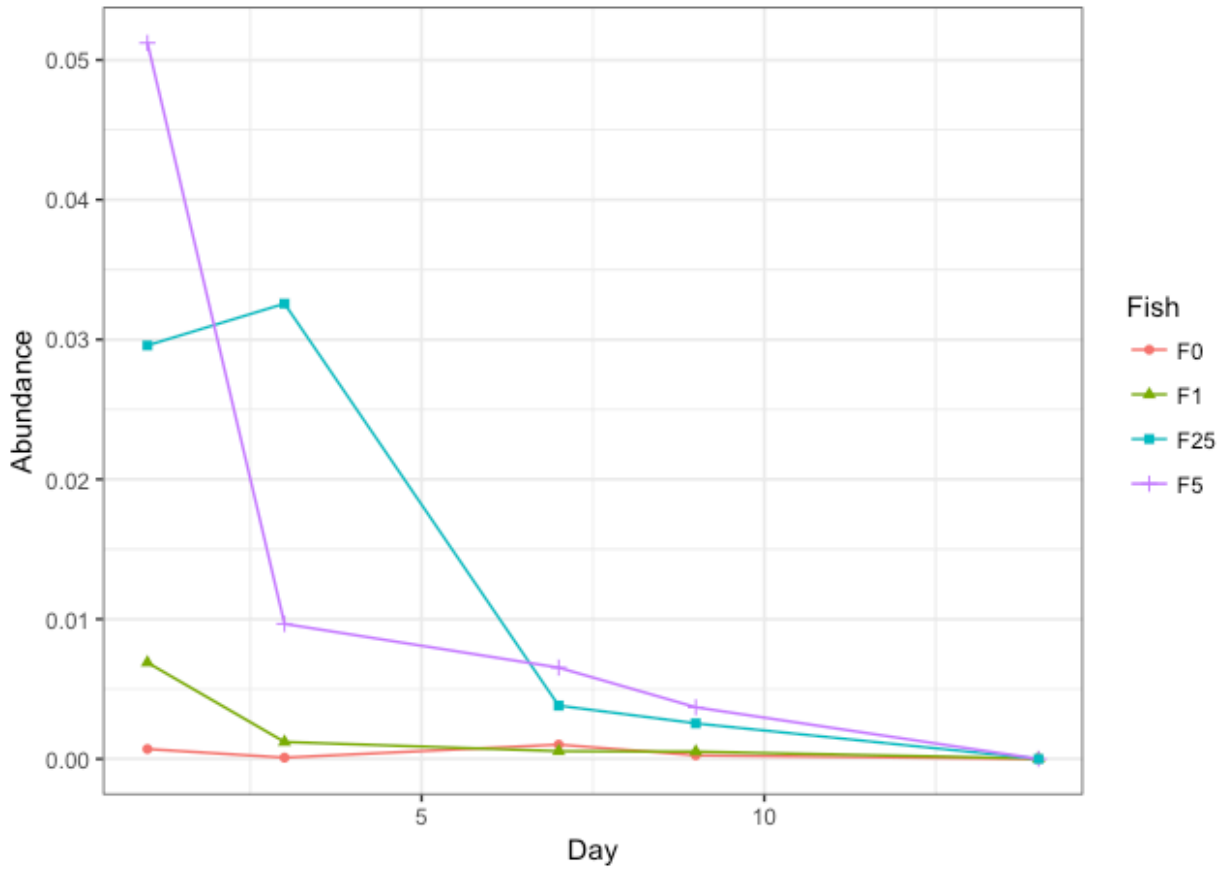

Figure 13. Changes of relative abundance of *Leptotrichia* species in water samples of time.

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APPENDIX A: SUPPLEMENTARY MATERIALS

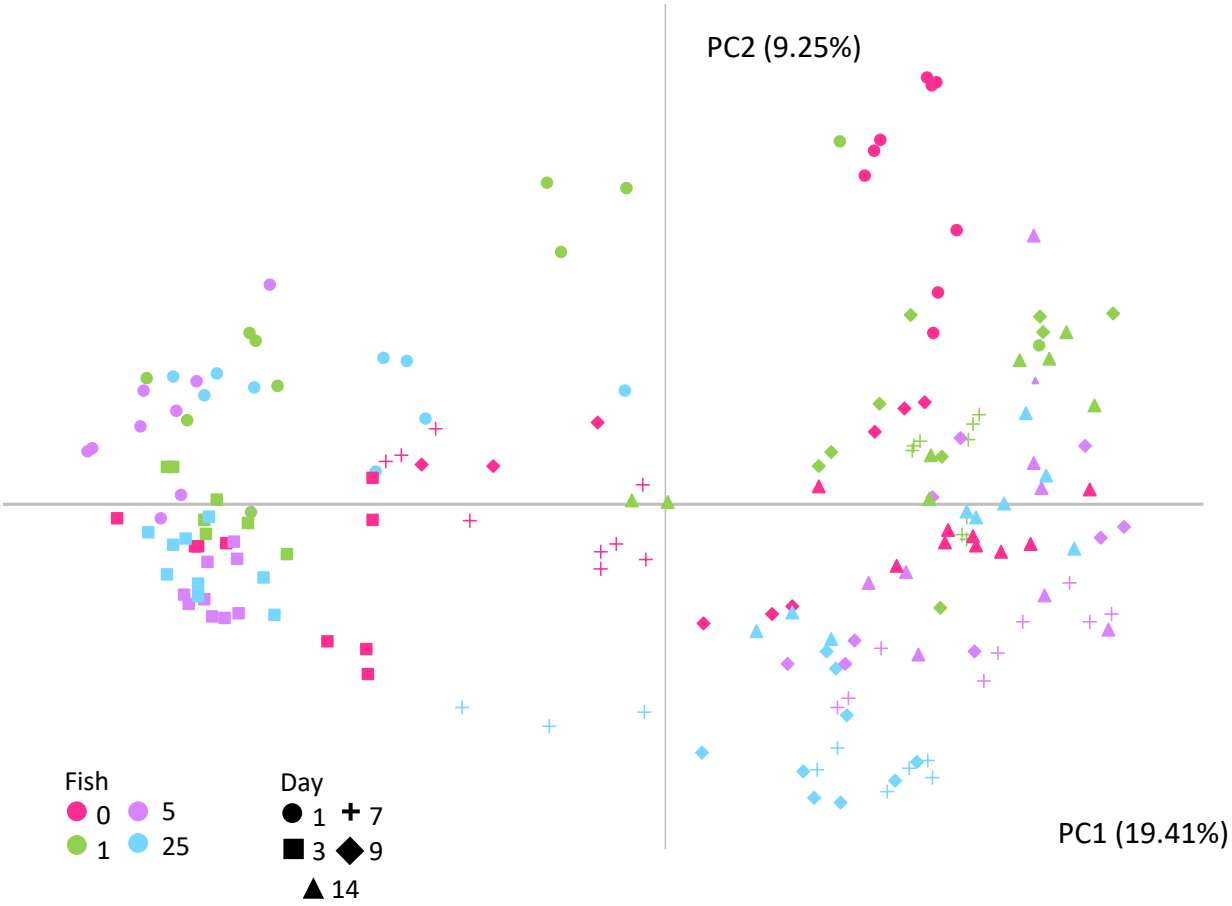

Figure 14. 2-D Principle Coordinate Plot analyzed through Bray Curtis statistical assignment.