Studies on the Microbiota of Fishes and the Factors Influencing Their Composition

by

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Abstract

In this dissertation, I aimed to characterize the microbiota of finfish including identification of the bacteria present as well as the factors that influence their composition. I examined the microbiota of the skin, gill, gut, and/or blood of 7 marine and 3 freshwater fish species. I looked at the impact of fish species, geographic location, season, DNA extraction protocol, and oil exposure on bacterial community structure. Methods used included ribosomal intergenic spacer analysis (RISA) for identification of patterns in bacterial community structure followed by sequencing techniques to determine the bacteria responsible for observed differences. My research indicated that fish species exerts a stronger influence on fish microbiota structure than environmental parameters. Studies included on the skin and gut indicated a species-specific portion of the microbiota that remained stable across various locations and seasons as well as exposure to varying levels of oil. These results challenge the current paradigm that the microbiota of fishes simply reflects that of the surrounding water. My results also indicate that choice of DNA extraction protocol can greatly influence the observed diversity in the fish gut microbiota. Finally, analysis of the microbiota from the blood of apparently healthy fish revealed high bacterial diversity that was similar in structure to that of the gill. Thus isolation of bacteria from the blood or internal organs of a diseased fish may not be sufficient to identify the causative agent of disease.

Overall my work identified Proteobacteria as the dominant bacterial phyla in marine fish samples whereas Fusobacteria dominated the gut microbiota of freshwater fish species included in these studies. My results indicate that fish harbor microbial communities that are distinct from that of the surrounding water and that this community is primarily influenced by fish species. Species-specific microbiota were seen in both fish skin and gut, with influence from the surrounding environment on bacterial community structure indicating the presence of both core and transient members of these bacterial communities.

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List of Abbreviations

ACE	Abundance-based coverage estimation
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
bp	Base pair
CFU	Colony forming unit
GOM	Gulf of Mexico
MDS	Multidimensional scaling
OTU	Operational taxonomic unit
ppt	Parts per thousand
RDP	Ribosomal database project
RISA	Ribosomal intergenic spacer analysis
SIMPER	Similarity percentages
UPGMA	Unweighted pair group method with arithmetic mean
YOY	Young-of-year

CHAPTER 1. LITERATURE REVIEW

The Fish Microbiota

Microbes play an essential role in the development and health of mammals. Alongside an increase in scientific literature reporting the functions of bacteria in mammals is a resurgence in interest in the microbial communities or microbiota of fishes. Mammalian studies often focus on the gut but researchers now recognize that bacteria inhabit all tissues with an epithelial membrane, including the skin, respiratory tract, and urogenital tract [1-4]. Early studies on the microbiota of fishes also concentrated on the gut, but investigators suggested the bacterial communities were simpler than those of mammals with skin and gill microbiota reflecting that of the surrounding water [5, 6] and gut microbiota reflecting that of food items [7, 8]. However these studies used culture-dependent methods requiring growth of the bacteria on nutrient media. Even then scientists understood that choice of media and incubation temperature could impact the results of a study [5, 9, 10]. Currently research indicates that only about 1% of microscopically visible bacteria can be grown using established culture techniques [11], a phenomenon known as the “great plate count anomaly” [12]. As a result of this known bias, more recent studies employed a large variety of culture-independent methods and determined that, 1) the microbiota of fishes is highly diverse; 2) bacteria play an important role in fish health similar to their role in mammals; and 3) a variety of factors

influence fish microbiota structure. A review by Austin [13] addressed these points in relation to studies performed up to 2006, thus the contents of this literature review will include his findings as well as studies published after 2006.

Skin microbiota

Reports on bacterial loads associated with fish skin range from 10^2 to 10^5, but unfortunately units differed between studies (CFU/cm^2 [13], CFU/g [14], and CFU/mL [15]) making direct comparisons between investigations difficult. Researchers detect a high diversity of bacterial genera associated with fish skin (Table 1-1). A majority of the genera identified are members of the phylum Proteobacteria, specifically the class Gammaproteobacteria. However members of the phyla Bacteroidetes and Firmicutes are common as well. Fish sampled from freshwater harbor different bacteria than those sampled from seawater. Freshwater fishes commonly contain members of the genera *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Moraxella*, and *Pseudomonas* [13, 16, 17]. Less commonly identified genera include *Azospirillum*, *Kluyvera*, *Pantoea*, *Plesiomonas*, *Segniliparus*, *Anoxybacillus*, *Exiguobacterium*, and *Staphylococcus* [16, 17]. On the other hand, marine fish are more commonly associated with *Vibrio*, *Pseudoalteromonas*, and *Photobacterium* [13, 18-22]. Despite these differences, marine fish also harbor members of *Pseudomonas* [22, 23], *Aeromonas* [22, 23] and *Acinetobacter* [18] on their skin and mucus. Other genera identified in studies on marine fish skin include *Roseobacter*, *Alteromonas*, *Marinomonas*, *Shewanella*, *Bacteroides*, and *Clostridium* [18-20, 22, 23].

Gill microbiota

Research on fish gill microbiota is limited, but aerobic counts indicate bacterial loads similar to that of skin (10^3 to 10^6 CFU/g). Gill also harbors a wide diversity of bacterial genera (Table 1-2) but similar to skin the members are primarily of the class Gammaproteobacteria and phylum Bacteroidetes. Again, microbiota varies between freshwater and marine fish species. Many of these differences are due to the same genera discussed for skin with *Acinetobacter*, *Aeromonas*, *Enterobacter*, and *Pseudomonas* [13, 14, 17] associated with freshwater fish and *Photobacterium*, *Pseudoalteromonas*, and *Vibrio* [24, 25] identified on marine fish. Few studies examined skin and gill jointly. Wang et al. [17] detected lower bacterial diversity on the gill of gibel carp (*Carassius auratus gibelio*) and bluntnose black bream (*Megalobrama amblycephala*), whereas Feng et al. [23] found relatively similar diversity between sample types in yellow grouper (*Epinephelus awoora*). However both studies observed differences between communities suggesting that these surfaces harbor distinct microbiota.

Gut microbiota

When examining the gut microbiota of fishes, studies use the digesta (feces) to analyze the allochthonous (non-adherent) bacteria and rinsed intestine to identify the autochthonous (adherent) bacteria [26]. Some studies detected significant differences in the microbiota of feces and rinsed intestine in terms of diversity and species composition [27-31] while others did not [32]. For the sake of this review, I will consider these sample types separately. Culturable counts differ greatly between studies on the gut microbiota, with numbers for both feces and rinsed intestine ranging from 10^2 to 10^9 CFU/g [27-29,

33-42]. Direct microscopic counts range from 10^8 to 10^{11} CFU/g [34, 42, 43]. In studies that enumerated bacteria in both sample types, allochthonous bacteria typically outnumbered autochthonous bacteria by 1-2 log [27, 29, 31]. A large diversity of bacterial genera associates with the fish gut (Table 1-3 and 1-4). In general, feces harbor greater diversity than rinsed intestine samples, but many of the abundant genera are similar between sample types. For instance in freshwater fishes, *Aeromonas*, *Enterobacter*, *Pseudomonas*, *Micrococcus*, and *Bacillus* are common in both feces [27, 29-31, 40, 41, 44-50] and intestine [14, 27, 29, 30, 33, 51]. However frequent inhabitants of gut contents that are less prevalent in intestinal mucus include *Acinetobacter*, *Clostridium*, *Lactobacillus*, *Lactococcus*, and *Carnobacterium* [27, 29-31, 40-42, 44, 46-48, 52]. As compared to freshwater fish, the gut microbiota of marine fish maintains a greater abundance of *Pseudoalteromonas* and *Vibrio* in both feces and rinsed intestine [22, 35-38, 53-58], and a higher incidence of *Photobacterium* in feces samples [35, 53, 54]. Researchers detect *Pseudomonas* in both the allochthonous and autochthonous bacterial community of marine fish. However, studies identify *Acinetobacter* more commonly associated with feces samples [35, 36, 43] and *Psychrobacter*, *Carnobacterium*, and *Staphylococcus* more frequently in rinsed intestine samples [22, 33, 36-38, 58].

Internal organ microbiota

Despite the current belief that healthy fish blood and internal organs are sterile [5, 10], multiple studies isolated a number of bacterial genera from these tissues (Table 1-5 and [59, 60]). Although all studies to date have used culture-based techniques, there are

no specifications as to aerobic counts for the bacteria found in these samples. A majority of the genera identified belong to the class Gammaproteobacteria, but there are also representatives of the phyla Actinobacteria and Firmicutes. The most common genera isolated are *Pseudomonas*, *Aeromonas*, and *Micrococcus* [21, 22, 61-64], however studies on marine fishes have also reported the isolation of *Vibrio* and *Photobacterium* [21, 22, 25, 64, 65]. Thus many of the bacteria isolated from the blood and internal organs of healthy fishes are similar to those isolated from other tissues.

Factors Influencing Microbiota Structure

Early observations revealed that the same bacterial species found on fish skin were also present in the surrounding water [10, 13] and that the community structure of both the fish and water microbiota shifted due to changes in environmental parameters including salinity, precipitation, temperature, dissolved oxygen, pH, and abundance of particulate matter [5]. Thus scientists concluded that the bacteria that associated with and colonized the skin and gills of fishes also occupied the neighboring environment [13]. Investigations indicated that these environmental variables along with the microbiota associated with food items influenced gut microbial community composition as well [5, 10, 13]. Researchers further hypothesized that the micro-environment of the fish skin could impact microbiota structure and the mucus of fish could inhibit attachment or growth of some bacteria [5, 6]. These early studies on fish microbiota used culture-based methods and thus results were biased due to the “great plate count anomaly” described

previously. The use of culture-independent methods provided scientists with evidence that the microbiota is a complex community influenced by a wide variety of factors.

Environmental parameters

Results from more recent studies indicate that environmental parameters do in fact influence microbiota structure. Using culture-based techniques, Martin-Antonia et al. [56] observed changes in bacterial communities in the feces of Senegese sole *Solea senegalensis* due to changes in water temperature, specifically in the members of the class Alphaproteobacteria and the genus *Vibrio*. Culture-independent methods examining a large number of fish species also detected significant differences between marine and freshwater fish gut communities [70, 71] strengthening the literature on the influence of salinity. Other studies reported an influence of precipitation on the abundance of *Salmonella* in the digestive tract of warmouth *Lepomis gulosus* [32] and reduced diversity in the skin microbiota of smallmouth bass *Micropterus dolomieu* in sites containing a high abundance of large suspended solids [16].

Two similar studies on different fish species indicate complex interactions between the microbiota, geographic location, and season. Wilson et al. [18] monitored the skin microbiota of Atlantic cod *Gadus morhua* in the northeast Atlantic Ocean while Le Nguyen et al. [72] examined a combination of the skin, gill, and gut microbiota of Pangasius *Pangasius hypophthalmus* from Vietnam. Both studies sampled fish from multiple locations across multiple seasons, detecting similar overall patterns. The bacterial communities were both site and season-specific with subsets of bacteria that remained constant despite changes in the environment. This stable portion of the

community was also observed in skin, gill, and intestine mixtures from tilapia (species not specified) in Cameroon and Vietnam [73] and the skin of whiting caught from the Irish and Celtic Seas [19, 74]. The results of these studies demonstrate a significant influence of unidentified environmental parameters associated with season and geographic location on the fish microbiota.

Despite apparent influence from the environment, fish including loricariid catfish [50] and Atlantic salmon *Salmo salar* [66] harbor gut microbiota that are distinguishable from that of the surrounding water. A meta-analysis by Sullam et al. [67] determined that the bacteria present in the gut microbiota of fishes are more closely related to symbionts isolated from other animals including mammals and invertebrates than to free-living bacteria in the environment, supporting the notion that a portion of the gut community is stable despite changes in the environment. Interestingly the microbiota of the skin and gill of fishes are also significantly different from that of the water column. Both freshwater and marine fishes including Atlantic salmon [15], whiting *Merlangius merlangus* [19], gibel carp and bluntnose black bream [17], and lionfish *Pterois* sp. [20] exhibit these differences.

Fish age and growth

Age influences fish microbiota structure, particularly in the gut, as demonstrated in a number of species. Bacteria colonize eggs quickly [66, 67] and differences in glycoproteins on the egg surface may yield a species-specific microbiota at this stage [68]. Bacteria begin colonization of the digestive tract prior to first feeding as the fish larvae drink water in order to control osmoregulation [68]. At the onset of first feeding,

there is an increase in bacterial diversity [69] and change in community species composition [67]. Brunvold et al. [69] suggest this shift may be due to the colonization of bacteria associated with food items or to the development of the gut resulting in new micro-environments. Some studies indicate that the stable portion of the fish gut microbiota is not from feed [66] but derived from the egg microbiota or that of the surrounding water [67]. The juveniles establish a relatively stable gut bacterial community at around 50 d post-hatch [66], likely due to diet or mucus secretion that favors bacterial establishment [67], but bacterial diversity continues to increase as the fish ages [41].

Diet

Research strongly supports the hypothesis that diet significantly impacts fish microbiota composition. Researchers often perform diet manipulation studies in culture systems in an attempt to increase fish production or prevent disease. Most studies examine diet impacts on the gut microbiota alone, but some also investigate the relationship between diet and the bacterial communities of the skin, gill, and internal organs. Investigations on the gut microbiota focus on autochthonous and/or allochthonous microbiota. Studies on both the autochthonous and allochthonous microbiota often detect differences in bacterial load due to changes in diet [22, 23, 37, 38, 40, 41, 44, 56]; however a few studies observing only allochthonous communities detected no differences in bacterial populations [23, 53]. The bacterial genera that change with diet alterations are often similar between fish species and include *Pseudomonas* [37, 41, 44], *Carnobacterium* [22, 37, 38, 51], and *Vibrio* [41, 53, 56]. Diet may also

influence the microbiota of other fish tissues. For example, Atlantic salmon had increased bacterial loads associated with their skin with increased food intake as well as reduced numbers due to ingestion of food containing antibiotics [15]. Puffer fish *Takifugu obscurus* showed slight differences in skin bacterial load due to differing diets including changes in the abundance of *Pseudomonas* [22]. Although Feng et al. [23] found no differences in numbers of bacteria on the skin of yellow grouper, abundances of *Agrococcus* differed between diet treatments, and differences were also seen in abundances of *Pseudomonas* in gill samples. Dietary changes can also be detected in the microbiota of internal organs. Yang et al. [22] observed slight differences in the bacterial load as well as varying abundances of the genera *Lactococcus* and *Pseudomonas* in liver and ovary samples in correlation with dietary changes in puffer fish. Thus diet can impact not only gut microbiota, but also the bacterial communities found in association with skin, gill, and internal organs.

Fish species

A number of studies have demonstrated species-specificity of fish microbiota from a variety of tissues. Roeselers et al. [70] identified large similarities in the gut microbiota of zebrafish *Danio rerio* sampled from different culture facilities and wild populations and detected the presence of a shared microbiota within zebrafish that remained stable despite environmental influence. Smriga et al. [54] saw significant differences in the allochthonous microbiota of the parrotfish *Chlorurus sordidus*, surgeonfish *Acanthurus nigricans* and two-spot red snapper *Lutjanus bohar* from the same coral reef. The authors stated that microbiota differences may be due to variable

diets, but may also indicate species-specificity. Similarly, Atlantic salmon and sea trout *Salmo trutta trutta* harbored distinguishable gut microbiota at the bacterial genus and species levels [49]. In a comparison of the microbiota of gibel carp and bluntnose black bream, Wang et al. [17] detected significant dissimilarities between gill and skin bacterial communities within each species as well as in both sample types among species. This study analyzed fish held within the same pond on the same diet thus these changes were likely due primarily to factors associated with the fish species and not environmental parameters. In conclusion it seems likely that the host (fish species) influences the structure of the internal and external microbiota although exact mechanisms for host selection of bacteria are unknown. Thus early hypotheses on the influence of the micro-environment of fishes are supported and portions of the microbiota may in fact be species-specific.

Disease

Studies investigating the influence of disease on fish microbiota are limited in the scientific literature. Although disease alters the microbial communities of corals [75-79], its impacts on the bacteria associated with fish are underexplored. Cipriano [16] demonstrated reduced bacterial diversity with dominance shifting to opportunistic pathogens on the skin and mucus after *Aeromonas salmonicida* infection in Atlantic salmon. Similarly, bacterial diversity decreased dramatically with community dominance shifting to *Pseudomonas* and *Vibrio* during disease caused by a variety of organisms in turbot *Scophthalmus maximus* [65]. Recent studies conducted by our group revealed that treatment with potassium permanganate resulted in disruption of the skin microbiota of

channel catfish and increase susceptibility to columnaris disease (H. H. Mohammed, unpublished data). With the desire to formulate probiotics that manipulate the microbiota to reduce disease in fishes, further studies should explore the interactions between pathogens and the commensal microbiota.

Roles of Fish Microbiota

Bacteria play a vital role to host development and health beginning early in development. Studies on gnotobiotic or germ-free individuals allow for the determination of the functions of the microbiota in fish. Although many of the studies included in this section focused on the gut microbiota, it is possible that similar interactions occur in the fish skin and other tissues. The influence of diet on the external microbiota of fishes ([15]; further discussed in "Factors influencing microbiota structure") as well as the ability of fish skin to produce immune responses similar to those found in the gut [80] indicates an unexplored connection between fish skin and gut. Thus there is potential for the microbes present on the fish surface to provide benefits to the host as well and future studies should explore these interactions. Current knowledge indicates that commensal bacteria play a part in proper development of the gastrointestinal tract and immune system, provide essential nutrition, and prevent disease.

Gastrointestinal tract and immune system

Studies on germ-free zebrafish demonstrated the role of the microbiota in development of the gastrointestinal tract of fishes. The morphology of gut epithelial cells

differs significantly between germ-free zebrafish and those with a commensal microbiota. Specifically, vacuoles in the cells of germ-free fish are clear whereas those from normal fish contain eosinophilic and electron-dense material [81]. Also, interactions with the commensal microbiota accelerates epithelial cell proliferation [81]. Bates et al. [82] demonstrated that commensal bacteria are necessary for proper differentiation and maturation of the gut epithelium. Although the exact mechanisms are unknown, the gut microbiota is also necessary for proper development and function of the immune system. For example, up-regulation of genes involved in innate immunity occurs in the presence of commensal microbes [81, 83]. Microbes influence proper development and maturation of the GALT (gut-associated lymphoid tissues) which protects against pathogens as well as controls immune functions in the gastrointestinal tract [84-86].

Nutrition

The gut microbiota plays an important role in host nutrition from metabolizing compounds and producing vitamins to aiding the host in uptake of nutrients. Microbes found in the gut are capable of metabolizing cellulose and other complex polysaccharides that fish are unable to digest on their own due to the lack of endogenous cellulase production [87]. Members of the microbiota of freshwater fishes produce vitamin B_{12} that can be used by the fish host [88, 89]. Also some bacteria in gut produce short-chain fatty acids as the end product of metabolism that the host can use for energy or lipid synthesis [90]. Commensal microbes can also increase the intestinal absorption of compounds [81] including fatty acids and protein macromolecules [82], as well as reduce expression of enzymes that aid in cholesterol metabolism [81].

Drag reduction

Bacteria associated with the mucus of fish skin reduce drag in response to turbulent flow [91, 92]. The genera responsible for drag-reduction are *Pseudomonas* as well as members of the family Micrococcaceae, all of which are normal inhabitants of the skin microbiota of many fish species (see "The Fish Microbiota"). These microbes are hydrophobic, a characteristic which is hypothesized to assist the bacteria in adhesion to the fish skin while producing a smoother surface at the mucus-water interface [91]. The compounds responsible for drag-reduction were identified as extracellular polymers. *Acinetobacter calcoaceticus* can also reduce drag *in vitro* [93] and it is interesting to note that the genus *Acinetobacter* is a frequent inhabitant of the external microbiota of fishes.

Disease

Bacteria associated with fish mucosal surfaces also play an important role in fish health as they can compete with pathogens for space and nutrients. I discuss this function in length as well as its implications for development of probiotics in the following section entitled "Probiotics".

Probiotics

In 2010, production of food fish from aquaculture reached 60 million metric tons worth over $119 billion [94] and aquaculture is set to overtake capture fisheries as a source of food fish [95]. However, disease remains a limiting factor to the expansion and profitability of the aquaculture industry [96-99]. Treatment options for bacterial diseases

in the United States are limited to four Food and Drug Administration (FDA) approved drugs, three of which are antibiotics [100]. Consumer concern over the presence of antibiotics in food as well as an increase in the number of antibiotic resistant microbes has sparked interest in probiotics as alternatives to antibiotics for treatment and prevention of diseases [101, 102].

The World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [103]. The goal of administering probiotics is manipulation of the gut microbiota to improve the beneficial impacts of the commensal microbes through exclusion of opportunistic pathogens and stimulation of the immune system (for reviews on probiotics in aquaculture, see [96, 101, 102, 104]. The ideal candidates for probiotics are dominant members of the normal microbiota of the species of interest [104, 105]. Thus characterization of the healthy microbiota of a species is beneficial to choosing an ideal probiotic. Aside from inhibition of pathogens and stimulation of the immune system, probiotics may also impact fish growth, digestion, and composition of the gut microbiota (see following examples). Thus interpretation of the success of a probiotic involves investigation of a large number of factors.

Researchers often select probiotics for trials due to their ability to inhibit opportunistic pathogens. Bacteria may produce antimicrobials against pathogens of concern or exclude pathogens through competition for space or nutrients [96, 102, 104]. Many times inhibition occurs without identification of the mechanism of exclusion. One commonly tested probiotic is *Bacillus subtilis* which reduces mortality due to *Aeromonas hydrophila in vivo* [106] as well as *A. hydrophila* and *Pseudomonas fluorescens in vitro*

[107]. In tilapia (*Oreochromis niloticus*), strains of *Micrococcus luteus* and *Pseudomonas* sp. were antagonistic to *A. hydrophila* in the lab, but only *M. luteus* provided protection against infection *in vivo* [108]. Son et al. [109] demonstrated the effectiveness of *Lactobacillus plantarum* in reducing mortalities due to *Streptococcus* infections as well as its ability to inhibit iridovirus in grouper (*Epinephelus coioides*). Similarly, administration of *Roseobacter* strain 27-4 to turbot (*Scophthalmus maximus*) reduced mortalities due to *Vibrio anguillarum* [110] and two species of *Carnobacterium* provided protection against *Aeromonas salmonicida* and *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*) [111]. Thus studies validated the ability of a variety of probiotics to protect against infections in a number of fish species.

Probiotics also enhance digestion, leading to increased fish growth. *B. subtilis* increased nutrients and digestion enzymes including amylase and protease in the gut of ornamental fishes, leading to better food digestion and absorption, resulting in better growth [106]. Similarly, use of *Lactobacillus* in gilthead sea bream (*Sparus aurata*) led to higher digestive enzyme activity which the authors hypothesized resulted in better digestion and nutrient absorption, increasing the growth rate in individuals treated with probiotic [112]. Although many studies do not examine both enzyme activity and growth, some studies observe an increase in food conversion and growth rates in treatments with probiotics [107-109, 113-117].

A successful probiotic is capable of colonizing the gut where it either produces antimicrobials or competes for space and nutrients with opportunistic pathogens [96, 102] and promotes a stable healthy microbiota that is resistant to invasion by pathogens [85, 102]. Kim and Austin [111] demonstrated the ability of two *Carnobacterium* species to

persist in gut microbiota samples. Similarly, investigations detected multiple probiotic strains in the gut of brown trout *Salmo trutta*, but the numbers reduced dramatically after probiotic treatment stopped [118]. Also bacterial strains persisted not only in the feces of rainbow trout *Oncorhynchus mykiss* but also in rinsed intestine samples [113]. On the other hand, *Roseobacter* strain 27-4 did not colonize the gut of turbot [110]. Other studies monitoring gut microbiota composition alongside probiotic treatment achieved mixed results. Although Etzion et al. [119] detected no impact of one probiotic, the other led to a change in microbiota that remained stable during the course of treatment. While Bagheri et al. [114] observed an increase in total bacterial load in the gut while administering probiotics, other studies [106, 120] saw an overall reduction. Impacts on specific genera are also variable as Barbosa et al. [120] detected fewer *Vibrio* after treatment with probiotics while Tapia-Paniagua et al. [55] saw an increase. Thus impacts on the gut microbiota differ depending on choice of probiotic and fish species under investigation.

One of the most commonly investigated parameters in probiotic trials is the impact on immune function. Studies monitor a variety of markers to demonstrate immune performance including: 1) respiratory burst activity referring to the rapid release of reactive oxygen species by phagocytic immune cells including neutrophils and macrophages during the initiation of phagocytosis [121], 2) abundances and activity of phagocytic cells, and 3) abundance and activity of lysozyme which is an enzyme capable of lysing bacteria as well as activating components of the immune system [122]. Many studies recorded boosts in immune function due to probiotic treatment including increase in respiratory burst, phagocytic, and lysozyme activities, as well as higher abundances of

phagocytic cells and lysozyme [107, 109, 111, 113, 117, 118, 120, 123, 124]. Although knowledge of the function of probiotics in fish is still limited, studies indicate promise for the use of probiotics in boosting host digestion and growth, immune function, and stabilizing the microbiota to help prevent disease.

Fish Species Profiles

All fish used in the following studies are ray-finned, bony fishes (class Actinopterygii). This dissertation examined 6 marine and/or estuarine species (striped mullet *Mugil cephalus*, pinfish *Lagodon rhomboides*, Atlantic croaker *Micropogonias undulatus*, sand seatrout *Cynoscion arenarius*, spotted seatrout *Cynoscion nebulosus*, red snapper *Lutjanus campechanus*, and Gulf killifish *Fundulus grandis*) and 3 freshwater species (largemouth bass *Micropterus salmoides*, bluegill *Lepomis macrochirus*, and channel catfish *Ictalurus punctatus*). Species are listed by order, family, and species.

Order Mugiliformes

Family Mugilidae

Mugil cephalus Linnaeus, 1758

Mugil cephalus is a member of the family Mugilidae which contains 71 valid species [125]. It is described as a cosmopolitan species as it inhabits coastal waters of every major ocean in the world, particularly from 42°N to 42°S latitudes [126, 127]. As a result of its wide distribution, it has many common names including common mullet, grey mullet, flathead mullet, sea mullet, and striped mullet [128]. Although *M. cephalus*

is tolerant of a wide range of salinities (freshwater to 80 ppt [129]) and temperatures (13°C to 33°C [126]), the species prefers subtropical waters and oligohaline to mesohaline salinities [126]. Males and females reach sexual maturity at 3 [130] to 6 years of age at lengths of about 375 mm [131]. Mature individuals move from estuaries and marshes to offshore waters during late fall and winter to spawn [132-135]. Eggs and larvae drift on ocean currents for one month [126, 132] before inshore migration occurs at a length of 18-22 mm [135]. Larval fish are planktivorous but consume small invertebrates at 10-20 mm. As the fish grow they feed upon copepods. Juveniles and adults eat primarily detritus, algae, and diatoms [126, 130]. Young fish reside in estuaries and marshes until sexual maturity when they move into more open water [132]. Individuals can live up to 13 years [136] and the largest on record was caught in Texas in 2009, weighed 4.71 kg and was 743 mm long [137].

Order Perciformes

Family Sparidae

Lagodon rhomboides (Linnaeus, 1766)

Lagodon rhomboides, commonly known as the pinfish, is one of the 139 valid species [125] of the family Sparidae which includes the breams and porgies. Its native range reaches along the Atlantic coast of the United States from Woods Hole, Massachusetts, and throughout the GOM to the Yucatan Peninsula [138, 139]. Individuals are found in temperatures ranging from 10-35°C and salinities of zero to 75 ppt [140]; however studies indicate fish abundances increase with salinity [141] and preference is between 30-40 ppt in waters around 30°C [142, 143]. Adults move into

estuaries during the winter and spring where they are often found associated with vegetation [144] or other structures including rocks and docks [138]. Pinfish have been reported from depths up to 73 m [138, 139], but highest abundances are found in depths of less than 30 m [139, 145]. Their presence in the estuary is both size and temperature dependent. In general, larger individuals live in deeper waters [139, 145]. Fish migrate to deeper waters during the winter [139] and return to the shallower estuaries in spring [138, 146] suggesting a preference for warmer temperatures. Mature adults are typically 130 mm long [145] and move offshore to spawn during late fall and winter [138, 139, 144-146]. Larval fish enter the estuaries in late winter to spring [147] where they settle out of the plankton and live among vegetation or other habitats providing cover [139, 148] at depths of less than 5 m [146]. The optimum salinity for young pinfish is 15-30 ppt [149]. Studies describe 4 ontogenetic shifts in diet: 1) individuals <20 mm are planktivorous, 2) 20-30 mm eat copepods, shrimp postlarvae, and amphipods, 3) 30-80 mm are omnivorous, ingesting about 30% plants as well as shrimp and fish, 4) >100 mm eating 90% plants or mussels in areas where vegetation is scarce [147, 150-152]. Thus *L. rhomboides* shifts from carnivory to herbivory as it grows [150], although Carr and Adams [152] reported individuals greater than 80 mm acting as omnivores as they still consumed significant amounts of shrimp and fish. Individuals as old as 7 years have been reported in the literature [145] but most do not live longer than 2-3 years [144, 153]. The largest *L. rhomboides* on record was 1.51 kg, 387 mm long, and was caught off Horn Island, MS in 1992 [137].

Order Perciformes

Family Sciaenidae

***Micropogonias undulatus* (Linnaeus, 1766)**

Micropogonias undulatus, commonly referred to as the Atlantic croaker, belongs to the family Sciaenidae which includes 291 valid species [125] of croakers and drums. *M. undulatus* inhabits coastal waters from the Gulf of Maine southward to Argentina, as well as throughout the GOM [154]. The species can tolerate a wide range of temperatures (9-42°C) and salinities (0-25 ppt) [155]. Adults enter bays and estuaries during the spring and remain there during the summer months [156] where they primarily feed on arthropods and fishes [144, 157, 158]. Adults migrate offshore to spawn [144, 159, 160] in late fall and winter [144, 158] with peak spawning in October/November [159, 160]. The larvae enter bays from the GOM during winter and spring on tidal currents [160, 161]. The juveniles remain in the upper estuary where they are planktivorous until a length of 15 mm [158]. As they grow, their diets shift to include annelids and insect larvae [158], followed by crustaceans and mollusks, then fishes [144, 157]. Juveniles are more abundant inshore and there is an increase in fish size along the salinity gradient from the upper estuary toward the GOM [144, 159, 162]. YOY fish are often found in association with soft substrates while age-1 and older fishes are often found near hard substrates including oyster reefs [159]. Larger individuals (85-100 mm) move further offshore [159]. Sexual maturity is reached in both males and females between 140-170 mm total length [159] with individuals in the GOM reaching maturity by the end of their second year [160, 163] although it may be as late as 3-4 years among the Atlantic coast distributions [164]. *M. undulatus* can live to be 8 years old [165] although many do not

survive past 2 years [160]. The current world record for *M. undulatus* was set in 2007 in Virginia with an angler catching an individual weighing 3.94 kg and measuring 673 mm in length [137].

Cynoscion arenarius Ginsburg, 1930

The sand seatrout *Cynoscion arenarius* is a coastal species ranging from Florida Bay at the southern tip of Florida throughout the GOM to Campeche Bay and the Yucatan Peninsula [166]. Adults and juveniles can tolerate a large range of temperatures and salinities (5-37°C, 0-37.2 ppt) but have a preference for temperatures between 20-30°C [166, 167]. *C. arenarius* utilize both inshore and offshore environments of the GOM [168] but mature fish spend more time in the Gulf than in the upper bay [169]. Adults (>160 mm total length) feed mostly on fish and crustaceans [166, 170-172]. Fish move offshore to overwinter [173] and return inshore in spring and summer [166, 174]. The inshore migration coincides with spawning which takes place from March to September with peaks in March/April and August/September [166, 174]. Spawning starts in deeper waters (15-80 m depth, about 175 km offshore) in the early part of the season and moves inshore as the season continues [175] with a majority of spawning occurring at depths of 7-15 m [166]. Larval fish move further into estuaries during the spawning season [175] and remain there until reaching 50-60 mm total length at which point they start to migrate outward [166], resulting in a size gradient from the upper estuary to the Gulf [173]. Larval fish eat copepods and other plankton-sized organisms [176]. As the fish grow, they shift to piscivory of smaller fishes including anchovy and small *C. arenarius*, followed by larger menhaden at lengths >100 mm [177]. Fish reach maturity

at 140-180 mm total length at the end of their first year of life [174] and have a total lifespan of about 3 years [166]. The largest *C. arenarius* on record weighed in at 2.78 kg with a length of 635 mm and was caught off Dauphin Island, Alabama in 2007 [137].

Cynoscion nebulosus (Cuvier, 1830)

The range of the spotted seatrout, *Cynoscion nebulosus*, reaches from the western Atlantic at New York, around Florida, and through the GOM to Mexico [178]. Adults are commonly present in upper estuaries associated with bottom vegetation [179], however they move offshore during winter [179] and when salinity is below 5 ppt [180]. *C. nebulosus* is tolerant of a wide range of salinities (0-60 ppt) and temperatures (above 12°C) but is more abundant at salinities of about 20 ppt and temperatures around 30°C [181, 182]. Spawning occurs from March to October with peaks in both the spring and late summer [160, 179, 183-185]. Spawning most often occurs at night over vegetation in areas out of the reach of the tide [180, 183] but has also been reported near shorelines [186] and at depths up to 15 m [160]. Size at maturity is 240 mm and is reached at 2-3 years of age [160, 179]. Males of the species emit a croaking sound during the mating process [180, 184]. Eggs sink into the vegetation [160] and hatch in about 40 hours [180] where larvae feed primarily on copepods [185]. Postlarvae and young-of-the-year occur most often at depths less than 3.7 m and are typically found amongst vegetation [160, 187]. Juveniles eat crustaceans including mysid and caridean shrimp, as well as fish [171, 179, 185]. Adults eat primarily larger fish including croaker, spot, and mullet, as well as penaeid shrimp [160, 179, 180, 188, 189]. *C. nebulosus* have small home ranges with most tagged fish being recaptured within 30 miles of the tagging site [180, 190]. Fish

typically start schooling at an age of 6 to 8 weeks and continue until 5-6 years of age which is the expected lifespan of males. However, females can live longer and are often solitary at these older ages [180]. The world record for *C. nebulosus* comes from Florida where an individual measuring 1003 mm and weighing 7.92 kg was caught in 1995 [137].

Family Lutjanidae

Lutjanus campechanus **(Poey, 1860)**

The red snapper *Lutjanus campechanus* is a member of the 109 valid species of the family Lutjanidae [125]. This species is distributed from North Carolina, throughout the continental shelves of the GOM [191]. Adults *L. campechanus* are often found associated with structures including natural and artificial reefs and oil and gas platforms [192-196]. All ages of *L. campechanus* can be found at a large range of depths (7-240 m) on the continental shelf [197]. Fish mature after their second year [198] at a length of about 290 mm [192]. Mature fish move into shallower waters to spawn (24-67 m depth [197]) in late spring to early fall [192, 198, 199]. The eggs remain in the pelagic zone where they hatch after about 1 day, releasing 2.2 mm long larvae [200]. After 26-27 days, fish settle onto shell or sand substrates [193, 201-203]. As juveniles grow, they move further offshore towards structures that provide a greater level of relief and form schools [192, 194, 195, 201, 204, 205]. Diets of juveniles consist primarily of copepods, shrimp, and some fish [195, 205]. Adults live solitarily among reefs [192] and feed mostly on fish, crabs, and squid [193, 195]. *L. campechanus* can live to be 50 years old [206]. In 1996 off the coast of Louisiana, a *L. campechanus* weighing 22.79 kg and measuring 965.2 mm was caught, setting a new world record for this species [137].

Family Centrarchidae

Micropterus salmoides (Lacepède, 1802)

The family Centrarchidae includes 38 valid species including *Micropterus salmoides* or commonly the largemouth bass. *M. salmoides* was originally distributed from the southern Quebec westward to Minnesota and southward to the Gulf, continuing to southern Florida and northward through the Atlantic slope drainages to Virginia. However it has been introduced throughout the western United States as well as into other countries including Mexico, Central America, southern Canada, Asia, Africa, and Europe [207]. *M. salmoides* prefer shallow (<3 m), low current waters with submerged vegetation or other structures that provide cover [208-211], although in the northern ranges they move to deeper waters to overwinter [212]. Adults are solitary, whereas juveniles may form aggregations for feeding in open waters [208]. Although individuals have been found in salinities as high as 24 ppt, *M. salmoides* is most abundant in salinities less than 4 ppt and grows fastest at temperatures between 24-30°C [212]. Both sexes mature between 2-4 years of age at a length of 250-300 mm total length [213-215], but some populations have demonstrated sexual maturity as early as age-1 or as late as age-5 [216, 217]. Spawning season differs throughout the geographic range of the species, with earlier spawning in the southern ranges (March-June [207, 214, 216]) and later in the northern ranges (May-July [207, 216]). In Kenya and Puerto Rico, spawning has been reported in January [217, 218]. Males construct nests in areas protected by vegetation, rocks, or other structures in shallow waters (<2.5 m) protected from wind activity [207, 218]. Females may spawn in more than one nest. After the release of egg and sperm, fertilized eggs settle into the nest and attach to the substrate [207]. Males

guard the nests and hatched fry for several weeks, aerating the eggs during incubation, and do not eat during this process [207, 218]. After hatching, fry remain in the nest for a couple of days before moving into shallower shoreline waters amongst vegetation and other protective cover [207]. Small fish (up to 40 mm) feed on copepods and other microcrustaceans. By 100 mm, *M. salmoides* eats primarily fish and crayfish [207, 219-222]. The maximum age for the species is about 11 years in the southern ranges versus 15 years in the northern reaches and populations often contain individuals between 7-8 years of age [207]. The largest *M. salmoides* on record was caught in 2009 in Japan, weighing in at 10.12 kg and 691 mm in length [137].

***Lepomis macrochirus* Rafinesque, 1819**

The bluegill *Lepomis macrochirus* is native to the St. Lawrence and Great Lakes systems and the Mississippi River basin from Quebec to Minnesota, southward to the GOM and in the Atlantic and Gulf slope drainages from North Carolina to Mexico. Recent introductions have increased the species' distribution throughout the United States as well as into other countries including South Africa, Korea, and Japan [216]. The species can tolerate salinities as high as 18 ppt [223] and temperatures up to 40°C [224]. *L. macrochirus* undergo ontogenetic shifts in habitat that are reflective of shifts in prey as well as season and predation risk. Mittelbach [225] described the behavior of this species as it relates to optimal foraging efficiency. In short, individuals select the most energetically favorable prey: they choose larger over smaller prey which is typically found in the open water in summer but in vegetation in spring (in the form of large insects). This is the pattern that large individuals (>100 mm) follow. However, smaller

individuals (<80 mm) are also faced with predation by fish such as *M. salmoides*. Thus small *L. macrochirus* remain in the vegetation feeding on the larger prey options during the day and move into more open waters to feed on larger prey at night when predation risk is smaller. This pattern has been supported by other studies [226-228]. *L. macrochirus* also undergoes an ontogenetic shift in diet, consuming mostly microcrustaceans, filamentous algae, and insects at small sizes, shifting to a diet higher in macroinvertebrates, plants, and fish [229-231]. Also it is common for individuals to eat eggs from the nests of other *L. macrochirus* [232, 233]. The species overwinters in deeper areas among tall vegetation [225-227]. Aside from the complicated habitat and prey selection, *L. macrochirus* has alternative reproductive strategies. Parental males build nests and protect the eggs from predators [234, 235]. As *L. macrochirus* are colonial breeders (building nests in groups), the territories of parental males often overlap, providing extra protection to the eggs and the females can deposit eggs in multiple nests [233, 236]. There are also cuckold males which are sexually mature males that do not build a nest, but sneak into the nest of a parental male and deposits sperm while a female is in the nest, thus reproducing without guarding a nest [237]. Hatched fry move out of the nest and into vegetation [238]. Age and size at maturity varies depending upon reproductive strategy, with cuckolds maturing at 2-3 years at a length of 60-80 mm, parental males at 5-7 years and 130-160 mm, and females maturing at 4-5 years [217, 235, 239, 240], although Swingle [232] reported maturity for this species at 1 year. Spawning takes place in spring and summer [241-243]. Average life span for the species is 5-6 years [136] but an individual was reported that was 10 years old [244]. The world

record for *L. macrochirus* was set in 1950 in Alabama when an individual weighing 2.15 kg was caught [137].

Order Cyprinodontiformes

Family Fundulidae

Fundulus grandis Baird & Girard, 1853

There are 41 valid species in the family Fundulidae, including *Fundulus grandis* or the Gulf killifish [125]. The Gulf killifish ranges from Marco Island located in southwestern Florida throughout the northern GOM to the Laguna Tamiahua in Veracruz, Mexico [245]. This species is abundant in salt marshes [246-248] but are also been found in association with non-vegetated areas and oyster reefs [249]. In times of high tides, individuals move higher into the marsh in waters as shallow as 5 cm [247]. Although juveniles are present in the marsh all year, larger adults <100 mm are in higher abundances in spring and summer [248] which coincides with the spawning season (March to August) [250, 251]. Spawning occurs biweekly among vegetation inundated with water during the higher spring tides [250, 251]. Eggs are attached to marsh grasses [132] and are often above the water line during neap tides which protects against predation and burying by sediments [250]. Eggs incubate on the marsh grass and hatch in 2 weeks [252] at the next spring tide [250]. Fish move into deeper marsh ponds or creeks during the winter and thus are more abundant in the spring through fall [253]. Young fish (30-50 mm) eat primarily detritus, polychaetes, amphipods, and gastropods. As they grow, the proportion of crustaceans and mollusks increase. By the time they reach adult

size they eat mostly shrimps, crabs, and fishes [248, 254, 255]. *F. grandis* reach a maximum size of about 145 mm standard length [256].

Order: Siluriformes

Family Ictaluridae

Ictalurus punctatus (Rafinesque, 1818)

Ictalurus punctatus commonly called the channel catfish is one of 51 valid species of the family Ictaluridae [125]. This species was originally distributed throughout the St. Lawrence and Great Lakes systems and Mississippi River basins from southern Quebec to Montana, spreading southward to the GOM [257]. It has since been introduced throughout the United States [258, 259]. *I. punctatus* migrates to deeper waters to overwinter [260, 261]. Fish are often found associated with structure that provide cover including logs and boulders in depths of 1-2 m [262] but often come to the surface or inshore to feed [259, 263]. An ontogenetic shift in diet occurs between 300-400 mm where smaller individuals feed on detritus, plankton, and aquatic insects [259] and larger individuals eating primarily fish and crayfish [264-266]. Individuals mature between the ages of 5 and 9 years at a length of 330-500 mm [259, 267, 268]. Spawning occurs from May to August [269-271] in secluded areas such as burrows or rock cavities [259]. The males guard the nests, eggs hatch in a week and the fry remain in the nests for a week before swimming to shallow water near cover [259, 272]. Individuals 22 years of age have been reported in the literature [264]. The largest *I. punctatus* on record weighed 26.3 kg and measured 1200 mm in length and was caught in South Carolina in 1964

[137]. *I. punctatus* is the main aquaculture species in the USA with more than 150,000 metric tons/year of production [273].

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Table 1-1. Genera identified in studies on fish skin and mucus microbiota.

Phylum	Class of Proteobacteria	Freshwater Fish	Marine Fish
Proteobacteria	α-proteobacteria	*Azospirillum*	*Brevundiimonas* , *Caulobacter* , *Hyphomicrobium* , *Loktanella* , *Nautella* , *Paracoccus* , *Ponticoccus* , *Prosthecomicrobium* , *Roseobacter* , *Ruegeria* , *Thalassobius* , *Tropicibacter,* Unidentified Rhodobacteraceae
	β-proteobacteria	*Alcaligenes* , *Comamonas*	*Achromobacter* , *Alcaligenes*
	γ-proteobacteria	*Acinetobacter* , *Aeromonas* , *Enterobacter* , *Escherichia* , *Kluyvera* , *Moraxella* , *Pantoea* , *Plesiomonas* , *Pseudomonas* , *Psychrobacter* , *Vibrio* , Unidentified Enterobacteriaceae	*Acinetobacter* , *Aeromonas* , *Algicola* , *Alteromonas* , *Amprhite* , *Aranicola,* *Cobetia* , *Enterobacter* , *Escherichia* , *Ferrimonas* , *Marinimonas* , *Oleibacter* , *Pantoea* , *Photobacterium* , *Pseudoalteromonas* , *Pseudomonas* , *Psychrosphaera* , *Psychrobacter* , *Serratia,* *Shewanella* , *Thalassomonas* , *Vibrio* , Unidentified Pseudomonadales
Actinobacteria		*Micrococcus* , *Segniliparus*	*Agrococcus, Kocuria* , Unidentified coryneforms

Table 1-1 continued

Phylum	Class of Proteobacteria	Freshwater Fish	Marine Fish
Bacteroidetes		*Flavobacterium* , *Flexibacter*	*Bacteroides* , *Cytophaga, Flavobacterium, Mesoflavibacter, Nonlabens, Tenacibaculum,* Unidentified Bacteroidetes
Cyanobacteria		Unidentified genus	-
Firmicutes		*Anoxybacillus* , *Bacillus* , *Exiguobacterium* , *Lactobacillus* , *Staphylococcus*	*Bacillus, Clostridium* , *Lactobacillus* , *Cetobacterium* , *Propionigenium* , *Staphylococcus*
Verrucomicrobia		-	*Roseibacillus*
	Sources	Austin 2006 [13]; Latha and Mohan 2013 [14]; Cipriano 2011 [16]; Wang et al. 2010 [17]	Austin 2006 [13]; Wilson et al. 2008 [18]; Smith et al. 2007 [19]; Stevens and Olson 2013 [20]; Mylniczenko et al. 2007 [21]; Yang et al. 2007 [22]; Feng et al. 2010 [23]; Balcazar et al. 2010 [275]

Table 1-2. Genera identified in studies on fish gill microbiota.

Phylum	Class of Proteobacteria	Freshwater Fish	Marine Fish
Proteobacteria	α-proteobacteria	*Agrobacterium*	*Oceanicola*
	β-proteobacteria	-	*Achromobacter* , *Alcaligenes*
	γ-proteobacteria	*Acinetobacter* , *Aeromonas* , *Aliivibrio* , *Enterobacter* , *Erwinia* , *Pantoea* , *Pseudomonas* , *Vibrio* , Unidentified Enterobacteriaceae	*Aeromonas, Aranicola, Enterobacter, Ferrimonas, Idiomarina, Klebsiella, Oceanisphaera, Pantoea, Photobacterium, Proteus, Pseudoalteromonas, Pseudomonas, Psychrobacter, Shewanella,*
Actinobacteria		*Micrococcus* , *Mycobacterium* , *Segniliparus* , Unidentified coryneforms	*Agricoccus, Micrococcus*
Bacteroidetes		*Cytophaga*	*Chryseobacterium, Cytophaga, Flavobacterium, Joostella, Psychroflexus, Salinimicrobium, Tenacibaculum, Winogradskyella*
Cyanobacteria		-	-
Firmicutes		*Exiguobacterium* , *Lactobacillus* , *Staphylococcus*	*Bacillus, Bagococcus*
Verrucomicrobia		-	*Roseibacillus*
	Sources	Austin 2006 [13]; Latha et al. 2013 [14]; Wang et al. 2010 [17]	Austin 2006 [13]; Feng et al. 2011 [23]; Svanevik and Lunestad 2011 [24]; Valdenegro-Vega et al. 2013 [25]

Table 1-3. Genera identified in studies on freshwater fish gut microbiota.

Phylum	Class of Proteobacteria	Non-adherent Microbiota	Adherent Microbiota
Actinobacteria		*Corynebacterium, Kocuria, Microbacterium, Micrococcus, Mycobacterium, Rhodococcus, Rothia,* Unidentified Actinobacteria, Unidentified coryneforms	*Micrococcus*
Bacteroidetes		*Bacteroides, Chryseobacterium, Flavobacterium, Prevotella, Sporocytophaga*	*Flavobacterium*
Cyanobacteria		Unidentified Cyanobacteria	-
Firmicutes		*Anoxybacillus, Bacillus, Carnobacterium, Clostridium, Enterococcus, Eubacterium, Exiguobacterium, Kurthia, Lactobacillus, Lactococcus, Staphylococcus, Streptococcus, Veillonella*	*Bacillus, Carnobacterium, Clostridium, Lactobacillus, Staphylococcus, Streptococcus*
Fusobacteria		*Cetobacterium*	*Cetobacterium*
	Sources	Wu et al. 2010 [27]; Merrfield et al. 2009 [29]; Heikkinen et al. 2006; Kim et al. 2007 [30]; Namba et al. 2007 [31]; [40]; Dimitroglou et al. 2009 [41]; Navarrette et al. 2010 [42]; He et al. 2011 [44]; Wu et al. 2012 [46]; Pond et al. 2006 [47]; Han et al. 2010 [48]; Skrodenyte-Arbaciauskiene et al. 2008 [49]; Di Maiuta et al. 2013 [50]; Zhou et al. 2009 [52]	Latha and Mohan 2013 [14]; Ringo et al. 2006 [26]; Wu et al. 2010 [27]; Merrifield et al. 2009 [29]; Kim et al. 2007 [30]; He et al. 2009 [51]

Table 1-4. Genera identified in studies on marine fish gut microbiota.

Phylum	Class of Proteobacteria	Non-adherent Microbiota	Adherent Microbiota
Proteobacteria	α-proteobacteria	*Ochrobactrum*	*Ehrlichia/Neorickettsia, Phaeobacter*
	β-proteobacteria	-	*Burkholderia*
	γ-proteobacteria	*Acinetobacter, Aeromonas, Aranicola, Enterobacter, Enterovibrio, Ewingella, Ferrimonas, Glaciecola, Halomonas, Pantoea, Photobacterium, Pseudoalteromonas, Pseudomonas, Psychrobacter, Shewanella, Vibrio,* Unidentified Vibrionaceae	*Acinetobacter, Aeromonas, Aliivibrio, Aranicola, Enterobacter, Enterovibrio, Ferrimonas, Klebsiella, Photobacterium, Providencia, Pseudoalteromonas, Pseudomonas, Psychrobacter, Shewanella, Stenotrophomonas, Vibrio*
Actinobacteria		*Agrococcus, Arthrobacter, Brachybacterium, Micrococcus, Nocardia*	*Agrococcus, Arthrobacter, Microbacterium, Micrococcus, Mycobacterium, Nesterenkonia*
Bacteroidetes		Unidentified Bacteroidetes, Unidentified Flavobacteriaceae	*Chryseobacterium, Gelidibacter,*
Cyanobacteria		Unidentified Cyanobacteria	-

Table 1-4 continued

Phylum	Class of Proteobacteria	Non-adherent Microbiota	Adherent Microbiota
Firmicutes		*Bacillus, Brochothrix, Bulleidia/Erysipelothrix, Carnobacterium, Clostridium, Eubacterium, Jeotgalicoccus, Lactobacillus, Lactococcus, Marinilactibacillus, Sporosarcina, Staphylococcus, Streptococcus*	*Bacillus, Brevibacillus, Brochothrix, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Macrococcus, Planococcus, Staphylococcus, Trichococcus*
Fusobacteria		Unidentified Fusobacteria	-
Spirochaetes		*Brevinema*	Unidentified Spirochaetes
Verrucomicrobia		*Roseibacillus*	*Roseibacillus*
	Sources	Feng et al. 2011 [23]; Ringo et al. 2006 [28]; Hovda et al. 2007 [35]; Bakke-McKellep et al. 2007 [36]; Shiina et al. 2006 [43]; Silva et al. 2011 [53]; Smriga et al. 2010 [54]; Tapia-Paniagua et al. 2010 [55]; Martin-Antonio et al. 2007 [56]; Balcazar et al. 2010 [275]; Clements et al. 2007 [276]	Yang et al. 2007 [22]; Feng et al. 2011 [23]; Ringo et al. 2006 [33]; Bakke-McKellep et al. 2007 [36]; Ringo et al. 2006 [37]; Ringo et al. 2008 [38]; Ward et al. 2009 [57]; Tanu et al. 2012 [58]

Table 1-5. Genera identified in studies on the internal organs of fish.

Phylum	Class of Proteobacteria	Genera Identified
Proteobacteria	α-proteobacteria	*Sphingomonas*
	β-proteobacteria	*Achromobacter, Alcaligenes*
	γ-proteobacteria	*Aeromonas, Chyrseomonas, Citrobacter, Enterobacter, Escherichia, Moraxella, Morganella, Pasteurella, Photobacterium, Plesiomonas, Proteus, Pseudoalteromonas, Pseudomonas, Serratia, Shewanella, Stenotrophomonas, Vibrio*
Actinobacteria		*Brevibacterium, Corynebacterium, Microbacterium, Micrococcus,* Unidentified Actinomycetales
Bacteroidetes		*Flavobacterium*
Firmicutes		*Bacillus, Carnobacterium, Exiguobacterium, Lactobacillus, Lactococcus, Sarcina, Staphylococcus, Streptococcus*
	Sources	Austin 2006 [13]; Mylniczenko et al. 2007 [21]; Yang et al. 2007 [22]; Valdenegro-Vega et al. 2013 [25]; Evelyn and McDermott 1961 [61]; Allen and Pelczar 1969 [62]; Bullock and Snieszko 1969 [63]; Arias et al. 2013 [64]; Toranzo et al. 1993 [65]

CHAPTER 2. COMPARISON OF DNA EXTRACTION PROTOCOLS FOR THE ANALYSIS OF GUT MICROBIOTA IN FISHES

Abstract

In this study, I investigated the influence of methodology on bacterial DNA extraction from fish gut samples for microbial community downstream analysis. Samples were taken from feces and intestine of three sympatric freshwater fish species with varying diets. Samples were processed immediately (fresh), stored at -20°C for 15 days, or preserved in RNA*later*® reagent for 15 days. Samples were then extracted with commercial kits either designed for animal tissues or specifically formulated for stool samples. Microbial community fingerprints were generated using ribosomal intergenic spacer analysis (RISA). Factors considered in this study were DNA quantity and quality, fingerprint quality, diversity as depicted by band number, repeatability, and practicalities such as cost and time. Method of extraction (including both storage condition and kit used) had a larger impact on the structure of bacterial community profiles than the sample type (feces *versus* intestine). Aside from frozen samples which were consistently outperformed by other storage methods, treatment performance differed by sample type. We recommend extraction of bacterial DNA from fresh samples using the tissue kit when analyzing the intestine, and fresh samples in combination with the stool kit when analyzing feces. If samples cannot be processed immediately, preservation in RNA*later*®

is preferred to freezing. Microbial community analysis is influenced by extraction methodologies and that should be taken into consideration for metadata analysis.

Introduction

The gut microbiota of fish is critical to host survival as it plays an active role in metabolism of nutrients, enhancement of the immune system, and inhibition of potential pathogens (for a review on this topic, see [1]). The essential relationship between gut microbiota and fish health has prompted studies describing the gut bacterial community composition of fishes, especially in aquaculture. While much research has focused on the influence of diet, probiotics and prebiotics on the overall health and gut microbiota of farmed fish species [2-13], a few have considered the gut microbiota of wild fishes [14-19]. Alterations in the gut microbiota of fishes in captivity as compared to those in the wild [20] may have important implications for the health of the animals, and thus factors that modify the gut microbiota are of great interest.

The gut poses a difficult environment for microbial community studies due to the presence of a variety of PCR inhibitors, including bile salts [21] and complex polysaccharides [22] which may impact the results of bacterial community analyses. A variety of commercial DNA extraction kits are available and some have been compared in terms of associated impact on downstream analysis in human gut microbiota [23-26]. However the influence of methodology on microbiota results is often ignored and has not been investigated in fish thus making comparisons between studies using different methodologies challenging. As most studies focus on one or a few fish species,

similarities between species in terms of bacterial community composition and the impact of various factors on this configuration are hard to interpret.

To that end, the purpose of this study was to compare bacterial community analysis methods for three common recreational species found throughout the United States, the channel catfish *Ictalurus punctatus* (Rafinesque 1818), bluegill *Lepomis macrochirus* Rafinesque 1819, and largemouth bass *Micropterus salmoides* (Lacepède 1802). Although these species live sympatrically, their diets differ as bluegill are generalists [27-29], largemouth bass are carnivores [30-32], and channel catfish are omnivores [33, 34]. These characteristics allow us to diminish the impact of the local environment on variations in gut microbiota [16, 35-38] while maintaining those associated with diet [7, 11, 12, 16, 39-42] and physiology [16, 43]. Feces and intestinal wall samples were collected, stored under 3 conditions, and subjected to 2 commercial DNA extraction kits to compare DNA quantity, quality, fingerprint pattern (reflecting bacterial community structure), and repeatability. These results will help to identify potential biases inherent in the various steps included in bacterial community analysis methods.

Methods

Sample Collection

Fish were collected in February, 2012 at the Auburn University Aquaculture Research Station pond S8 (Auburn, Alabama; 32°40’18.7”N, 85°30’36.00”W). Five individuals of each fish species (largemouth bass, bluegill, and channel catfish) were

captured and kept alive in aerated pond water until processing (approximately 4 hours). Channel catfish were caught on baited catfish jugs which were set to fish overnight and collected in the morning (approximately 15 hours). Largemouth bass and bluegill were caught using standard baited hooks on spinning reels. Average size (mm) of individuals sampled were 602±40.0, 361±37.4, 178±18.0 for channel catfish, largemouth bass, and bluegill, respectively. Prior to gut sampling, fish were euthanized with 300 mg/L tricaine methanesulfonate. The lower third of the intestine was aseptically removed from each individual. Gut content (feces) was squeezed from the intestine and saved for further analysis. The remaining intestinal tissue (intestine) was collected separately. The respective samples were pooled for all 5 individuals of the same fish species.

Sample Preparation

Feces and intestine were homogenized separately for 2 minutes using a hand-held tissue homogenizer. Each sample was aliquoted into the following series: 9 tubes with 25 mg each were prepared for DNA extraction using the tissue kit and 9 tubes with 220 mg each were prepared to be used with the stool kit. Each set of 9 tubes was divided in triplicate for each storage method tested. Three were frozen at -20°C for 15 days, 3 were placed in RNA*later*® overnight at 4°C to allow thorough tissue penetration and then placed at -20°C for 15 days, and 3 were immediately subjected to DNA extraction. Prior to extraction, samples stored in RNA*later*® were thawed and centrifuged at 5000 *g* for 10 minutes. Supernatant was discarded. The sample was washed with sterile phosphate buffered solution three times to remove excess salts present in RNA*later*®.

DNA Extraction

DNA was extracted from samples of 25 mg with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA; tissue kit) according to manufacturer's spin-column protocol for purification of total DNA from animal tissues. These extractions were preceded by pretreatment for Gram-positive bacteria as per manufacturer's instructions. DNA was extracted from samples of 220 mg using the DNA isolation for pathogen detection protocol included in the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA; stool kit). This procedure includes lysis and protein degradation within its protocol, so no pretreatment was necessary.

Ribosomal Intergenic Spacer Analysis (RISA)

RISA was performed as previously described [44] with the following alterations. To prepare samples for gel loading, 10 µL PCR products were diluted with 10 µL AFLP® Blue Stop Solution (LI-COR), and 0.6 µL of sample was loaded into the wells. RISA images were analyzed using Bionumerics v. 7 (Applied Maths, Austin, TX) as previously described [45] including background subtraction, normalization, and cluster analysis.

Results Interpretation

The following six treatments were included in all analyses: 'FT' = fresh, tissue kit; 'FS' = fresh, stool kit; 'RT' = RNA*later*®, tissue kit; 'RS' = RNA*later*®, stool kit; 'ZT' = frozen, tissue kit; 'ZS' = frozen, stool kit. Cost of materials and time required to extract DNA were recorded for each treatment. After DNA extraction, a NanoDrop ND-1000 (Thermo Scientific, Rochester, USA) was used to quantify total DNA. DNA

quantities were adjusted for the amount of tissue used (adjusted quantity = ([ng/μL]/mg). Protein contamination (260/280 ratio) was recorded from each sample.

As the first decision in analysis of fish gut microbiota is which sample type to choose, we first determined whether sample type (feces *versus* intestine) had a significant impact on the bacterial community structure as depicted by RISA analysis. For each species, dendrograms based on percent RISA fingerprint similarity were formed using Pearson product moment correlation and 0.5% optimization. To visualize grouping patterns based on each factor (sample type, treatment), multidimensional scaling (MDS) was performed. In order to statistically quantify groupings seen in MDS plots, analysis of similarities (ANOSIM) was performed using Primer v6 [46]. The results of these analyses were used to determine whether sample type or extraction method had a larger influence on bacterial community structure.

Repeatability was calculated based on the fingerprint similarity among replicates. Percentage similarity was determined from the similarity matrix obtained from Pearson product-moment correlations and UPGMA analysis. For each set of replicates, three percent similarities were recorded. For example, samples 31, 32, and 33 were three replicates from bluegill intestine, preserved by freezing, and extracted with the stool kit. For these samples, similarities were recorded between 31 & 32, 31 & 33, and 32 & 33. These numbers would be analyzed for overall similarity between replicates, with higher percentages representing better repeatability.

Fingerprint patterns were visually inspected for quality. All fingerprints were aligned by factor and the best overall pattern was determined with optimal images having dark, distinct bands that stood out well against the background. Consistencies in banding

pattern between replicates as well as presence/absence of bands in comparison with other treatments were also considered. The number of bands in a RISA fingerprint reflects the bacterial diversity within a sample [47]. Thus to determine which method revealed the most diversity, we compared the number of bands for each factor. Only bands with greater than five times background intensity were included in the analysis.

Statistical Analysis

For each dependent variable, each combination of fish species and sample type was analyzed separately. All significance levels were determined at $\alpha = 0.05$. An ANOVA followed by Tukey's post-hoc pairwise comparisons where significant was performed on each variable (adjusted DNA quantity, 260/280 ratio, number of bands, and repeatability). Transformations were performed where necessary to meet the assumptions of normality and homogeneity of variances. In all variables analyzed, higher numbers meant better performance.

Treatment Scoring

To determine final performance, each treatment was given a score for each dependent variable. As there were 6 treatments, the highest possible score for each category was 6. For adjusted DNA quantity, 260/280 ratio, number of bands, and percent similarity, scores were based off groups resulting from Tukey's pairwise comparisons. Treatments in the highest groups received a score of 6, those in the second highest a 5, etc. If the ANOVA showed no significant effect of treatment, all treatments were given a score of 6. For general use, cheaper and faster treatments received higher scores. Better fingerprint patterns determined by visual inspection received higher scores.

Results and Discussion

Sample Type

MDS plots suggested that treatment (Figure 2-1B) had more influence on bacterial community structure than sample type (Figure 2-1A) in all fish species. These results are supported by ANOSIM in all cases. For sample type, R values were 0.027, 0.084, and 0.117 for largemouth bass, bluegill, and channel catfish, respectively. R values for treatment were 0.555, 0.682, and 0.606 for largemouth bass, bluegill, and channel catfish, respectively. The small R values (< 0.25) associated with sample type indicate high overlap in communities, rendering them nearly indistinguishable [46]. However, treatment R values were > 0.5 in all cases, suggesting slight overlap but clear separation. Thus, choice of sample treatment appears to have more influence over microbiota results than does choice of sample type.

It is common practice to use rinsed intestinal tissue to examine the autochthonous gut bacteria and fecal material to analyze allochthonous bacteria [12]. Few studies have looked at both comparatively [12, 15, 20, 48, 49] and each of these analyzed only one fish species. Findings differed between adherent and non-adherent communities in terms of bacterial abundance and diversity, but most studies found distinct communities in feces versus intestinal tissue. This study showed that differences among extraction methods were higher than those between sample types; however our final results do not refute the notion that these communities are different. Various studies have found differences in extraction efficiency based upon bacterial species [50, 51]. Particularly, the thick layer of peptidoglycan in Gram-positive bacteria may make them more difficult to

lyse [50], thus reducing their detection in downstream analysis. Although both kits include treatments for the lysis of cell walls of Gram-positive bacteria, manufacturer instructions indicate that longer incubation times [52] or increased temperature at the lysis step [53] may improve lysis of difficult bacteria. As for storage method, fresh and RNA*later*® samples may have maintained the integrity of weaker and more easily lysed cells until time of extraction thus detecting their presence where frozen samples could not. Therefore it may be the bacterial species composition of the sample types themselves that caused discrepancies in the efficiency of the treatments. Future studies could examine the reasons behind these differences through identification of bacterial species whose extraction efficiencies differ between methodologies.

General Use

Treatments involving RNA*later*® and/or the stool kit were more expensive due to cost of materials. The added cost of RNA*later*® depended on sample size (mass) as the manufacturer recommends a 10:1 ratio of reagent to sample. As a result, storage in RNA*later*® adds more cost per sample when used with the stool kit (requires 220 mg tissue) then when used with the tissue kit (requires 25 mg). In addition, the stool kit costs more per sample than the tissue kit. As a result, RS samples were the most expensive while FT and ZT samples were the least expensive. There is no difference in time for the extraction steps of each storage method, however the tissue kit takes approximately an hour longer to execute than the stool kit because of lysis and proteinase K digestion required for Gram-positive bacteria DNA extraction whereas these steps are included in the stool kit standard extraction protocol. The extra time required to extract samples using the tissue kit does not prevent extractions from being completed within a few hours and

the increased cost of the RNA*later*® buffer and the stool kit are not likely to restrict use of these products except in the case of very large sample sets. Thus these factors are likely not critical when choosing extraction methods for a study.

DNA Quantity

Total DNA yield ranged from 13.3-84.3 ng/µL in feces samples and 10.9-237.1 ng/µL in intestine samples. An ANOVA for adjusted DNA quantity extracted by each treatment showed a significantly higher amount of DNA was consistently extracted by the tissue kit than by the stool kit regardless of storage method. Within each kit, fresh samples had the tendency to yield more DNA than other storage methods. Although treatments including the tissue kit consistently resulted in higher DNA yields, it is worth noting that these quantities include bacterial DNA as well as that from fish tissue and other organisms present in the gut at the time of sampling. Also the differences in yields did not appear to impact downstream analysis (higher yields were not indicative of higher diversity).

DNA Quality

DNA quality was analyzed in terms of 260/280 ratios, where lower ratios indicate contamination by protein or reagents [54]. Ratios ranged from 1.61-2.33 in feces and 1.63-2.18 in intestine. Although there were significant differences in ratios in bluegill and channel catfish, all ratios were higher than 1.6 and these differences did not seem to impact downstream analysis.

Repeatability, Diversity and Fingerprint Quality

Only largemouth bass fecal samples showed a significant difference in repeatability with the RT treatment being significantly less repeatable than FT and ZS.

Average number of bands in RISA fingerprint patterns (reflecting bacterial diversity) indicated the RS treatment always detected the highest diversity in feces but results were more variable in intestine samples (see Tables 2-1 & 2-2). Number of bands was not significantly different in intestine samples from either bluegill or catfish. FS and RS were in the highest group in all significant tests despite sample type, while ZT was always in the lowest group. The RISA profiles differed in band presence, intensity, and profile consistency between treatments (Figure 2-2 through 2-7) but the fingerprint quality results correlated well with band number as high molecular weight bands were typically present in both RS and FS treatments, but were commonly reduced in intensity or missing in other treatments. RS and FS were also relatively consistent between replicates. Overall RS and FS profiles were often rated as the top two treatments in feces and were in the highest group in number of bands in both sample types. However in intestine, FT profiles maintained the high molecular weight bands and consistency, followed or matched by RS. In both sample types RT samples were often inconsistent, while ZT and ZS samples were lacking in profile intensity. ZT, ZS, and RT treatments consistently lost the higher molecular weight bands as well as much of the diversity in the low to mid ranges.

Our results, showing a distorted bacterial community associated with frozen samples in both feces and intestine, are consistent with other studies [55-57]. However storing samples in RNA*later*® prior to freezing seemed to eliminate this bias and produced results more similar to fresh samples extracted with the same kit. The better performance of the stool kit in feces samples is consistent with the presence of PCR inhibitors as the stool kit has reagents specially formulated to remove fecal PCR inhibitors whereas the tissue kit is namely for use in tissues and blood. Similarly, the

better performance of the tissue kit in intestine may be due to the lack of inhibitors present in the intestinal wall, although we have not explored this possibility. It should be noted that storage of intestine samples in RNA*later*® led to reduced visual fingerprint quality. This buffer has been demonstrated to bias bacterial community structure results [58] but the mechanism behind these impacts and why they were only apparent in intestine samples warrants further exploration.

In this study, the relationship between fresh and RNA*later*® buffered samples seems to be dependent upon sample type, as both storage methods performed similarly in feces, but FS outperformed other treatments in intestine. Other studies that examined the influence of DNA extraction methods on bacterial community analysis only looked at fecal samples [25, 59-61] or fresh *versus* cold storage [55, 57, 62]. In human studies, fecal samples are commonly used due to the low-invasive nature of the sampling as well as its reflection of the bacteria associated with the gastrointestinal tract [26, 59]. To our knowledge, no study has examined the impact of methodology on gut wall samples. Similarly the relationship between fresh samples and those stored in RNA*later*® is underexplored. Interestingly the results of this study suggest a possible interaction between storage/extraction and sample type that future studies should investigate further.

There are inhibitors inherently found in feces [63], but these may differ by individual [50], diet [60, 64], and extraction method [50]. Monteiro *et al*. [64] found that plants in the diet increased the number of PCR inhibitors found in fecal material. Our analysis included three fish species with varying diets, although channel catfish of the size we sampled (on average 602 mm total length) have a diet mainly consisting of fish [33] so their diets may have overlapped highly with the largemouth bass. However we

may have expected differences in fish species in terms of treatment performance. Although there were slight differences in treatments between fish species, overall patterns were similar to those of the final results.

Recommendations and Conclusion

Final score tabulations (Tables 2-3 & 2-4) show obvious differences in treatments. ZT and ZS scored lowest among all treatments in both feces and intestine. RS and FS both received the highest score for feces. For intestine samples FT samples scored highest. Overall, we recommend use of fresh samples extracted with the stool kit in analysis of fish feces. If the sampling environment does not allow for fresh samples, RNA*later*® is a reliable substitute. For intestine samples, we recommend using fresh samples extracted with the tissue kit.

This study demonstrated the impact of storage method and DNA extraction kit on fish gut microbiota analysis using RISA. Although intestine and feces are believed to contain different microbial communities, methods of extraction had more of an impact on the structure of the communities than did sample type. Treatment impacted downstream RISA analysis with overall results differing between feces and intestine samples. These differences have the potential to bias results of community analysis, and thus should be carefully considered when planning these types of studies.

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Table 2-1. ANOVA and Tukey's post-hoc analysis results of feces sample type from each fish species. Data was transformed where necessary to meet ANOVA assumptions. Averages included in the table are derived from the raw data. Visual fingerprint groups are based off visual inspection of fingerprint patterns. Significant P values are indicated in bold type.

	Storage Method	Extraction Kit	Adjusted DNA Quantity		260/280		# bands		Repeatability		Visual pattern
			Average	Group	Average	Group	Average	Group	Average	Group	Group
Largemouth bass	Fresh	Tissue	2.69	A	1.93	A	3.33	BC	95.4	A	B
		Stool	0.13	B	1.92	A	8.67	AB	79.5	AB	A
	RNA*later* ®	Tissue	1.94	A	2.02	A	4.33	BC	70.1	B	D
		Stool	0.13	B	2.08	A	13.3	A	79.3	AB	A
	Frozen	Tissue	2.03	A	2.15	A	2.33	C	85.7	AB	C
		Stool	0.08	C	2.09	A	2	C	93	A	C
		P Value	**< 0.0001**		0.197		**0.002**		**0.015**		NA
Bluegill	Fresh	Tissue	2.61	A	1.78	D	7	B	79.9	A	C
		Stool	0.15	C	2.06	A	17.7	A	81.2	A	A
	RNA*later* ®	Tissue	1.67	B	1.92	BC	14.3	AB	79.6	A	B
		Stool	0.13	C	2.08	A	18.7	A	81.2	A	A
	Frozen	Tissue	1.9	AB	1.82	CD	8.33	B	77.3	A	E
		Stool	0.12	C	2.02	AB	14.3	AB	83.1	A	D
		P Value	**< 0.0001**		**< 0.0001**		**0.005**		0.968		NA
Channel catfish	Fresh	Tissue	2.78	A	1.74	CD	5.33	B	87.9	A	B
		Stool	0.14	C	1.91	B	14.7	A	73.9	A	A
	RNA*later* ®	Tissue	1.35	B	1.89	BC	8.33	AB	76.3	A	C
		Stool	0.08	CD	2.09	A	15	A	77.4	A	A
	Frozen	Tissue	2.82	A	1.65	D	4.67	B	72.7	A	E
		Stool	0.08	D	1.97	AB	6	B	74.8	A	D
		P Value	**< 0.0001**		**< 0.0001**		**0.001**		0.292		NA

Table 2-2. ANOVA and Tukey's post-hoc analysis results of intestine sample type from each fish species. Data was transformed where necessary to meet ANOVA assumptions. Averages included in the table are derived from the raw data. Visual fingerprint groups are based off visual inspection of fingerprint patterns. Significant P values are indicated in bold type.

	Storage Method	Extraction Kit	Adjusted DNA Quantity		260/280		# bands		Repeatability		Visual pattern
			Average	Group	Average	Group	Average	Group	Average	Group	Group
Largemouth Bass	Fresh	Tissue	1.94	A	1.79	A	6.33	ABC	83	A	A
		Stool	0.18	B	2.04	A	9.33	A	87.7	A	A
	RNA*later* ®	Tissue	1.71	A	1.87	A	3	BC	67	A	C
		Stool	0.07	C	1.87	A	7	A	88.2	A	A
	Frozen	Tissue	1.43	A	1.89	A	2	C	66.4	A	D
		Stool	0.07	C	1.89	A	4.33	BC	86.8	A	B
		P Value	**< 0.0001**		0.129		**0.003**		0.317		NA
Bluegill	Fresh	Tissue	1.78	A	1.84	B	5.33	A	72	A	A
		Stool	0.36	B	2.06	A	4	A	50	A	D
	RNA*later* ®	Tissue	1.24	A	1.99	AB	5	A	57	A	D
		Stool	0.12	C	2.09	A	11	A	73.6	A	A
	Frozen	Tissue	1.28	A	1.93	AB	3.67	A	68.5	A	D
		Stool	0.08	C	1.96	AB	6.67	A	71.9	A	C
		P Value	**< 0.0001**		**0.012**		0.102		0.412		NA
Channel catfish	Fresh	Tissue	2.26	A	1.75	BC	11	A	85.8	A	A
		Stool	0.3	BC	2.02	A	7	A	79.4	A	C
	RNA*later* ®	Tissue	4.15	AB	1.92	AB	11.3	A	77.3	A	B
		Stool	0.16	CD	2	A	10.7	A	71.8	A	B
	Frozen	Tissue	2.02	AB	1.72	C	4.33	A	65.8	A	E
		Stool	0.08	D	2.01	A	4.67	A	69.4	A	D
		P Value	**< 0.0001**		**0.001**		0.349		0.521		NA

Table 2-3. Final scores for feces sample type from all fish species. Scores are determined by Tukey's post-hoc analysis for all variables except visual quality whose scores are based off of visual comparison of RISA fingerprints. Higher scores indicate better performance.

		Fresh, Tissue kit	Fresh, Stool kit	RNA*later* ®, Tissue kit	RNA*later* ®, Stool kit	Frozen, Tissue kit	Frozen, Stool kit
	Cost per sample	6	4	5	3	6	4
	Time to extracted DNA	5	6	5	6	5	6
Largemouth bass	Adjusted DNA quantity	6	5	6	5	6	4
	260/280 ratio	6	6	6	6	6	6
	# bands	5	6	5	6	4	4
	Repeatability	6	6	5	6	6	6
	Visual quality	5	6	3	6	4	4
Bluegill	Adjusted DNA quantity	6	4	5	4	6	4
	260/280 ratio	3	6	5	6	4	6
	# bands	5	6	6	6	5	6
	Repeatability	6	6	6	6	6	6
	Visual quality	4	6	5	6	2	3
Channel catfish	Adjusted DNA quantity	6	4	5	4	6	3
	260/280 ratio	4	5	5	6	3	6
	# bands	5	6	6	6	5	5
	Repeatability	6	6	6	6	6	6
	Visual quality	5	6	4	6	2	3
	Total	89	94	88	94	82	82

Table 2-4. Final scores for intestine sample type from all fish species. Scores are determined by Tukey's post-hoc analysis for all variables except visual quality whose scores are based off of visual comparison of RISA fingerprints. Higher scores indicate better performance.

		Fresh, Tissue kit	Fresh, Stool kit	RNA*later*®, Tissue kit	RNA*later*®, Stool kit	Frozen, Tissue kit	Frozen, Stool kit
	Cost per sample	6	4	5	3	6	4
	Time to extracted DNA	5	6	5	6	5	6
Largemouth bass	Adjusted DNA quantity	6	5	6	4	6	4
	260/280 ratio	6	6	6	6	6	6
	# bands	6	6	5	6	4	5
	Repeatability	6	6	6	6	6	6
	Visual quality	6	6	4	6	3	5
Bluegill	Adjusted DNA quantity	6	5	6	4	6	4
	260/280 ratio	5	6	6	6	6	6
	# bands	6	6	6	6	6	6
	Repeatability	6	6	6	6	6	6
	Visual quality	6	3	3	5	3	4
Channel catfish	Adjusted DNA quantity	6	5	6	4	6	3
	260/280 ratio	5	6	6	6	4	6
	# bands	6	6	6	6	6	6
	Repeatability	6	6	6	6	6	6
	Visual quality	6	4	5	5	2	3
	Total	99	92	93	91	87	86

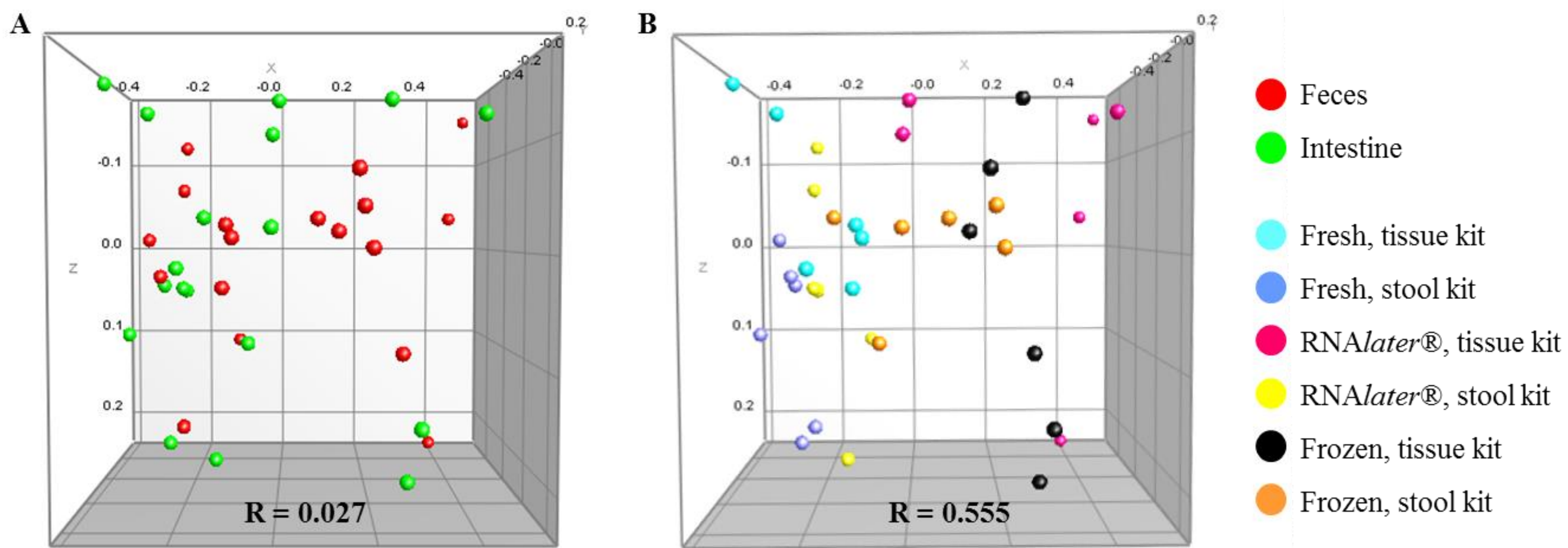

Figure 2-1. Multidimensional scaling (MDS) by factor for largemouth bass samples. Analysis of similarities results are indicated by the R value. Results were similar among all fish species. A, sample type; B, extraction method.

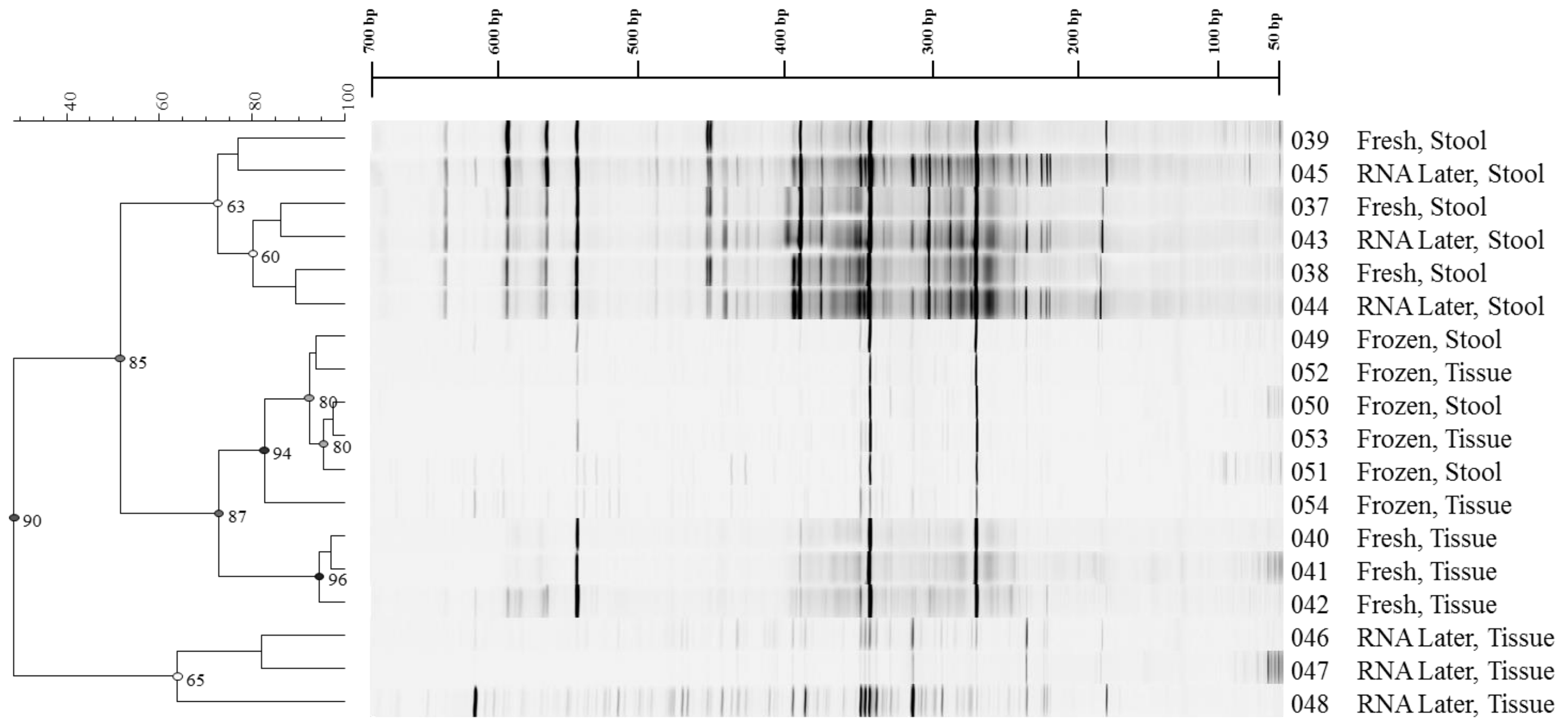

Figure 2-2. Dendrogram representing percent similarity between RISA fingerprints for largemouth bass sample type feces. Cophenetic correlations are indicated at the nodes.

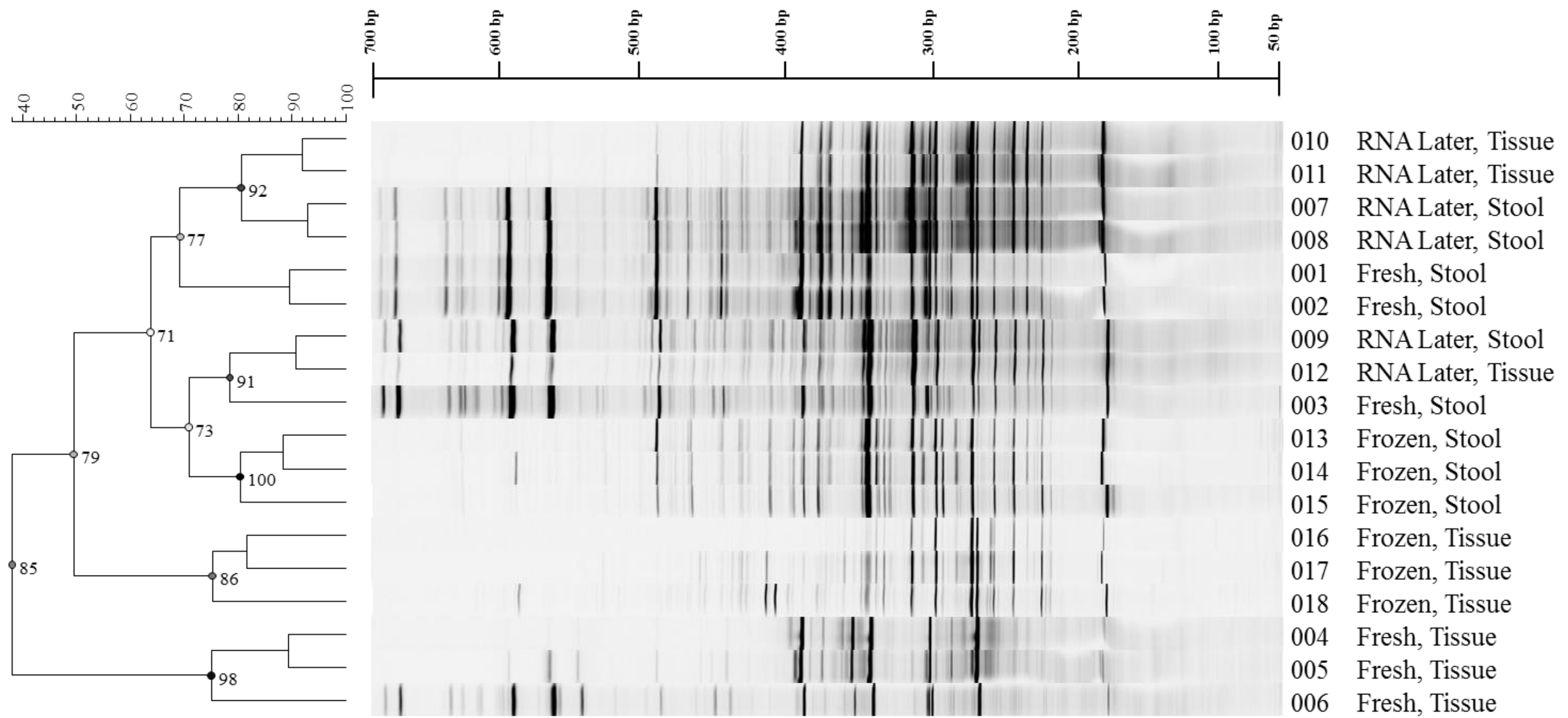

Figure 2-3. Dendrogram representing percent similarity between RISA fingerprints for bluegill sample type feces. Cophenetic correlations are indicated at the nodes.

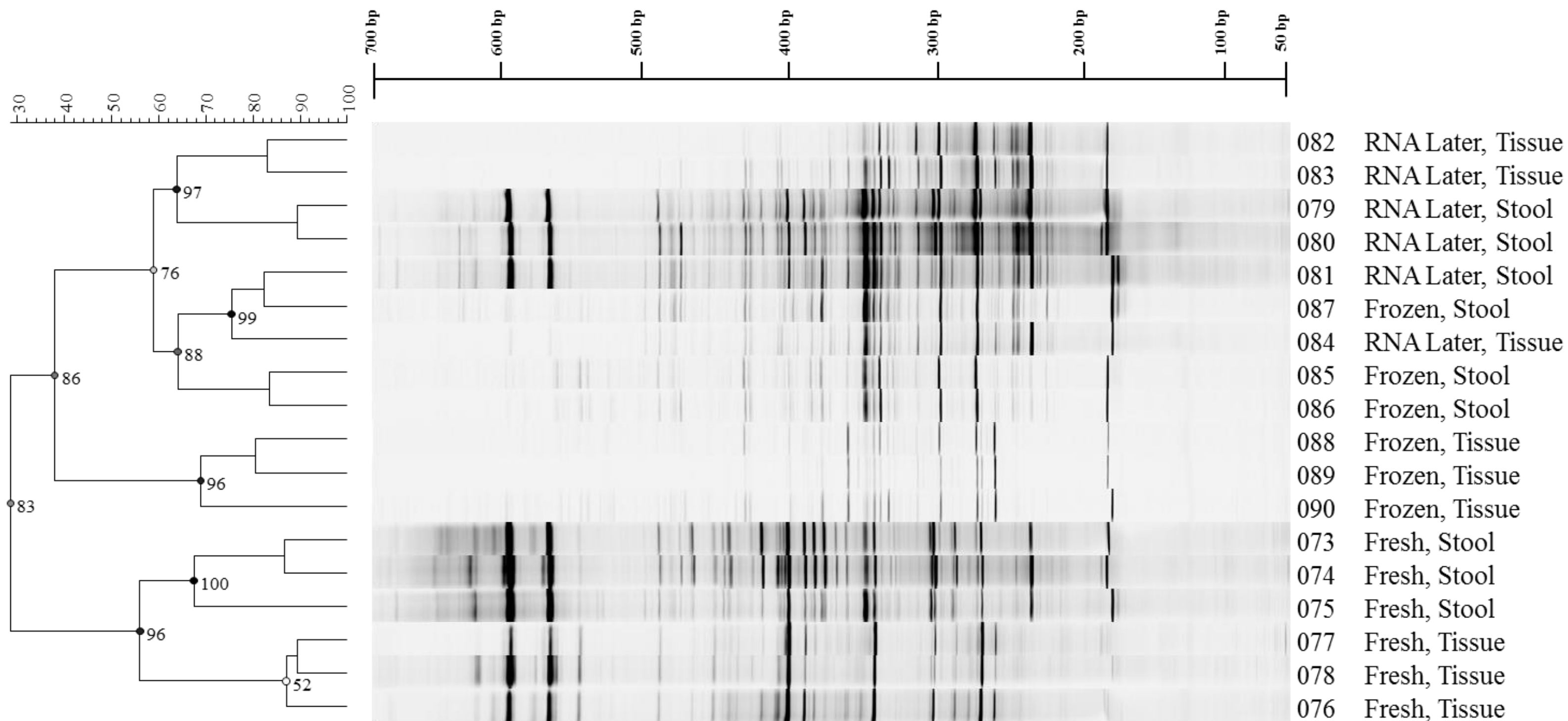

Figure 2-4. Dendrogram representing percent similarity between RISA fingerprints for channel catfish sample type feces. Cophenetic correlations are indicated at the nodes.

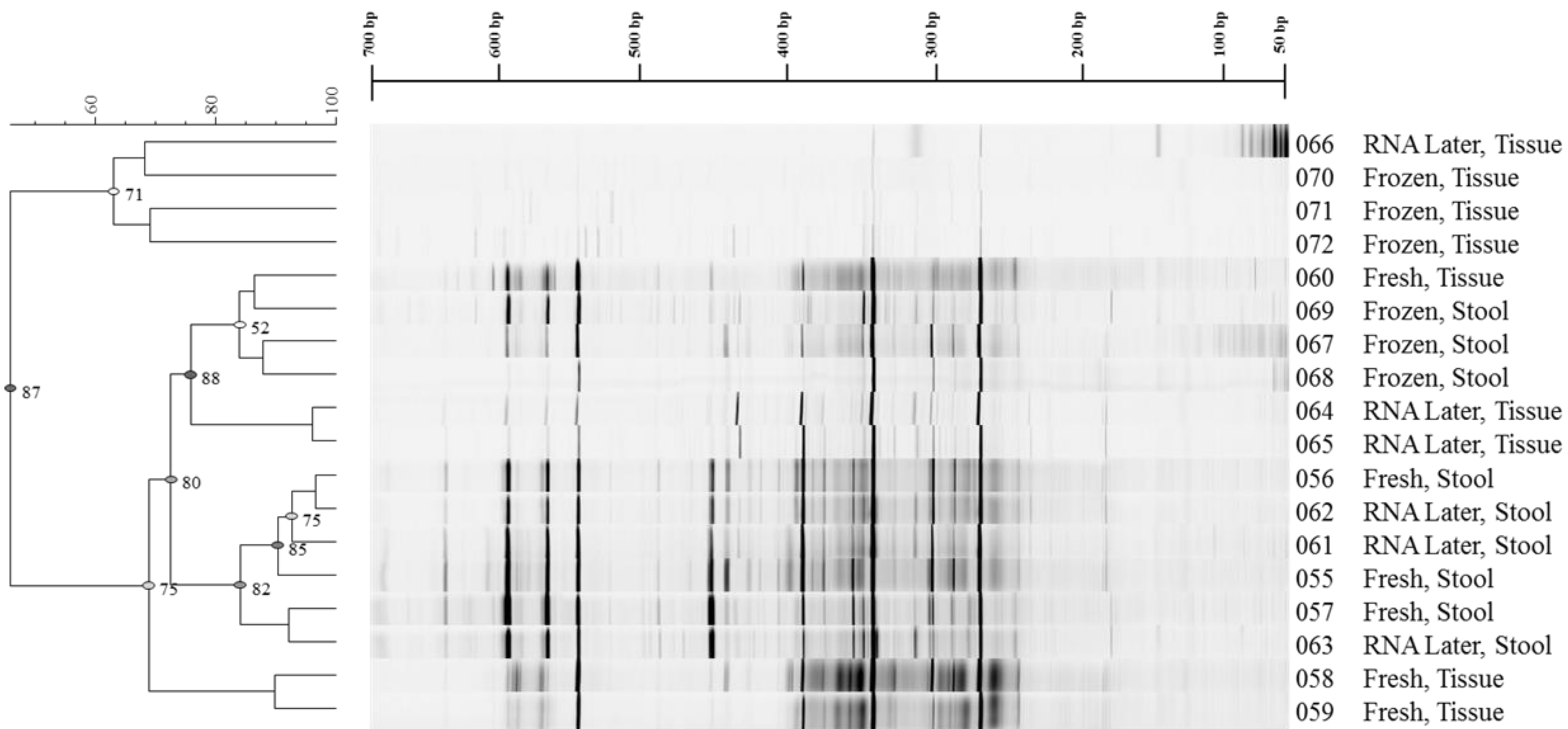

Figure 2-5. Dendrogram representing percent similarity between RISA fingerprints for largemouth bass sample type intestine. Cophenetic correlations are indicated at the nodes.

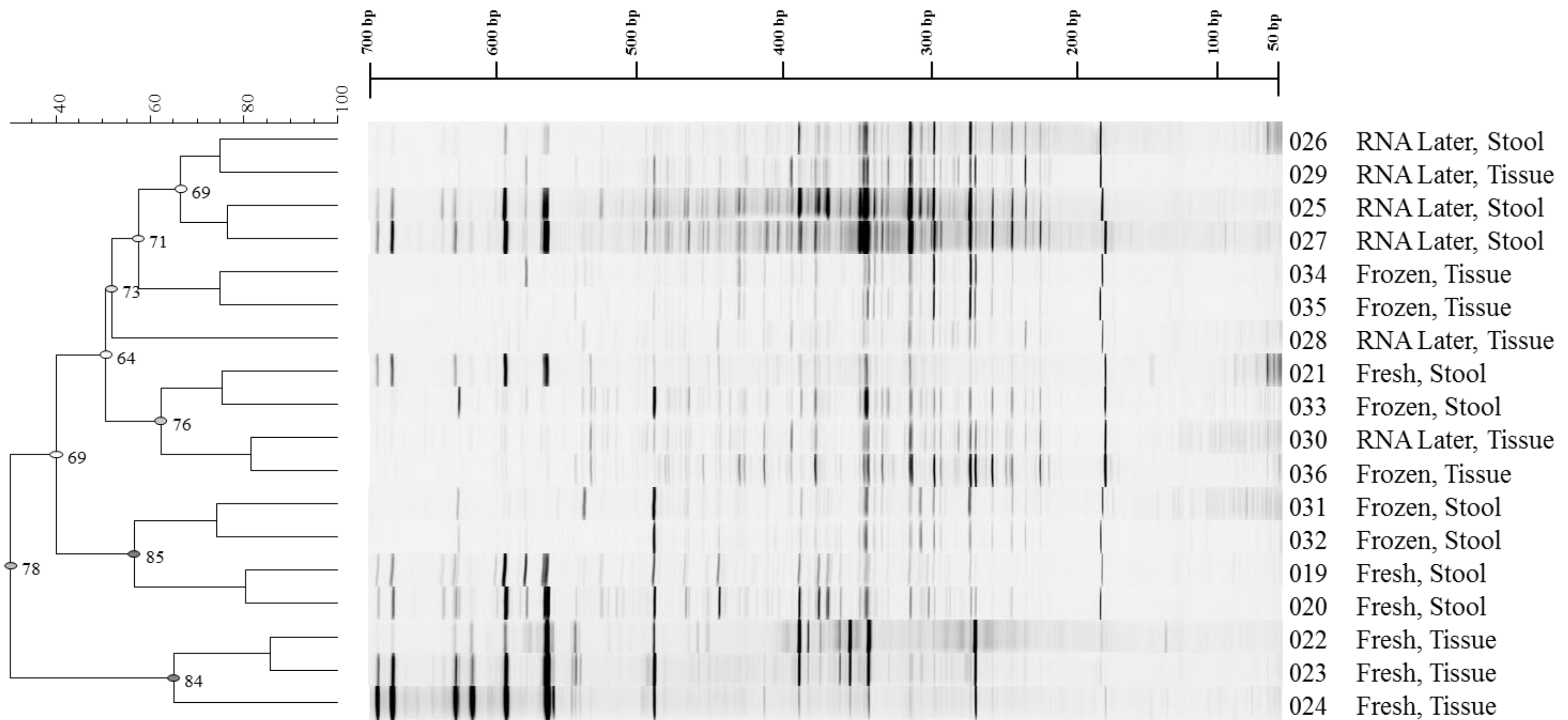

Figure 2-6. Dendrogram representing percent similarity between RISA fingerprints for bluegill sample type intestine. Cophenetic correlations are indicated at the nodes.

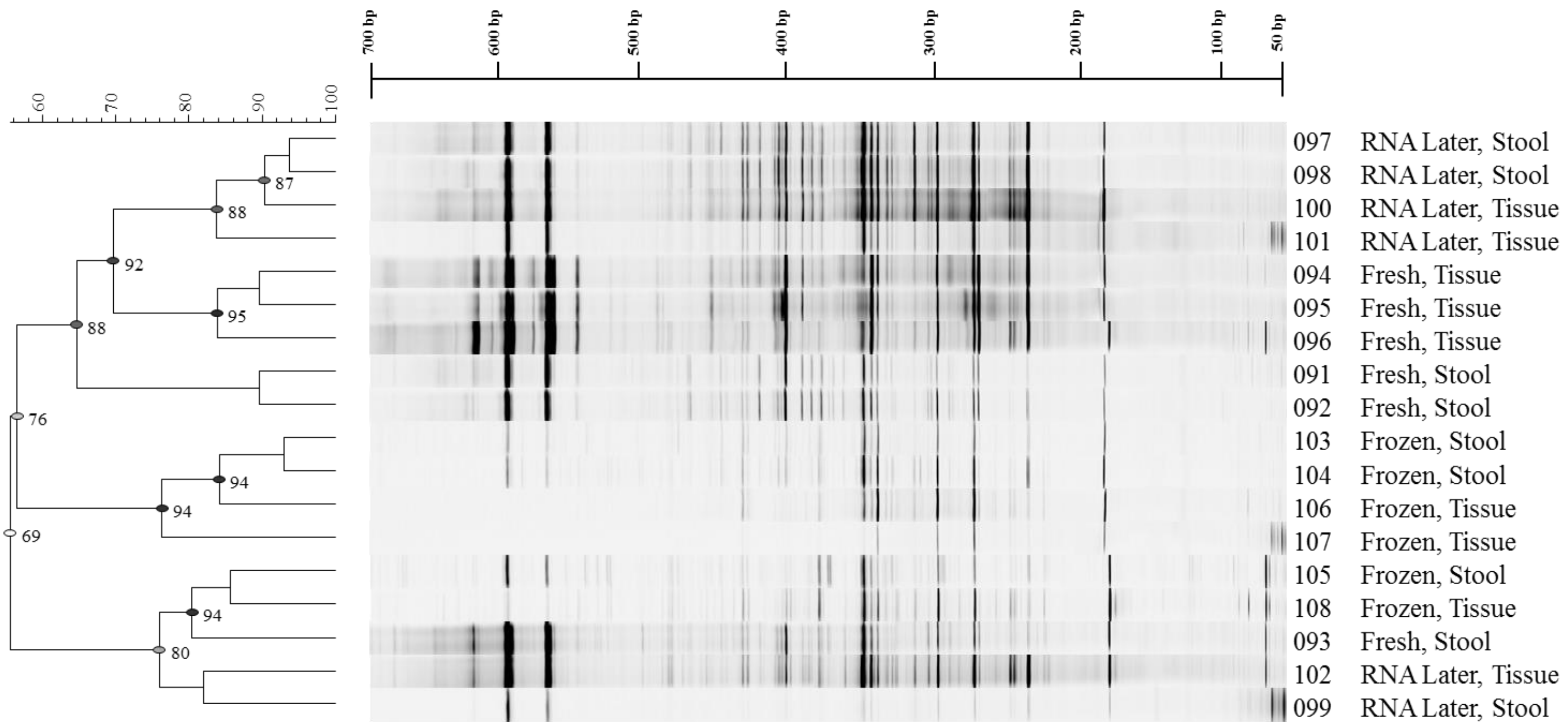

Figure 2-7. Dendrogram representing percent similarity between RISA fingerprints for channel catfish sample type intestine. Cophenetic correlations are indicated at the nodes.

CHAPTER 3. CHARACTERIZATION OF THE GUT MICROBIOTA OF THREE COMMERCIALLY VALUABLE WARMWATER FISH SPECIES

Abstract

Due to the strong influence of the gut microbiota on fish health, dominant bacterial species in the gut are strong candidates for probiotics. This study aimed to characterize the gut microbiota of channel catfish *Ictalurus punctatus*, largemouth bass *Micropterus salmoides*, and bluegill *Lepomis macrochirus* to provide a baseline for future probiotic studies. The gut microbiota of five pooled individuals from each fish species was identified using 16S rRNA pyrosequencing. Microbiota differed significantly between fish species in terms of bacterial species evenness. However, all gut communities analyzed were dominated by the phylum Fusobacteria, specifically the species *Cetobacterium somerae*. Relatively high abundances of the human pathogens *Plesiomonas shigelloides* and *Fusobacterium mortiferum*, as well as members of the genus *Aeromonas*, suggest these species are normal inhabitants of the gut. The overwhelming dominance of the genus *Cetobacterium* in all species warrants further investigation into its role in the fish gut microbiota. This study provides the first characterization of the gut microbiota of three economically significant fishes and establishes a baseline for future probiotic trials.

Introduction

Aquaculture is the fastest growing animal food-producing sector and is set to overtake capture fisheries as a source of food fish [1]. Currently, one of the main factors limiting expansion and profitability of aquaculture is lack of disease control [2]. In the USA, treatment options against bacterial diseases are limited to four US Food and Drug Administration (FDA) approved drugs, three of which are antibiotics [3]. Growing concern over the presence of antibiotic compounds in foods and an increase in antibiotic resistant microbes has led to an interest in alternatives to antibiotics such as probiotics for treatment and prevention of diseases [4, 5]. Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal balance. The goal of administering probiotics is to manipulate the gut microbiota to improve the fitness of the host, mainly through the exclusion of opportunistic pathogens [5]. However, the gut microbiota strongly influences fish health in other ways such as assisting in the development of the gut epithelium, providing essential nutrients, and stimulating the innate immune system [6]. Thus, alteration of gut bacterial communities with probiotics may prevent disease through a variety of mechanisms.

In choosing potential probiotics, dominant strains from fish species of interest are often good candidates [7]. Nevertheless, most probiotics used in aquaculture include lactic acid bacteria and members of the genus *Bacillus* isolated from mammals or terrestrial environments [7]. Although there are several examples where exogenous bacteria proved beneficial for fish, many studies using lactic acid bacteria or *Bacillus* were inconclusive or showed no beneficial effect on the host [8, 9]. Recent studies [10-12] have shown that 'host species' and not the environment is the primary

driving force shaping microbial communities in fishes. Hence, the microbial communities of each aquaculture species should be fully characterized to identify significant changes produced by the administration of probiotics, and to provide targets for the development of new probiotics.

In the USA, the top aquaculture species is channel catfish *Ictalurus punctatus* (Rafinesque 1818) with an estimated value of over $390 million annually [13]. In addition, aquaculture not only provides fish for the food market but also individuals for stocking of recreational fishing ponds. When it comes to recreational fishing, angler surveys indicate the top targeted species in the USA include largemouth bass *Micropterus salmoides* (Lacepède 1802), and panfish such as bluegill *Lepomis macrochirus* Rafinesque 1819 [14]. Despite their economic significance, the normal gut microbiota of *I. punctatus*, *M. salmoides*, and *L. macrochirus* have not been characterized, and thus dominant community members that are potential targets for the development of probiotics aimed at these fish species have not been determined.

The purpose of this study was to compare the bacterial diversity associated with the gut of three commercially valuable warmwater fish species *I. punctatus*, *M. salmoides*, and *L. macrochirus* using pyrosequencing to provide a baseline for future probiotic studies. We collected sympatric individuals from an experimental recreational fishing pond to minimize the effect of the local environment on the gut microbiota [15-17].

Methods

Sample collection

Sampling for all fish species occurred at Auburn University's E. W. Shell Research Station pond S8 in Auburn, Alabama (32°40'18.7"N, 85°30'36.00"W) in February 2012. Pond S8 is an experimental recreational fishing pond with limited access that was stocked in 1991 with *M. salmoides* and *L. macrochirus* reared at a state hatchery (Dr. DeVries, Auburn University, personal communication). Yearling *L. macrochirus* were stocked at 2500 fish/ha in spring and age-0 *M. salmoides* were stocked in fall at 250 fish/ha. *Ictalurus punctatus* individuals reached pond S8 as escapees from nearby aquaculture ponds and have maintained a constant population since the late 90s. Post-stocking, fish were allowed to exist naturally without artificial feeding. Five individuals each of *I. punctatus*, *L. macrochirus*, and *M. salmoides* were captured as follows. Catfish jugs were baited, set in the evening and allowed to fish overnight for approximately 15 h to collect *I. punctatus*. *Micropterus salmoides* and *L. macrochirus* were caught on baited hooks and spinning reels. Fish were kept alive in separate aerated coolers filled with lake water until processing (approximately 3 hours). Total lengths of sampled fish are given in Table 3-1.

DNA extraction

Upon arrival at the laboratory, fish were immediately euthanized with an overdose of tricaine methanesulfonate (300 mg/L). The lower one-third of the intestine was aseptically removed and the contents squeezed into a sterile tube. All five individuals of each species were pooled to form a single sample. This sample was homogenized for 2 min with a hand-held homogenizer. To account for intrinsic variability associated with DNA extraction and downstream nucleic acid analysis,

each fish sample was divided into 3 subsamples of 25 mg. These replicates were immediately subjected to DNA extraction with the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) following manufacturer's instructions, including pretreatment for lysis of Gram-positive bacteria. DNA was quantified using a NanoDrop ND-1000 (Thermo Scientific, Rochester, USA).

Bacterial community composition determined by sequencing

Samples were subjected to Roche titanium 454 sequencing of the 16S rRNA using individual barcodes and primer 27F (5'-AGRGTTTGATCMTGGCTCAG-3'). Resulting sequences were processed using an exclusive analysis pipeline (MR DNA, Shallowater, TX). Barcodes and primers were removed from the sequences. Sequences with <Q25 (base call error rate less than 0.3%), <200 base pairs in length, ambiguous base calls, and stretches of identical bases longer than 6 base pairs were removed. Denoising was performed, followed by removal of chimeras and singleton sequences. Cutoff for operational taxonomic units (OTUs) was defined at a <3% sequence difference in agreement with the current accepted prokaryote species concept [18]. OTUs were taxonomically identified using BLASTn against the Greengenes database [19]. As species richness and evenness can only be compared between samples when sample sizes are equal [20], resulting sequences were randomly selected so as to standardize to the sample with the least number of obtained sequences (N = 2109). Rarefaction curves, Good´s coverage, abundance-based coverage estimation (ACE), Chao1, Shannon evenness, and shared OTUs based on defined OTUs were generated using Mothur v.1.30.0 [21]. Rarefaction curves were standardized to the sample yielding the least number of total sequences. A one-way ANOVA was performed on all diversity indices, followed by a Tukey's post hoc test where significant ($P < 0.05$). A genera abundance table was loaded into Primer v6

[22] and similarity percentages (SIMPER) analysis was performed to determine the genera responsible for differences between fish species. Cut-off for low contributions was set at the default 90%.

Results

Diversity analysis

Pyrosequencing yielded a total of 58,164 bacterial sequences and 311 OTUs. After standardizing for sample size, 18,981 sequences remained with a total of 278 OTUs. Sequence coverage was ≥97% in all cases (Table 3-2), supported by the rarefaction curves generated by Mothur (Figure 3-1). Total expected richness as calculated by ACE and Chao1 did not differ significantly by fish species. However, the Shannon evenness index was significantly higher in *L. macrochirus* and *I. punctatus* than in *M. salmoides*. Over 38% of all OTUs were shared by all three fish species (Figure 3-2). *M. salmoides* shared the least with the other two species, and also had the highest number of unique OTUs.

Gut microbiota composition

Eight bacterial phyla were identified from the gut content of all fish species (Figure 3-3). From each fish species, the phylum Fusobacteria made up the majority of all sequences (82.6% in *L. macrochirus*, 90.6% in *M. salmoides*, and 94.9% in *I. punctatus*). Proteobacteria was the second most common phylum, varying in abundance from 5-16%. Within the Proteobacteria, each fish microbiota was composed of mostly Gammaproteobacteria, followed by Betaproteobacteria and Alphaproteobacteria. The less common phyla varied in abundances between fish species, with some unique members of each community. For example, only *M.*

salmoides contained Actinobacteria while *L. macrochirus* and *I. punctatus* lacked representatives from the Cyanobacteria and from the “Spring Alpine Meadow” candidate division, respectively.

The gut microbiota of each fish species was composed of 11 shared genera and 2-4 unique genera per fish species (Table 3-3). Of these, most sequences were identified as *Cetobacterium*. Other relatively abundant genera included *Aeromonas* and *Fusobacterium* in *L. macrochirus, Aeromonas* in *I. punctatus*, and *Plesiomonas* in *M. salmoides*. The 11 shared genera made up >98% of all identified sequences in each species, suggesting highly similar bacterial composition in the gut of these fishes at the genus level.

SIMPER analysis (Table 3-4) by bacterial genera indicated the largest difference in gut community composition between *L. macrochirus* and *M. salmoides*. A majority of the differences between all fish species were due to varying abundances of the genus *Cetobacterium*. Within this genus, all identified sequences shared high 16S sequence similarity with the bacterium *C. somerae*. High numbers of *Fusobacterium* in *L. macrochirus* as compared to *I. punctatus* contributed to the dissimilarity in gut microbiota between these two species. *Fusobacterium mortiferum* contributed the majority of sequences from this genus in *L. macrochirus*, while *I. punctatus* had approximately equal abundances of *F. mortiferum* and *F. gonidiaformans.* Relatively high abundances of *Plesiomonas* spp. in *M. salmoides* contributed to the clearer separation between this species and the other two fish species. All sequences within the genus *Plesiomonas* were closely related to *P. shigelloides*. Similarly, relatively high abundances of *Aeromonas* spp. in *L. macrochirus* separated this fish species from the others. Most *Aeromonas* species

cannot be differentiated by 16S alone [23], and thus species composition of this genus cannot be described in this study.

Discussion

Studies characterizing the microbiota associated with wild fish are often limited to analysis of one fish species [16, 17, 24-28] but results from studies that compared several fish species collected from the same area showed a marked specificity between fish species and microbiota [10, 29, 30]. Our results showed significant differences in bacterial species evenness between the gut microbiota of *L. macrochirus*, *M. salmoides*, and *I. punctatus* that were sharing the same environment and were not artificially fed. These fish species are known to have varying diet preferences and pond S8 offers a balanced ecosystem to satisfy their feeding requirements. Adult *M. salmoides* are primarily piscivorous, consuming fish, including *Lepomis* spp. and crayfish [31-33]. Although *I. punctatus* are omnivorous [34, 35], individuals larger than 300-400 mm are reported to be primarily piscivorous [35, 36]. As all of our *I. punctatus* were larger than 500 mm, they were likely consuming fish as their main diet. Adult *L. macrochirus* are generalists, eating primarily macroinvertebrates and zooplankton, as well as plants and small fish [37-39]. Although stomach content was not analyzed in this study, our patterns potentially reflect these diet differences.

Previous studies have seen differences in fish gut microbiota due to diet [12, 40-43], and an increased diversity from carnivores to omnivores to herbivores has been demonstrated in mammals [44, 45]. We observed an increase in the number of predicted OTUs from carnivory (*M. salmoides* > *I. punctatus*) to herbivory (*L.

macrochirus) but this trend was not statistically significant. Alternatively, species evenness was significantly higher in *L. macrochirus* and *I. punctatus* as compared to *M. salmoides*. McKinney [46] saw an increased evenness in the gut microbiota of omnivorous primates as opposed to carnivorous ones. However, this statistic is rarely reported in these types of studies thus, the commonality of this pattern is to the best of our knowledge unknown.

Pyrosequencing identified differences between fish species' gut microbiota in terms of bacterial abundances at each taxonomic level. The phylum Fusobacteria was the main component of all three species' gut communities followed by the Proteobacteria. A few studies have shown Fusobacteria as dominant members of the gut microbiota of freshwater fishes [43, 47] but not at the abundances observed in this study. The Fusobacteria are anaerobic, Gram-negative bacilli that produce butyrate [48], a short chain fatty acid that is often the end-product of the fermentation of carbohydrates including those found in mucins [49, 50]. In mammals, butyrate provides many benefits to the host, including providing a majority of the energy supply to gastrointestinal cells [50, 51], enhancing mucus production, acting as an anti-carcinogen and anti-inflammatory, as well as playing a role in satiation [50, 52-54]. This fatty acid has been found in the gut of herbivorous and omnivorous fishes [55, 56], but is not expected to be present in carnivorous species because of their low carbohydrate diets [57]. Nuez-Ortin et al. [58] demonstrated the ability of butyric acid to inhibit potential freshwater fish pathogens, and sodium butyrate is currently sold as a food additive to promote fish health and growth. However, trials using blends of sodium butyrate and other additives have not proven beneficial [59, 60]. Due to the large proportion of Fusobacteria in all three of these species, future investigations should determine their role in the fish gut microbiota.

Sequences closely related to the bacterium *C. somerae* constituted over 70% of sequences from each fish species. This was a surprising result as it has been demonstrated that bacterial communities whose relative species abundances are near equal are more resilient to environmental stress than those that rely on dominant species for certain functions [61]. *C. somerae*, formerly classified as *Bacteroides* type A [62] are poorly known, microaerotolerant, Gram-negative rods with fermentative metabolism that were originally described from children with late-set autism [63]. Since the original description, *C. somerae* has been found in a variety of freshwater herbivorous fish species [42, 43, 62]. In this environment, the bacterium produces high amounts of vitamin B12 [64]. *C. somerae* is also capable of inhibiting the growth of other bacterial strains [65]. The presence of this bacterium in a number of other freshwater fishes as well as its high abundance in this study warrants further studies into its function in the fish gut.

Interestingly, human pathogens including *F. mortiferum, P. shigelloides* [66, 67], and *Aeromonas* sp. were the second most commonly identified genera in *L. macrochirus*, *M. salmoides*, and *I. punctatus*, respectively. This is the second known study to isolate *F. mortiferum* from the gut of a fish [68] and its role has yet to be examined in fish, however it has also been isolated from wounds caused by catfish spines [69]. On the other hand, *P. shigelloides* seems to be a normal component of the gut of other fishes [65, 68, 70-72]. The genus *Aeromonas* not only includes opportunistic human pathogens but also fish pathogens such as *A. hydrophila* [73].

In summary, this study provides the first characterization of the gut microbiota of the economically significant *I. punctatus*, *M. salmoides*, and *L. macrochirus*. These bacterial communities were isolated from wild individuals from the same lake. The microbiota composition, despite sharing a high percentage of the same bacterial

genera, differed in evenness between fish species. Despite their differences, all three fish species harbored by vast majority the species *C. somerae,* sparking interest in its role in the fish gut. Studies are currently underway to isolate and further characterize this bacterium.

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Table 3-1. Total length (mm) of individual fish sampled for each fish species.

Fish Species	Individual Number	Total Length (mm)
Ictalurus punctatus	1	660
	2	584
	3	559
	4	584
	5	625
Micropterus salmoides	1	356
	2	381
	3	356
	4	406
	5	305
Lepomis macrochirus	1	152
	2	179
	3	203
	4	179
	5	179

Table 3-2. Diversity indexes as calculated by Mothur software (v.1.30.0). Operational taxonomic units (OTUs) are defined at 97% sequence similarity. ACE, abundance-based coverage estimation. Significance among total values for each fish species was determined by a one-way ANOVA followed by Tukey's post hoc test.

Sample ID	Fish Species	# Observed OTUs	# Predicted OTUs ACE	# Predicted OTUs Chao1	Shannon evenness	Good's Coverage
4	*Lepomis macrochirus*	146	198	218	0.757	0.978
5	*Lepomis macrochirus*	160	250	208	0.727	0.975
6	*Lepomis macrochirus*	141	187	199	0.732	0.979
40	*Micropterus salmoides*	137	177	173	0.696	0.981
41	*Micropterus salmoides*	120	156	164	0.683	0.982
42	*Micropterus salmoides*	131	180	189	0.679	0.978
76	*Ictalurus punctatus*	148	208	218	0.726	0.975
77	*Ictalurus punctatus*	116	183	153	0.729	0.982
78	*Ictalurus punctatus*	143	192	183	0.714	0.978
Totals	*Lepomis macrochirus*	149	212	208	0.739	0.977
	Micropterus salmoides	129	171	175	0.686*	0.980
	Ictalurus punctatus	136	194	185	0.719	0.979

*Statistically significant (ANOVA: $F_{2,6} = 17.52$, $P < 0.01$).

Table 3-3. Genera identified in each fish species represented by percentage of total sequences. Genera accounting for ≥0.01% of sequences in at least one fish species are included. Shared genera are present in all three fish species. Unique genera are present in only one or two fish species.

	Classification				Abundance (%)		
	Phylum	Class	Family	Genus	*Ictalurus punctatus*	*Micropterus salmoides*	*Lepomis macrochirus*
Shared Genera	Fusobacteria	Fusobacteria	Fusobacteriaceae	*Cetobacterium*	94.0	89.9	72.0
	Fusobacteria	Fusobacteria	Fusobacteriaceae	*Fusobacterium*	0.90	0.66	10.56
	Proteobacteria	α-proteobacteria	Methylobacteriaceae	*Methylobacterium*	0.01	0.02	0.03
	Proteobacteria	β-proteobacteria	Neisseriaceae	*Laribacter*	0.03	0.11	2.56
	Proteobacteria	γ-proteobacteria	Aeromonadaceae	*Aeromonas*	3.18	0.06	10.2
	Proteobacteria	γ-proteobacteria	Enterobacteriaceae	*Plesiomonas*	0.39	7.64	2.84
	Proteobacteria	γ-proteobacteria	Enterobacteriaceae	*Serratia*	0.05	0.01	0.18
	Firmicutes	Clostridia	Clostridiaceae	*Clostridium*	0.03	1.24	0.57
	Firmicutes	Clostridia	Lachnospiraceae	*Epulopiscium*	0.02	0.08	0.01
	Bacteroidetes	Bacteroidia	Porphyromonadaceae	*Parabacteroides*	0.15	0.06	0.56
	Tenericutes	Mollicutes	Mycoplasmataceae	*Mycoplasma*	0.04	0.03	0.04
Unique Genera	Actinobacteria	Actinobacteria	Micrococcaceae	*Kocuria*	-	0.01	-
	Proteobacteria	α-proteobacteria	Unspecified	*Pelagibacter*	-	-	0.03
	Proteobacteria	β-proteobacteria	Neisseriaceae	*Deefgea*	< 0.01	-	0.02
	Proteobacteria	γ-proteobacteria	Shewanellaceae	*Shewanella*	0.01	-	< 0.001
	Proteobacteria	γ-proteobacteria	Xanthomonadaceae	*Xanthomonas*	-	-	0.01
	Firmicutes	Bacilli	Staphylococcaceae	*Salinicoccus*	-	0.01	-

Table 3-4. SIMPER analysis between each combination of fish species. Average abundances include all three replicates within each fish species.

Fish Species	Bacteria Genus	Average Abundance		Contribution to Dissimilarity (%)
		Species 1	Species 2	
1. *Lepomis macrochirus* 2. *Micropterus salmoides*	*Cetobacterium*	3244	8669	76.6
	Plesiomonas	128.0	737.0	8.40
	Aeromonas	461.0	6.000	5.80
1. *Lepomis macrochirus* 2. *Ictalurus punctatus*	*Cetobacterium*	3244	4927	71.1
	Fusobacterium	476.0	47.00	11.4
	Aeromonas	461.0	167.0	8.50
1. *Micropterus salmoides* 2. *Ictalurus punctatus*	*Cetobacterium*	8669	4927	75.0
	Plesiomonas	737.0	20.00	15.5

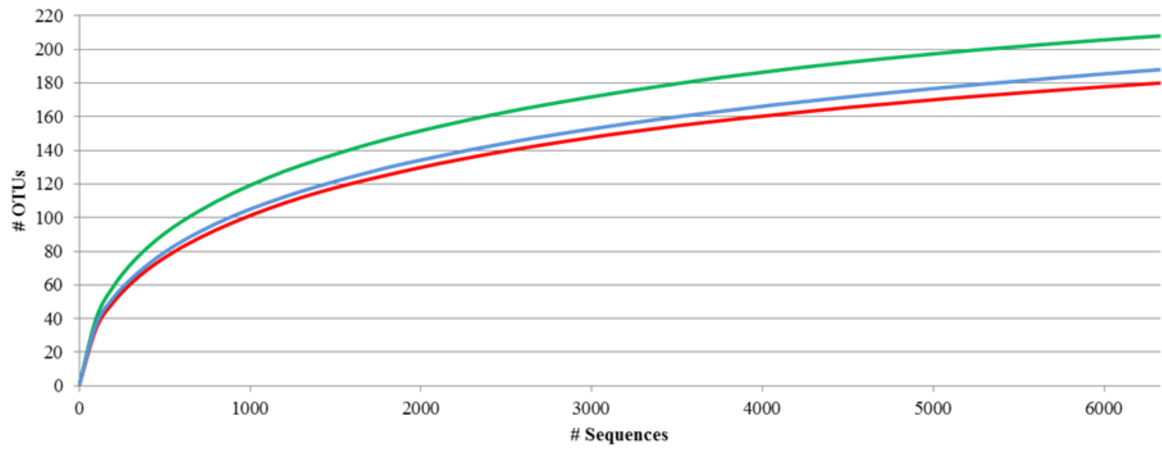

Figure 3-1. Rarefaction curves by fish species. Sequences were standardized to equal sample sizes for direct comparison. Red, *L. macrochirus*; green, *M. salmoides*; blue, *I. punctatus*.

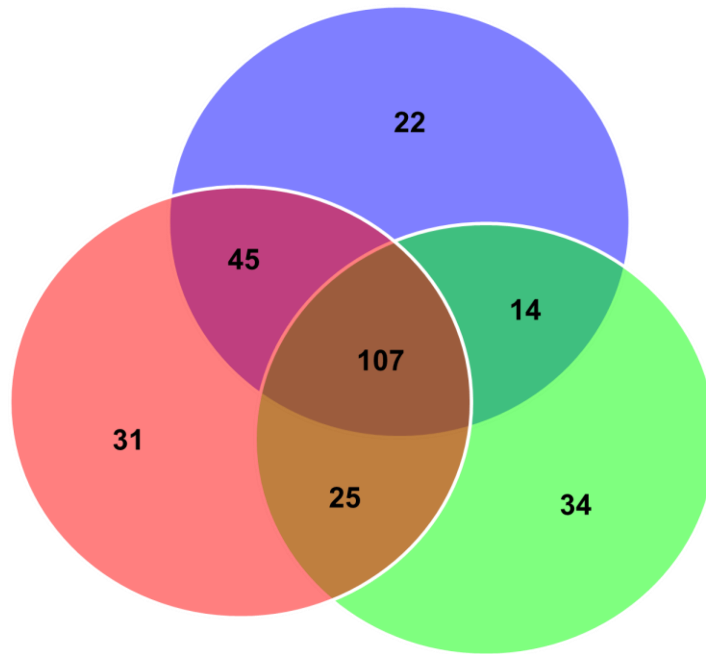

Figure 3-2. Venn diagram representing shared operational taxonomic units (OTUs) between fish species. Red, *L. macrochirus*; green, *M. salmoides*; blue, *I. punctatus*.

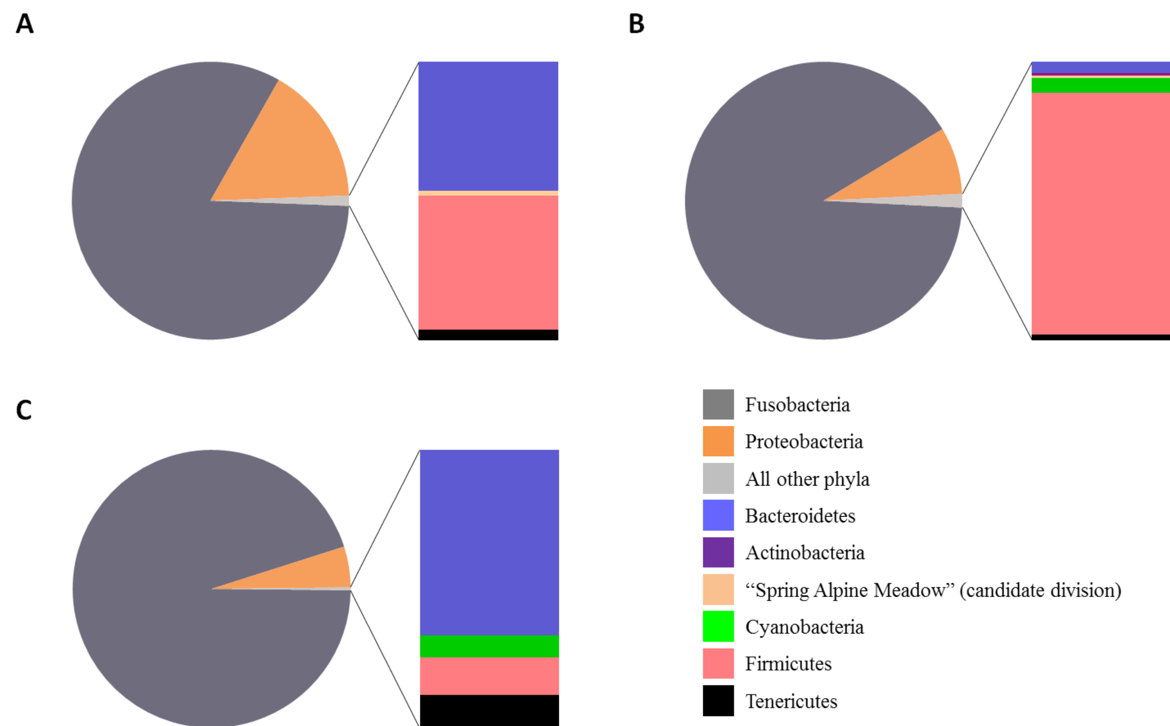

Figure 3-3. Phylum composition of each fish species as obtained through sequencing. A, *L. macrochirus*; B, *M. salmoides*; C, *I. punctatus*.

CHAPTER 4. DIVERSITY OF THE SKIN MICROBIOTA OF FISHES: EVIDENCE FOR HOST SPECIES SPECIFICITY

Abstract

Skin microbiota of Gulf of Mexico fishes were investigated by ribosomal internal spacer analysis (RISA) and 16S rRNA gene sequencing. A total of 102 fish specimens representing 6 species (*Mugil cephalus*, *Lutjanus campechanus*, *Cynoscion nebulosus*, *Cynoscion arenarius*, *Micropogonias undulatus*, and *Lagodon rhomboides*) were sampled at regular intervals throughout a year. The skin microbiota from each individual fish was analyzed by RISA and produced complex profiles with 23 bands on average. Similarities between RISA profiles ranged from 97.5% to 4.0%. At 70% similarity, 11 clusters were defined, each grouping individuals from the same fish species. Multidimensional scaling (MDS) and analysis of similarity (ANOSIM) correlated the RISA-defined clusters with geographic locality, date, and fish species. Global R-values indicated that fish species was the most indicative variable for group separation. Analysis of 16S rRNA gene sequences (from pooled samples of ten individual fish for each fish species) showed that the Proteobacteria was the predominant phylum in skin microbiota, followed by the Firmicutes and the Actinobacteria. The distribution and abundance of bacterial sequences were different among all species analyzed. *Aeribacillus* was found in all fish species representing 19% of all clones sequenced, while some genera were fish species-specific

(*Neorickettsia* in *M. cephalus* and *Microbacterium* in *L. campechanus*). Our data provide evidence for the existence of specific skin microbiota associated with particular fish species.

Introduction

Microbiota refers to the community of microorganisms occupying a specific ecosystem. Focused primarily on human gut microbiota, recent studies have promulgated a new microbiological paradigm that posits a 'healthy' or 'normal' microbiota is critical to human health [1, 2]. Such a 'healthy microbiota' concept applies to the bacteria associated with any tissue that has an epithelial membrane, e.g., epidermis and olfactory, respiratory, and urogenital mucosas [3-6]. In humans, the maintenance of 'healthy' microbiota reportedly is linked with the prevention of infectious diseases, production of amino acids and vitamins essential for host homeostasis, and lower predisposition to diabetes, allergies and, in some instances, cancers [5]. Such host-bacteria mutualism has been documented in mammal models such as the mouse [7], domesticated animals including chicken [8], pig [9], and cow [10], insects [11], and marine invertebrates [12, 13].

The largely ectothermic aquatic vertebrates (= fishes), which comprise the majority of vertebrate species [14], and their associated microbiota are vastly underexplored. Specifically, scant information exists on the biodiversity, geographic

distribution, and seasonality of microbiota on marine, estuarine, and freshwater fishes. The majority of published studies treating fish microbiota focus on gut microbiota. Most of these studies experimentally modified and/or supplemented diets with probiotics towards promoting a gut microbiota optimal for fish growth and disease resistance [15-19]. Mouchet et al. [20] characterized the genetic diversity of the gut microbiota associated with 15 fish species of the southwestern Atlantic Ocean off Brazil and showed that the genetic diversity of the fish gut microbiota was significantly influenced by geographic locality, diet, and fish species while the functional diversity was mainly determined by diet and fish species. The microbiota present on the skin of fishes are far less studied, but do show some level of specificity [21, 22]. Some have been used as biological tags indicating where fish were originally captured from the wild [23] and where they were cultured prior to being processed and packaged for market [24]. Seasonal shifts in the composition of fish skin microbiota are known in wild Atlantic cod (*Gadus morhua*) [22] and aquacultured catfish (*Pangasius* sp.) [24].

The evolutionary origins and ancestry of fish microbiota remains largely unstudied, and, as a result, whether or not fish species harbour unique microbiota is poorly understood. Horsley [25] used culture-based methods to conclude that microbiota of fish epidermis and mucus were representative of whichever bacteria occurred in the fish's water. However, culture-based surveys vastly underestimate microbiota diversity since an estimated <1% of bacteria can be isolated and cultured under laboratory conditions [26]. Nevertheless, some of these pioneering studies surveyed the microbiota of various fishes [25, 27-29] and identified seasonal and biogeographic patterns of variation similar to those revealed by culture-independent methods [22]. None of these

studies supported a strong correlation between a fish species and a unique microbiota; however, few compared microbiota across fish species [27]. That bacteria can seemingly benefit their hosts, or persist as commensals on the surface of their fish hosts, and potentially show some level of specificity to certain fish lineages could together support the notion of a long-standing symbiosis. Perhaps these relationships have existed long enough to exhibit cophyly. Epidermis and mucus of fish constitute an immunologically active and dynamic barrier that prevents pathogen colonization and subsequent infections that may result in a disease condition. Therefore, it seems logical to hypothesize that the microbes of the fish skin microbiota have established a close relationship with their host, similar to those that have colonized the nasopharyngeal cavity in humans and other vertebrates [30]. These hypotheses remain largely untested using modern molecular approaches, principally due to a lack of foundational descriptive information on the species identities (community composition) of those bacteria that form the microbiota of fishes.

To that end, the objective of this study was to apply culture-independent methods to characterize and compare microbiota on skin of several teleostean fishes, i.e., striped mullet (*Mugil cephalus*) (Mugiliformes: Mugilidae), red snapper (*Lutjanus campechanus*) (Perciformes: Lutjanidae), spotted seatrout (*Cynoscion nebulosus*) (Perciformes: Sciaenidae), sand seatrout (*Cynoscion arenarius*), Atlantic croaker (*Micropogonias unduluatus*) (Perciformes: Sciaenidae), and pinfish (*Lagodon rhomboides*) (Perciformes: Sparidae), of the north-central Gulf of Mexico. Based on previous studies, we hypothesized that season (temperature) will be the primary force shaping the diversity and structure of fish skin microbiota.

Methods

Sample Collection

Sampling began in June 2010 and continued monthly through December 2010 with one additional sampling during September 2011. Sampling locations including coastal waters of Dauphin Island (DI) (30°14’55”N 88°04’29”W) and Orange Beach (OB) (30°14’50”W 87°40’01”W) in Alabama and Ocean Springs (OS) (30°23’31”N 88°47’54”W) in Mississippi. The offshore site (28°57’20”N 89°44’37”W) was approximately 30 km west of the mouth of the Mississippi river in Louisiana (LA). Table 4-1 summarizes collection dates, locations, and numbers of fish analyzed per collection event. One L of seawater was collected at each location using a sterile container (except for the offshore location). Seawater surface temperature was measured at 1 m depth in situ using a mercury-in-glass thermometer (SargentWelch, USA). Salinities were measured with a handheld refractometer (Vital Sine™ Model SR-6). Fishing efforts lasted between 4 and 8 h, except for the offshore location wherein fish were collected as part of a 3-day fisheries research cruise. Fish were captured using standard baited hooks and 20 (100 for red snapper) pound test monofilament fishing line on spinning reels. Hooked fish were raised from the water, secured and suspended in air by the angler grasping the leader base or hook shaft, and then touched only by a second worker wearing sterile surgical gloves and equipped with flamed and ethanol-rinsed, heavy-gauge scissors. In coordination with raising the fish from the water, the second worker approached and immediately excised a portion (~1 cm^2) of the dorsal fin. The tissue was placed in a sterile 1.7 mL centrifuge tube and frozen at -20°C until further processing.

Species sampled were striped mullet (*Mugil cephalus*) (Mugiliformes: Mugilidae), red snapper (*Lutjanus campechanus*) (Perciformes: Lutjanidae), spotted seatrout (*Cynoscion nebulosus*) (Perciformes: Sciaenidae), sand seatrout (*Cynoscion arenarius*), Atlantic croaker (*Micropogonias unduluatus*) (Sciaenidae), and pinfish (*Lagodon rhomboides*) (Perciformes: Sparidae). All fish were identified according to Carpenter [31]. Ordinal and familial classifications of fishes follows Nelson [14]. Common names for fishes follows Eschmeyer [32].

DNA Extraction and PCR

The DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) was used for fish DNA extractions following manufacturer's instructions with the following adaptations: to ensure extraction from Gram-positive bacteria a treatment with lysozyme was incorporated as the first step in the protocol, followed by a proteinase K treatment that lasted for 15 hours, and DNA was eluted twice with 50 µL elution buffer. Water samples were centrifuged at 10,000 *g* for 20 min. Supernatants were discarded and DNA was extracted from pellets using the protocol described above. Extracted DNA was used as a template for PCR on the internal transcribed spacer region using the ITS-FEub (5'-GTCGTAACAAGGTAGCCGTA-3') and ITS-REub (5'-GCCAAGGCATCCACC-3') primers [33]. RISA was performed as previously described by Arias et al. [34] with the following modifications. The PCR mix contained 1X Taq buffer, 0.4 mM dNTPs (Promega, Madison, WI), 0.4 µM ITS-FEub primer, 0.2 µM ITS-R primer, 0.02 µM ITS-REub labeled primer, 5 mM $MgCl_2$, 1 U of Taq polymerase (5 PRIME, Inc., Gaithersburg, MD), and 100 ng of template DNA in a final volume of 50 µL. PCR conditions were as follows: initial denaturation at 94 ºC for 3 minutes, followed by 30

cycles of 94 ºC for 45 seconds, 55 ºC for 1 minute, and 68 ºC for 2 minutes, ending with a final extension at 68 ºC for 7 minutes. For water samples, a second round of PCR (as per above) was needed to visualize the RISA bands. Ten microliters of each PCR product was diluted with 5µL AFLP® Blue Stop Solution (LI-COR). Diluted samples were denatured at 95 ºC for 5 m followed by rapid cooling prior to gel loading to prevent reannealing. PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions. One microliter of sample was loaded into each well.

Sequencing

To identify the predominant bacterial species on the fish skin, we used a "shot-gun sequencing" approach using DNA extracted from selected individual fish. Equimolecular amounts of DNAs from 10 individuals from the same species were mixed and the 16S rRNA gene was amplified by PCR. In short, 16S rRNA amplification was done using the universal primers Bact-8F (5'- AGAGTTTGATCCTGGCTCAG -3') and UNI534R (5'- ATTACCGC GGCTGCTGG -3') that amplified the variable regions V1-V3. PCR reagents and conditions have been described elsewhere [35, 36]. Purified amplified products were cloned into the pCR-4-TOPO vector and transformed into competent *E. coli* One Shot TOP10 using the TOPO-TA cloning kit for sequencing (Invitrogen, San Diego, CA, USA). Ninety-six clones were randomly selected from each fish species. Clones were automatically sequenced using an ABI 3730xl sequencer at Lucigen Corp. (Madison, WI, USA).

Data analysis

RISA images were processed with BioNumerics v. 6.6 (Applied Maths, Austin, TX). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Pearson product-moment correlation coefficient (r). Cluster analysis was performed according to Arias et al. [34] using the unweighted pair-group method with arithmetic mean (UPGMA). Multidimensional scaling (MDS) was performed using optimized positions. Analysis of similarities (ANOSIM) was run from the similarity matrix generated in BioNumerics using Primer v6 (Primer-E Ltd., Plymouth, United Kingdom). DNA sequences were read and edited by the software Chromas version 1.45 (Conor McCarthy, School of Health Science, Griffith University, Gold Coast campus, Southport, Queensland, Australia) and loaded into the Ribosomal Database Project (RDP). The classifier tool was used to identify bacteria to the genus level [37]. Sequencing results were grouped taxonomically at the phylum level. Data was analyzed using SIMPER analysis in Primer v6.

Results

Fish captured

A total of 102 fish specimens representing 6 species, 5 genera, 4 families, and 2 orders were sampled monthly from June to December 2010 with an additional sampling point in September 2011 (Table 4-1). While red snappers were only captured in Louisiana waters during the month of September 2011 and striped mullets only in Ocean Springs,

MS, specimens of the other species were caught during a variety of months at multiple locations wherein temperatures and salinities were 16−32°C and 8−27 ‰, respectively (Table 4-2).

Individual fish external microbiota

The skin microbiota of each fish was fingerprinted by RISA. Each RISA profile consisted on average of 23 bands ranging from 50 to 700 bp. Figure 4-1 shows a typical RISA profile from each of the fish species analyzed. After creating a similarity matrix based on pair-wise comparisons using the Pearson product-moment correlation coefficient, a dendrogram was derived by UPGMA clustering analysis (Figure 4-2). The similarity between individual fish skin microbiota ranged from 97.5% to a minimum of 4.0%. In Figure 4-2, branches grouping profiles with ≥70% similarity from the same fish species were collapsed for ease of viewing. The cut-off point of 70% was chosen based on the reproducibility and repeatability of the RISA technique under our conditions. Previous studies from our group showed that up to 25-30% of the dissimilarity observed among RISA profiles can be due to variability introduced by the method [34]. At 70% similarity, 11 clusters representing 3 or more individual fish were defined. All 11 of those clusters grouped individual fish from the same species. These clusters contained a total of 52.9% of all individuals sampled. Seven clusters contained only one sampling month, representing 31.1% of the samples. Seven clusters also contained only one sampling location. These clusters included 26.9% of the individuals sampled. Seawater microbiota were also analyzed by RISA. However, the low amount of DNA obtained after extraction required two rounds of PCR amplification before the RISA profiles could be visualized. Therefore, side by side comparison of seawater samples along with fish samples was not

possible. The clustering analysis of the RISA seawater samples is shown in Figure 4-3. No clear correlation between sampling location or date and percent of similarity between seaweater samples could be inferred.

Variables affecting microbiota structure

Multidimensional scaling (MDS) was used to better visualize the groups defined by the RISA-based clustering analysis. Skin microbiota profiles were ascribed to groups based on the variables analyzed (date, location, and fish species) and their RISA similarities were represented by MDS plots. Figure 4-4 shows the MDS plots when fish species was used as a variable. Analysis of similarities (ANOSIM) was used to test the significance of the groupings for each variable. This analysis indicates the significance of groups based on a given factor; in this case we analyzed fish species, sampling date, and location. The results from the ANOSIM indicate whether the samples are statistically separated by a factor (significance at $p < 0.05$) and the extent of separation (given as a global R value). If $p<0.05$, the samples significantly group by the tested factor. Higher R values indicate less overlap in samples, or greater group separation. Thus, both the p value and R value must be interpreted to understand the extent to which a factor influences group separation [38]. In general, if an R value is less than 0.25, the groups have little separation, if it is greater than 0.5, there is some overlap but the groups are separated, and if the value is greater than 0.75 there is large separation between groups. Each variable was found to be significant ($p=0.001$) and global R values were above 0.25 in all cases, ranging from 0.338 to 0.549 (Table 4-3).

When skin microbiota were grouped by date, data showed that samples collected at different months differed from each other except in two cases. Microbiota from fish

collected in September 2010 were not statistically different from those collected in July, October, and November 2010. Similarly, samples from September 2011 could not be separated from those collected in October 2010. The global R value for date was 0.338 with pairwise comparison R values ranging from 0.189 to 0.635. When samples were assigned to groups based on location, all sites were found to be statistically different with the exception of Orange Beach, which could not be separated from the other locations. The global R value for location (R=0.362) was similar to the global R value for date as were the pairwise R values associated with date group comparisons (R values from 0.116 to 0.568).

Conversely, when groups were assigned based on fish species the global R value was much higher (R=0.549) indicating that fish species was the most indicative variable for group separation. Pairwise comparisons (not shown) indicate that each fish species group was significantly separated from each other group ($P < 0.05$) with R values ranging from 0.330 to 0.848.

Dominant microbiota

As fish species showed the highest significance for grouping the microbiota, 10 individuals from each species were pooled for sequencing. In order to obtain maximum bacterial diversity present within a fish species, representatives for each species that were scattered throughout the dendrogram were selected for analysis. Two plates of 96 samples each were sequenced for each fish species. However, we only obtained 69 high quality sequences (>400 bp) from spotted trout, thus we normalized the number of sequences to be compared by randomly selecting 69 sequences from each species. Sequences were identified at the genus level using the classifier tool of the RDP database.

All sequences have been deposited in GenBank under the accession numbers (JX543531 to JX543948).

When sequences were ascribed at the phylum level, each fish species returned a unique distribution of bacteria (Figure 4-5). The Proteobacteria was the predominant phylum and represented at least 42% of sequences from each fish species and 61% of all identified sequences. The second most predominant phylum in all fishes was the Firmicutes, with species of that phylum comprising 13-42% of the skin microbiota. Actinobacteria, Bacteroidetes, and Cyanobacteria were also identified, constituting 6%, 4%, and 1% of all sequences respectively. SIMPER analysis indicated that, based on phylum composition, pinfish and spotted seatrout were the most similar (89.8%), while sand seatrout and red snapper were the least similar (61%). Red snapper was the least similar on average to all other fish species (67.5% similarity), while spotted seatrout was on average the most similar to all other fish species (78.8% similarity).

In all species but striped mullet, members of the Gammaproteobacteria class constituted about 50% of all the Proteobacteria followed in abundance by Betaproteobacteria. Striped mullet presented a different composition of classes of the Proteobacteria, with the Alphaproteobacteria being the dominant group followed by the Gammaproteobacteria and Betaproteobacteria. Atlantic croaker and spotted seatrout showed the least diverse Proteobacteria group with representatives of only the Gamma- and Betaproteobacteria (Figure 4-5).

Figure 4-6 illustrates the most common (>5 representative sequences from at least one fish species) bacterial genera associated with all fish species. *Aeribacillus* was abundant on all fish species and accounted for 19% of all sequenced clones.

Pseudomonas was identified from all fish species except Atlantic croaker and represented 11% of all clones sequenced. *Janthinobacterium* was the third most frequently identified genus (10%) but absent on red snapper and Atlantic croaker. Unique bacterial genera associated with specific fish species included *Neorickettsia* on striped mullet and *Microbacterium* in red snapper. Spotted seatrout showed the least diverse bacterial population, with >75% of all the sequences recovered from this species belonging to three genera. Red snapper and striped mullet displayed the most diverse microbiota (at genus level), having representatives of 12 genera each.

Discussion

Oceans are oligotrophic environments wherein nutrients are scarce for heterotrophic bacteria. From that perspective, fishes are nutrient islands in a vast, predominantly nutrient-poor sea. From fish eggs to adults, fish surfaces are immersed in water and thereby susceptible to colonization by aquatic bacteria. This process appears to be selective since specific microbiota have been associated with wild fish larvae [39] as well as with hatchery-reared fish [40]. Based on laboratory experiments, adhesion to fish skin appears to be a widespread trait among bacteria, although these studies focused on fish pathogens like species of *Vibrio* [41] and *Flavobacterium* [42]. In addition, some bacteria are positively chemotactic to fish mucus [41, 43]. Because fish mucus is nutrient-rich [44] and bacteria are capable of growing in it [45], marine bacteria may benefit from attaching to fish skin; which is a surface that is normally covered by a contiguous layer of mucus (to the extent that some anatomical treatments of fish skin

refer to the mucus layer as a "cuticle" on the same functional anatomical footing as the keratinized epidermis of terrestrial vertebrates) [46]. However, from the host's perspective, bacterial adhesion to skin should be mediated to avoid over-colonization and disruption of integument functions. This is accomplished, probably in part, by the constant sloughing of the upper layers of the epidermis and the continuous secretion of mucus.

The equilibrium between bacteria that adhere to skin and the number of bacteria that an apparently healthy host can support will play a role in determining the 'normal skin microbiota' for a particular fish species. The diversity and structure of those microbiota can be studied at three levels: alpha diversity (within a host), gamma diversity (within a population), and beta diversity (that observed between hosts of the same population) [5]. The use of RISA, a rapid and inexpensive method, allowed us to compare the microbiota from each individual fish without the need for pooling samples and thus missing the host-to-host (beta) diversity. Although RISA does not provide phylogenetic information on particular amplified sequences, the complexity of RISA profiles reflects that of the microbiota [47]. Our RISA results revealed a broad range of similarities within all the samples analyzed at both intra- and interspecies levels (Figure 4-2). Not all microbiota from the same fish species clustered together, therefore we observed nonzero beta diversity among the populations examined. Based on our previous experience with RISA [34, 48], we concluded that the observed diversity was not due to the variability introduced by the technique with the set cutoff point for describing separate clusters at 70% similarity . Nonzero beta diversity can result from random or nonrandom colonization patterns; however, there is increasing evidence in support of the

latter [5]. In terms of relating the observed beta diversity with the variables examined, the defined clusters could not be assigned to a specific date or location. However, when all pair-wise similarities within a species were compared by ANOSIM, both variables (location and date) significantly influenced the microbiota profiles. Although our data does not refute the previously proposed hypothesis by which bacterial communities on fish are a result of the bacteria present in their surrounding waters [22-24], it suggests that fish species has greater influence on external microbiota.

The structure of marine bacterial communities is a result of both habitat (spatial) filtering [49] and temporal patterns influenced by both biotic and abiotic factors [50]. With exception of red snapper, an obligate marine species typically associated with offshore reefs [51], all fishes analyzed in this study are common residents of estuarine waters [31]. We expected that geographic location would not significantly determine the studied microbiota to the extent that season would (throughout the study water temperature fluctuated between 16°C to 32 °C). However, both variables exerted a similar influence on skin microbiota based on the global R-values obtained. Interestingly, red snapper microbiota were divided into 2 clusters: one cluster was the most basal group in the RISA dendrogram and the other clustered with a pinfish sample collected from Orange Beach 10 months earlier. Both fish species were collected from distinct environments (offshore vs. coast) yet their bacterial profiles shared up to 30% similarity. Nevertheless, red snapper microbiota were the least similar to all other fish species which may be explained by the different habitats in where those fish were collected (offshore vs. coast).

The variable 'fish species' had a global R-value of 0.549 and therefore most significantly shaped the structure of the fish microbiota. This result did not refute the notions that (A) the host plays an active role in selecting which microbial taxa can colonize and persist on it or that (B) the constituents of the microbiota are highly specific to particular host fish, similar to microbial species that only will grow on a particular kind of culture medium. A long list of physiological attributes of the bacterium or the fish could explain this specificity, and we did not specifically test any of them. We speculate that differences in mucus composition [44] and antimicrobial properties [52] between fish species may mediate adhesion interactions between fish and bacteria. Clearly, and contrary to our initial hypothesis, the variable 'fish species' determines the structure of the fish skin microbiota more so than the abiotic factors temperature or salinity; both of which reportedly are predictive of marine bacterioplankton microbiota [49, 50].

Since RISA does not provide phylogenetic information on the microbiota composition, sequencing was conducted to obtain information on the predominant bacteria associated with skin and mucus of the six species examined. Sequence data showed that each fish species had a unique microbiota. Overall, the Proteobacteria was the predominant phylum colonizing the external surface of fishes with 61% of all sequences belonging to this phylum. This result is in agreement with previous studies on other species regardless of the technique used for bacterial identification [22, 27, 29]. Within the phylum Proteobacteria, the Gammaproteobacteria was the most abundant class in all fish species except the striped mullet, and *Aeribacillus* was the most frequently identified genus. *Pseudomonas* was also frequently identified and it is noteworthy that previous studies using either culture or culture-independent methods

have also identified members of *Pseudomonas* as the main component of the skin microbiota of cod (*Gadus* spp.) [22, 28], salmon (*Salmo salar*) [25], skate (*Raja* spp.), lemon sole (*Microstomus* spp.), herring (*Clupea* spp.) [29], surgeon fish (*Acanthurus triostegus*), jack (*Caranx ferdau*) and grouper (*Epinephelus merra*) from the Pacific ocean [27]. Other frequently isolated genera such as *Janthinobacterium* and *Acinetobacter* have been previously reported from fish [39, 53].

Aeribacillus (ph. Firmicutes) was identified in all fish species we surveyed, providing the first report of it in association with a fish. The sequence identities obtained after BLAST identified the majority of our *Aeribacillus* sequences as *A. pallidus* (percent identity match at 98% or higher to type strain DSM 3670). This was a surprising result since this species is known to be a thermophilic, halotolerant bacteria found in hot springs. We queried the GenBank database with 16S rRNA gene sequences that were 400 bp in length or longer and the BLAST results were unequivocal. It is possible that our sequences may represent a new species of *Aeribacillus*, the full 16S rRNA gene sequence will be required to support this, or that we have discovered a new ecological niche for *A. pallidus*.

Noteworthy also was the presence of *Neorickettsia* sp. in striped mullet, an intracellular pathogen that causes severe illnesses in mammals and that is transmitted by flukes (Platyhelminthes: Digenea) that infect fishes [54]. The sequence identity was 95−96% with those found in GenBank (closest match was *N. risticii* type strain ACTT VR-986 in all cases), which suggested a potential new species of *Neorickettsia* associated with striped mullet. Similarly, *Microbacterium* sp. was found in red snapper only, yet represented up to 11% of all bacterial sequences from all red snappers sampled. Sequence

identities were high in most cases with percent identities over 98% matching *M. arborescens* (type strain DSM 20754)*, M. esteraromaticum* (type strain DSM 8609), and *M. paraoxydans* (type strain DSM 15019). However, five sequences shared less than 97% sequence identity with GenBank entries and may represent new *Microbacterium* species.

Predominant marine bacteria genera such as *Vibrio* and *Photobacterium* were identified in extremely low frequency (*Photobacterium*) or not detected at all (*Vibrio*). This contradicts previous studies in which both genera were abundant and common [21, 22, 39]. Interestingly, these studies used fingerprint techniques followed by 16S rRNA gene sequencing, similar to our methods. However, in those studies fish were collected by trawling, which increases bacterial densities on skin [53]. Differences in fishing gear may influence the recovery of those bacteria loosely associated with skin and mucus.

In conclusion, this study provides evidence for the presence of specific external microbiota associated with particular fish species. The composition and structure of those microbiota are likely to be impacted by several cofounding variables including abiotic factors linked to geographic locality and season as well biotic factors related to the nutrient potential or antimicrobial components of fish mucus. The bacterial profiles obtained from individual fish showed nonzero beta diversity indicating that the host influences the bacterial taxa associated with its external surfaces. In addition, and based on our sequence data, we suggest that the external surfaces of fish are colonized by a microbiota that is distinguishable between fish species.

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Table 4-1. Temporal and spatial distribution of fishing efforts summarizing number of fish analyzed in the study.

Fish species (Order: Family), common name		No. of fish	Locality	Date (mm/yy)
Mugil cephalus (Mugiliformes: Mugilidae), striped mullet		1	OS[a]	07/10
		14	OS	12/10
	Total	15		
Lutjanus campechanus (Perciformes: Lutjanidae), red snapper				
	Total	25	LA[b]	09/11
Lagodon rhomboides (Perciformes: Sparidae), pinfish		2	DI[c]	08/10
		2	DI	09/11
		1	DI	10/10
		3	DI	11/10
		1	MB[d]	06/10
		2	OS	07/10
		1	OS	09/10
		5	OB[e]	10/10
	Total	17		
Cynoscion arenarius (Perciformes: Sciaenidae), sand seatrout		1	MB	06/10
		9	OS	07/10
		6	OS	09/10
		8	OS	11/12
	Total	24		

Table 4.1 continued

Fish species (Order: Family), common name	No. of fish	Locality	Date (mm/yy)
Cynoscion nebulosus (Perciformes: Sciaenidae), spotted seatrout	1	DI	08/10
	9	MB	06/10
	1	OS	12/10
Total	11		
Micropogonias undulatus (Perciformes: Sciaenidae), Atlantic croaker	7	DI	08/10
	1	DI	09/10
	9	MB	06/10
	1	OS	07/10
	3	OS	09/10
	6	OS	12/10
Total	27		
Total fish sampled	102		

[a] OS, Ocean Springs, MS
[b] LA, offshore of Grand Isle, LA
[c] DI, Dauphin Island, AL
[e] MB, Mobile Bay, AL
[d] OB, Orange Beach, AL

Table 4-2. Water temperature and salinity of collection sites.

Date	Location	Temperature (°C)	Salinity (‰)
June 2010	Mobile Bay, AL	32	12
July 2010	Ocean Springs, MS	30	8
August 2010	Dauphin Island, AL	30	27
September 2010	Dauphin Island, AL	30	24
	Ocean Springs, MS	30	ND[a]
October 2010	Dauphin Island, AL	23	29
	Orange Beach, AL	22	20
November 2010	Dauphin Island, AL	18	27
	Ocean Springs, MS	20	17
December 2010	Ocean Springs, MS	16	17
September 2011	Offshore, LA	27	ND

[a] ND, not determined

Table 4-3. Analysis of similarity (ANOSIM) values obtained when skin microbiota profiles were ascribed to spatiotemporal variables and to host species.

Group	Global	Significance	Permutation ≥ Global
Date	0.338	0.001	0
Location	0.362	0.001	0
Species	0.549	0.001	0

Figure 4-1. RISA profiles obtained from one individual of each fish species, also indicating sampling location and date. Molecular weight marker indicates size range of the RISA profiles.

Cluster	Fish species	Date	Location
1 (2)	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
2 (3)	Croaker	Jul, Sept, Dec 2010	OS
3 (4)	Sand seatrout	Nov 2010	OS
	Pinfish	Oct 2010	OB
	Pinfish	Oct 2010	OB
	Pinfish	Oct 2010	OB
	Pinfish	Oct 2010	DI
	Sand seatrout	Sept 2010	OS
4 (3)	Striped mullet	Dec 2010	OS
	Striped mullet	Dec 2010	OS
5 (8)	Sand seatrout	Jun, Jul, Sept, Nov 2010	OS
	Sand seatrout	Jul 2010	OS
6 (2)	Spotted seatrout	Jun 2010	MB
7 (3)	Spotted seatrout	Jun 2010	MB
	Spotted seatrout	Jun 2010	MB
	Spotted seatrout	Aug 2010	DI
8 (4)	Croaker	Jun, Aug 2010	MB, DI
	Croaker	Sept 2010	OS
9 (2)	Croaker	Aug 2010	DI
	Croaker	Aug 2010	DI
	Croaker	Aug 2010	DI
	Croaker	Dec 2010	OS
	Croaker	Jun 2010	MB
	Croaker	Jun 2010	MB
	Croaker	Dec 2010	OS
	Croaker	Sept 2010	OS
	Striped mullet	Dec 2010	OS
	Striped mullet	Dec 2010	OS
	Striped mullet	Dec 2010	OS
	Striped mullet	Dec 2010	OS
	Spotted seatrout	Dec 2010	OS
	Striped mullet	Dec 2010	OS
	Striped mullet	Dec 2010	OS
	Striped mullet	Dec 2010	OS
	Striped mullet	Jul 2010	OS
10 (3)	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Red snapper	Sept 2011	LA
	Pinfish	Oct 2010	OB
	Croaker	Jun 2010	MB
	Red snapper	Sept 2011	LA
	Pinfish	Sept 2010	DI
	Croaker	Aug 2010	DI
	Pinfish	Nov 2010	DI
	Spotted seatrout	Jun 2010	MB
11 (3)	Pinfish	Jul, Sept, Nov 2010	OS, DI
	Pinfish	Jun 2010	MB
	Pinfish	Jul 2010	OS
	Pinfish	Aug 2010	DI
	Pinfish	Aug 2010	DI
	Pinfish	Sept 2010	OS
	Pinfish	Oct 2010	OB
	Croaker	Sept 2010	DI
	Croaker	Dec 2010	OS
	Red snapper	Sept 2011	LA

Figure 4-2. RISA patterns obtained from individual fish analyzed in the study. Fish species, location and date for each fish are specified. The scale represents the percent of similarity using the Pearson product-moment correlation coefficient. The dendrogram was constructed using UPGMA. Clusters were defined at 70% similarity; number of individual fish per cluster are shown in parentheses. Cophenetic correlation coefficients, reflecting the robustness of each node are indicated (only values over 75% are shown).

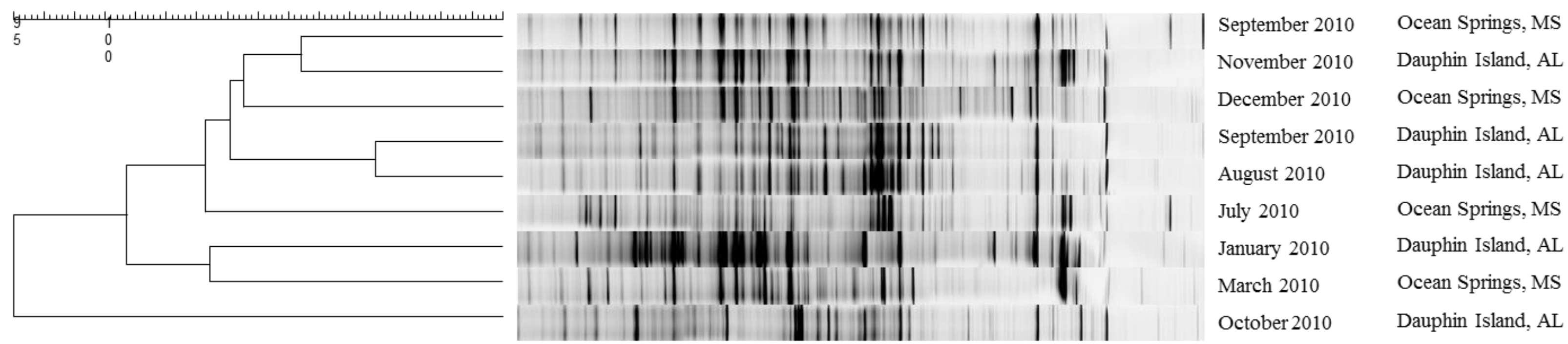
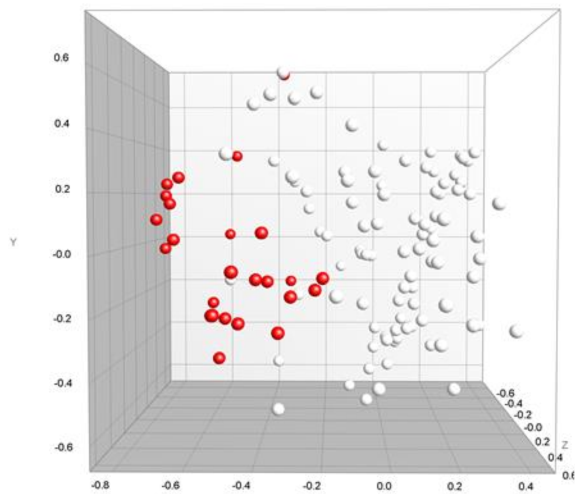
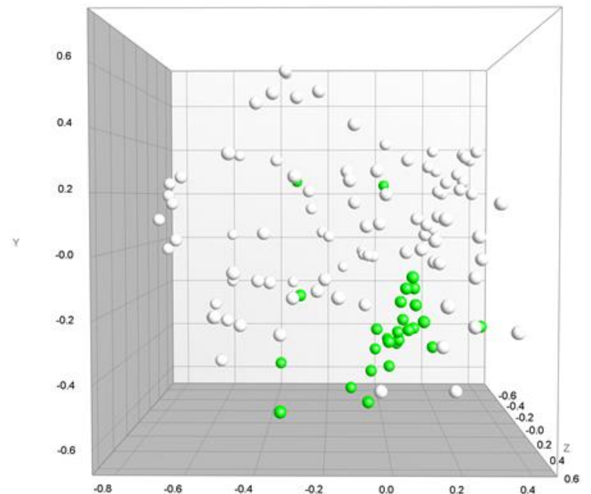
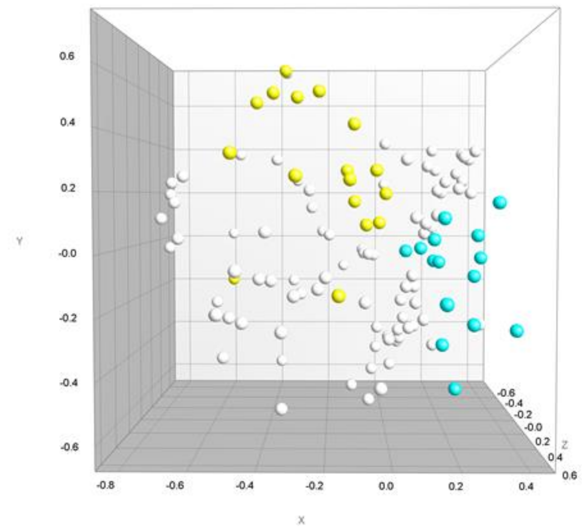


Figure 4-3. RISA profiles obtained from seawater samples indicating location and collection date.


Y
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
Z
-0.6
-0.4
-0.2
-0.0
0.2
0.4


Y
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
Z
-0.6
-0.4
-0.2
-0.0
0.2
0.4
0.6


Y
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
X
Z
-0.6
-0.4
-0.2
-0.0
0.2
0.4
0.6

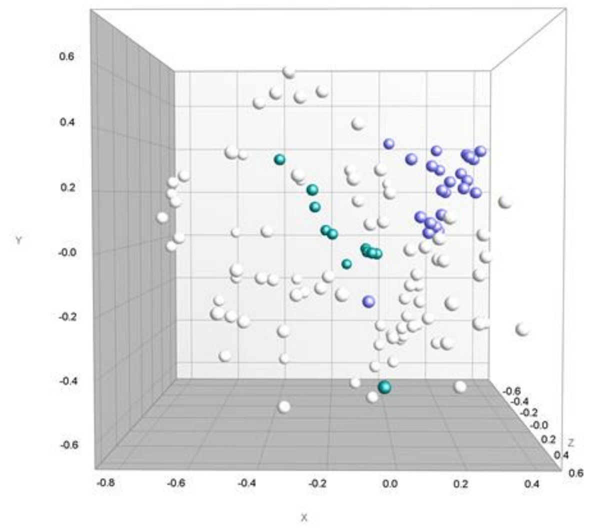

Y
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
X
Z
-0.6
-0.4
-0.2
-0.0
0.2
0.4
0.6

Figure 4-4. Multidimensional Scaling (MDS) representation of the similarity matrix generated by RISA cluster analysis. Each of the skin microbiota is represented by a dot and the distance between dots represents relatedness obtained from the similarity matrix. Isolates are coloured based on fish species. In Panel A, only the microbiota from red snapper (*Lutjanus campechanus*) are highlighted. Panel B displays the microbiota from Atlantic croaker (*Micropogonias undulatus*). Panel C shows the microbiota from striped mullet (*Mugil cephalus*) (yellow) and pinfish (*Lagodon rhomboides*) (turquoise). Panel D highlights the microbiota from spotted seatrout (*Cynoscion nebulosus*) (teal) and sand seatrout (*Cynoscion arenarius*) (purple).

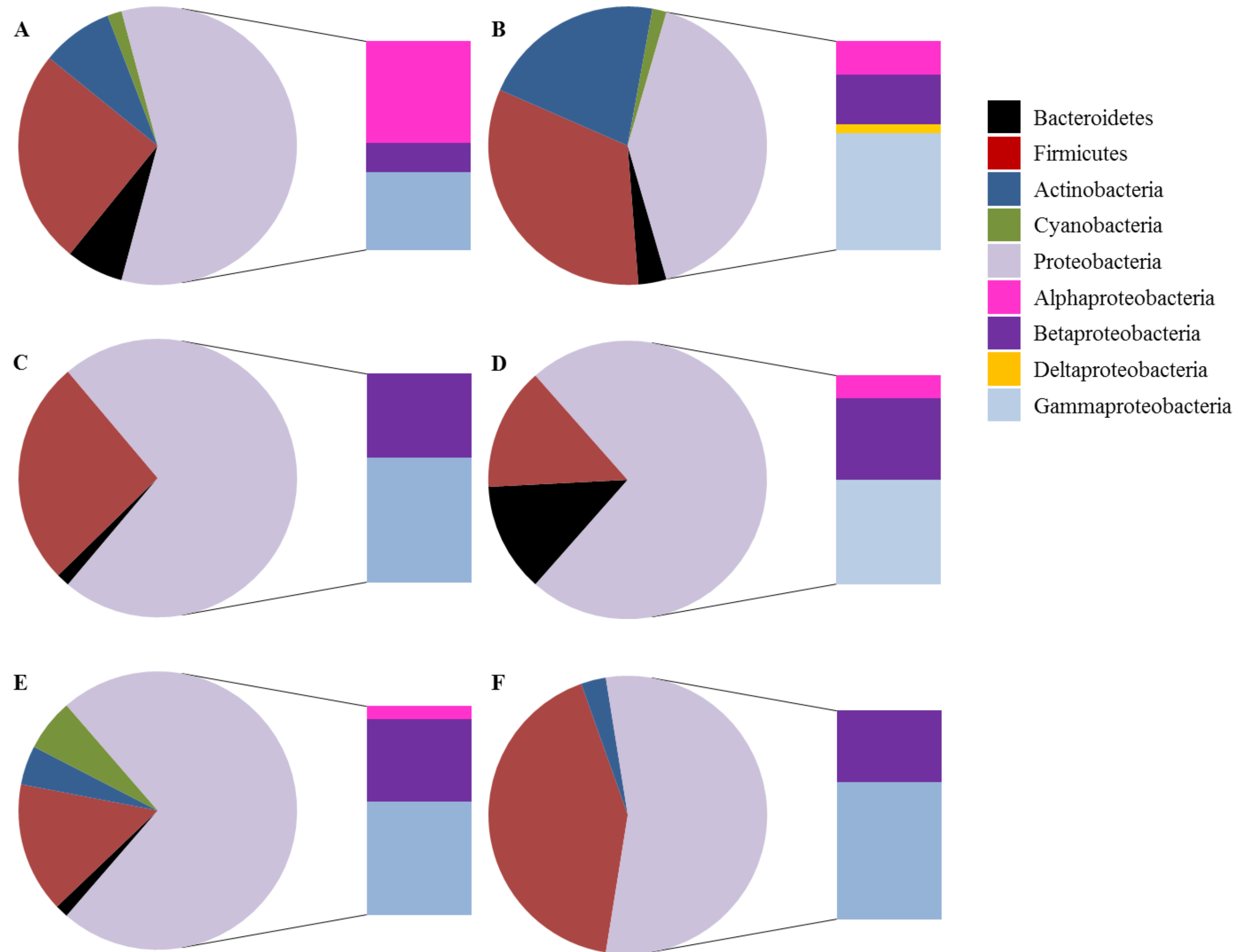

A
B
C
D
E
F
Bacteroidetes
Firmicutes
Actinobacteria
Cyanobacteria
Proteobacteria
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Gammaproteobacteria

Figure 4-5. Bacterial diversity at the phylum level (pie chart) and class level (bars) based on 16S rRNA gene sequencing. Pie diagrams show percent of sequenced clones belonging to different bacterial phylum from each fish species analyzed. Bar graphs represent the percentage of Proteobacteria classes detected in each fish species. Panel A, striped mullet (*Mugil cephalus*); panel B, red snapper (*Lutjanus campechanus*); panel C, spotted seatrout (*Cynoscion nebulosus*); panel D, sand seatrout (*Cynoscion arenarius*); panel E, pinfish (*Lagodon rhomboides*); panel F, Atlantic croaker (*Micropogonias undulatus*).

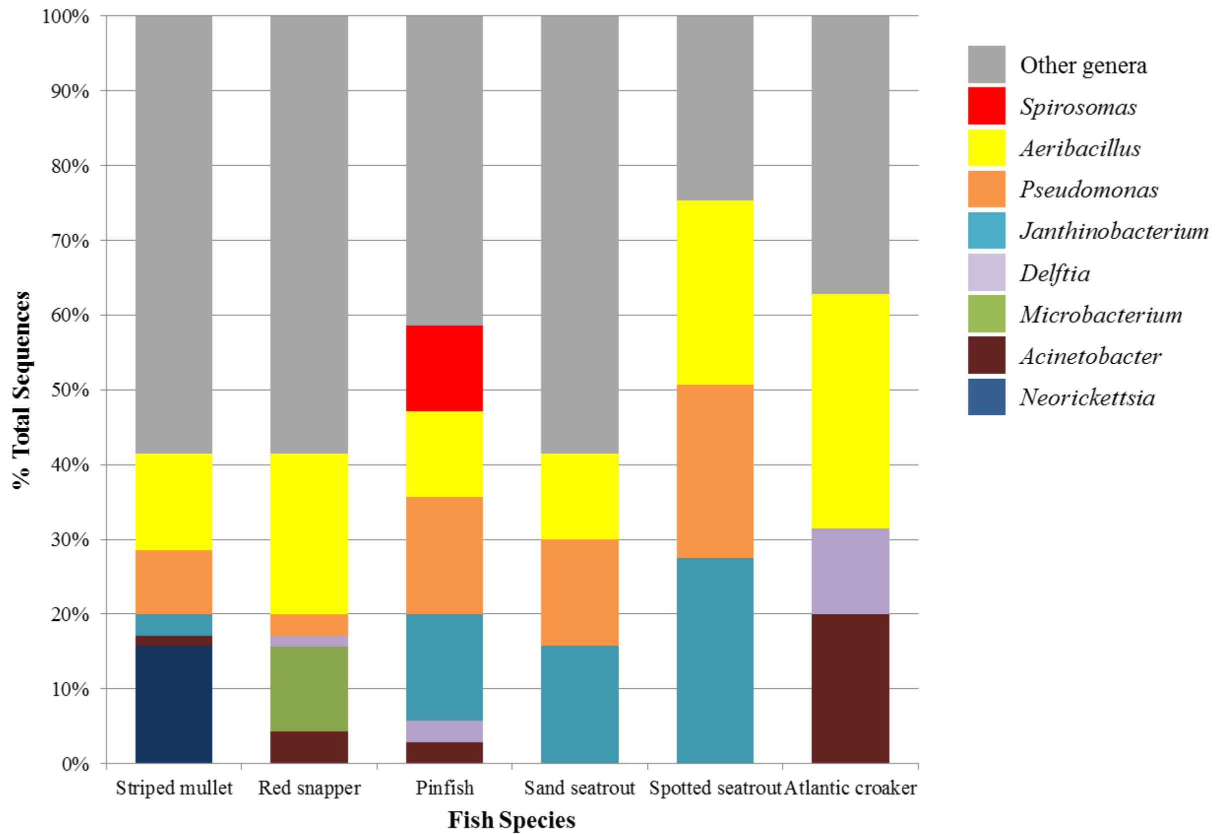


Figure 4-6. RISA profiles obtained from one individual of each fish species, also indicating sampling location and date. Molecular weight marker indicates size range of the RISA profiles.

CHAPTER 5. EXTERNAL BACTERIAL COMMUNITIES ASSOCIATED WITH THE GULF KILLIFISH (*FUNDULUS GRANDIS*) IN AREAS IMPACTED BY THE *DEEPWATER HORIZON* OIL SPILL

Abstract

Fish mucus contains complex bacterial communities that may be affected due to stress or disease. These stressors can result in a decrease in bacterial diversity, with opportunistic pathogens increasing in abundance relative to the healthy community. By observing changes in the bacterial assemblages over time, one can potentially monitor health of the fish. The *Deepwater Horizon* oil spill in the Gulf of Mexico in 2010 acted as a potential stressor to the marine ecosystem. Ribosomal Intergenic Spacer Analysis (RISA) and pyrosequencing were used to analyze the external bacterial assemblages of Gulf killifish (*Fundulus grandis*) collected from oil-impacted and non-impacted sites. Water samples and fin clips were collected to examine microbiota structure. *F. grandis* harbored a bacterial community significantly different from that of the surrounding water mainly due to differences between abundances in Cyanobacteria and Proteobacteria. *F. grandis* microbiota was dominated by members of the Gammaproteobacteria, specifically members of the genus *Pseudomonas*. *Rhodanobacter* and *Prochlorococcus* were reported

for the first time associated with the skin of fish. Results indicated no significant influence of oil exposure on microbiota composition. Conversely, season influenced the microbiota structure in the studied samples. Despite seasonal differences, high similarity between individual fish and community stability during oil exposure suggests the presence of a resilient core microbiota associated with the skin of *F. grandis*.

Introduction

On April 20, 2010 the *Deepwater Horizon* oil rig located approximately 50 miles southeast of Venice, LA, exploded releasing almost 5 million barrels of oil into the Gulf of Mexico [1] before the leak was capped on July 15, 2010. The spill resulted in the closure of 230,000 km^2 of federal waters to fishing (36.6% of the Gulf Exclusive Economic Zone) at its peak in June, 2010 [2] and is estimated to have cost Louisiana commercial fisheries up to $172 mil from 2011-2013 [3]. Economic losses in the Gulf states may be even higher considering a survey that indicated 23% of consumers reduced their Gulf seafood consumption due to safety concerns related to the oil spill [4].

The economic impacts were not the only cause for concern. The sudden influx of non-refined oil into the Gulf triggered alarm over its effects on coastal and marine environments and the organisms residing within them. Coastal wetlands are considered one of the most heavily impacted habitats during spills because oil can remain in these systems for many years, resulting in long-term exposure for the organisms [5]. These systems are extremely productive with 97% of commercial fish and shellfish species in

the Gulf of Mexico requiring this habitat during some portion of their life cycle [6]. The impacts on some species are exacerbated as they have small home ranges, meaning they move little throughout their lives and are in constant contact with the oil compounds found in exposed sites. The Atlantic killifish, *Fundulus heteroclitus* (Walbaum), exhibits small home ranges and high site fidelity [7-10]. *F. heteroclitus* is often used as a model species in the Atlantic to study the effects of pollutants on resident species [11-16] due to its sensitivity and fast physiological response to these stressors. Similarly, its sister species *F. grandis* (Baird and Gerard) exhibits high site fidelity [17] and can be used as a model to study toxicological effects on vertebrates in the Gulf of Mexico [18].

Many studies on the effects of oil on the health of fish are fatal to the animal as they involve the sampling of internal organs such as the liver and kidneys [19-22]. A non-lethal alternative for assessing fish stress is the study of their external bacterial assemblages. In the presence of external stressors, the autochthonous bacteria in the mucus of aquatic organisms changes in composition, resulting in lower species diversity with an associated increased prevalence of opportunistic pathogens [23-25]. Comparing the bacterial taxa in the mucus layer of *F. grandis* collected from oil impacted and non-impacted sites could become a long-term monitoring tool to evaluate the health of these organisms.

This study used ribosomal intergenic spacer analysis (RISA) and pyrosequencing to compare the bacteria associated with the skin and mucus of *F. grandis* one year after the *Deepwater Horizon* oil spill (DHOS). Fish and water samples were taken from impacted and non-impacted sites over the course of a year and samples were analyzed for differences in bacterial assemblages due to sample type, sampling date, and oil exposure.

Methods

Sample collection

Eight sites were chosen for sampling: four impacted (N1-4) and four non-impacted (O1-4) as determined by coordination with the Louisiana Department of Fisheries and the National Oceanic and Atmospheric Administration's (NOAA) Environmental Response Management Application (ERMA) (Figure 5-1). Samples were taken during February, May, August, and December 2011. *F. grandis* were captured using minnow traps placed in the marsh grass baited with cut-up menhaden (*Brevoortia patronus*; Goode). Traps were allowed to fish for approximately two hours. Twenty fish from each site were sampled each month by aseptically removing approximately 100 mm^2 clip of the dorsal fin using ethanol-sterilized tweezers and scissors. Fin clips were immediately placed into a sterile tube and set on ice. Upon arrival to the laboratory (less than 4 hours) all tubes were frozen at -20°C until further analysis. Water samples were collected using sterile 1 L glass bottles from the least (N1) and most (O4) impacted sites as determined from ERMA maps. As previous studies have demonstrated differences in bacterial communities between fish skin and the surrounding water [26-30], water samples were collected during February and May only to examine this pattern in *F. grandis*. In order to capture diversity, duplicate water samples (2) were taken at each point. Water samples were centrifuged for 20 min at 10,000 *g*, supernatant discarded, and pellets were analyzed as follows.

DNA extraction and RISA

DNA was extracted from fin clips and water samples using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). DNA was quantified with a spectrophotometer and diluted with RNase/DNase-free water to 5 ng/µL. PCR was performed using universal bacterial primers for the internal transcribed spacer region: ITS-F (5'-GTCGTAACAAGGTAGCCGTA-3') and ITS-R (5'-GCCAAGGCATCCACC-3') [31]. The PCR reaction used 100 ng of template DNA in the following mixture: 1X PCR buffer, 0.4 mM dNTPs (Promega, Madison, WI) 5 mM magnesium chloride, 0.4 µM ITS-F, 0.36 µM ITS-R, 0.04 µM fluorochrome-labeled ITS-R, and 0.2 U *Taq* polymerase (5 PRIME, Inc., Gaithersburg, MD) in a 50 µL reaction. PCR conditions were as follows: denaturation at 94°C for 3 min, 30 cycles of 94°C for 45 s, 55°C for 1 min, 68°C for 2 min, and a final extension of 68°C for 7 min. After amplification, 10 µL PCR product was combined with 5 µL AFLP Blue Stop Solution (LI-COR, Lincoln, NE). Diluted samples were denatured at 94°C for 5 min and immediately put on ice to prevent reannealing. RISA was performed using denatured products on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions. Water samples did not amplify with PCR, thus they were not included in RISA analysis.

Sequencing

Five *F. grandis* individual samples were randomly selected from samplings at sites N1 and O4 (reflecting maximum difference in oil impact) at each of the four months for pyrosequencing. DNA from all five fin clip samples were pooled in equimolecular amounts to form one sample from each site at each month (8 samples total). Similarly,

equimolecular amounts of DNA from duplicate water samples were combined. Roche titanium 454 sequencing was performed on 12 samples (4 water, 8 fin clips) using individual barcodes and primer 27F (5'-AGRGTTTGATCMTGGCTCAG-3') amplifying the variable V1-V3 region of the 16S rRNA gene. PCR conditions were as follows: initial denaturation at 94°C for 3 min and 30 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, including a final elongation at 72°C for 5 min. Resulting sequences were processed with an exclusive analysis pipeline (MR DNA, Shallowater, TX) including removal of barcodes and primers as well as sequences of less than 200 base pairs, a base call error rate of less than 0.3% (Q<25), ambiguous base calls, and long (>6 base pairs) stretches of identical bases. Following denoising and chimera and singleton sequence removal, operational taxonomic units (OTUs) were defined in agreement with the current accepted prokaryotic species concept (<3% sequence agreement; [32]) and taxonomically identified using BLASTn against the Greengenes database [33].

Data Analysis

RISA gels were analyzed with BIONUMERICS v. 7.0 (Applied Maths, Gent, Belgium). Following normalization and background subtraction, dendrograms depicting percent fingerprint similarity were generated using Pearson product-moment correlations. Cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA) as previously described [34]. The similarity matrix from BIONUMERICS was uploaded into Primer v6 (Primer-E Ltd, Plymouth, UK) where a two-way crossed analysis of similarities (ANOSIM) was performed using month and oil impact as factors.

Rarefaction curves, Good's coverage, observed OTUs, shared OTUs, diversity (using the catchall command), and Shannon Evenness Index (SEI) were calculated using Mothur v.1.33.3 [35] after standardization of each sample type to the sample yielding the least number of total sequences. Student's t-tests ($\alpha = 0.01$) were used to determine differences in species richness (in observed OTUs) and species evenness (SEI) between sample types (water *versus* fish). OTU tables including all samples were loaded into Primer v6 for clustering using group average and ANOSIM analysis (sample type). Fish-only OTU tables were used for multidimensional scaling (MDS; month and oil impact). Phyla and genera tables were also loaded into Primer for similarity percentages (SIMPER) analysis in order to determine specific taxonomical differences between communities.

Results

RISA

A total of 628 fin clips were analyzed in this study (Table 5-1). Dendrograms based on similarity in RISA fingerprints show a wide range of similarities between individual fish samples, falling between 2.3% and 98.6%. RISA profiles had on average 21 bands ranging from 50 bp to 700 bp. Average band numbers suggest higher diversity in the months of February and December, as well as higher diversity in non-impacted sites (Figure 5-2). There were 16 clusters containing 3 or more individuals with over 70% similarity (cutoff based on repeatability and reproducibility of RISA in our lab), however

427 individuals (68% of all samples) fell into a single cluster. ANOSIM analysis indicated only month significantly influenced fingerprint similarity ($P = 0.001$), however the R value was low ($R = 0.166$) suggesting high overlap in bacterial assemblages [36] among months.

Sample Type

Eleven of 12 samples were successfully pyrosequenced. Skin samples from fish taken from site O4 during December failed to amplify for unknown reasons and despite efforts to remove inhibitors. Resulting bacterial sequences ranged from 3,019-6,122 for fish samples and 20,806-53,251 for water samples with initial OTUs averaging 225 in fish and 981 in water. After standardization, OTU numbers decreased to an average of 212 and 878 in fish and water, respectively.

Good's coverage and rarefaction curves indicate sequencing reached >98% of the diversity in each sample type (Figure 5-3; Table 5-2). Species richness was significantly different between sample types ($P < 0.0001$) with fish samples containing less species (as indicated by observed OTUs) than water samples. Average predicted total richness was 300 OTUs for fish samples and 1,287 OTUs for water samples. Species evenness was not statistically different between sample types.

OTU-based analysis shows clear separation between water and fish samples (Figure 5-4) with sample types separating at 10.3% similarity. ANOSIM analysis was significant with an R value of 0.950 indicating distinct separation between groups [36]. SIMPER analysis attributed 90% of community differences to abundances in Cyanobacteria, Proteobacteria, and Firmicutes (Figure 5-5). At the genus level, overall

dissimilarity between sample types was 85.4%, with differences in abundances of *Prochlorococcus* and *Pseudomonas* alone responsible for over 30% of community differences (Table 5-3).

Fish skin microbiota

Sequencing seemed to show the opposite of RISA in terms of diversity (species richness), with May and the impacted site on average having a higher number of OTUs. Pooled samples showed a large amount of variability in OTU structure, ranging from 25.1 to 53.6% similarity (Figure 5-4). Overall, fish-associated bacterial taxa (Figure 5-5) were composed mainly of Proteobacteria (71.3%), specifically the Gammaproteobacteria and Betaproteobacteria. Fin clips also contained a large abundance of Cyanobacteria. *Pseudomonas* was by far the most abundant genus, followed by *Prochlorococcus* (Table 5-4). *Paracoccus*, *Acidovorax*, *Janthinobacterium*, *Rhodanobacter*, *Limnobacter*, *Acinetobacter*, and *Vibrio* were also present as >3% of all sequences.

Influencing factors

MDS analysis based on OTU abundances supported RISA data that season has more influence on bacterial assemblages associated with fish skin than oil (Figure 5-6). As both RISA and sequencing indicate no impact of oil exposure on bacterial taxa structure, only differences between months were analyzed further. A total of 37.9% of OTUs were shared by at least two months. February fish communities were most separated from other months (see Figure 5-4 and Table 5-5) mainly due to differences in *Pseudomonas*, *Janthinobacterium*, and *Prochlorococcus*. SIMPER analysis indicated the most differences in community structure between the months of February and May

(Figure 5-7). Proteobacteria and Cyanobacteria abundances were always major contributors to differences in community structure.

Discussion

This study described the external bacterial communities associated with the skin and mucus of the Gulf killifish *F. grandis*, as well as examined factors that may impact the structure of those communities using fingerprinting and sequencing techniques. Proteobacteria was the most abundant phylum identified with *Pseudomonas* being the predominant genus. Other abundant genera included *Rhodanobacter*, *Janthinobacterium*, *Acidovorax*, and *Paracoccus*, along with *Prochlorococcus* of the phylum Cyanobacteria. Despite many shared genera, these bacterial communities differed significantly from those found in the surrounding water column. Bacterial community structure did not differ between fish from exposed and non-exposed sites; however there were seasonal differences.

The presence of Gammaproteobacteria and specifically *Pseudomonas* are consistent with previous studies on the bacterial taxa associated with fish skin [27, 29, 37-41] and thus appear to be common residents of mucus microbiota. We also identified a relatively high abundance of the genera *Vibrio* and *Acinetobacter* which are often associated with fish external microbiota [29, 37-43]. *Janthinobacterium* has also been recorded in association with striped mullet, pinfish, sand and spotted seatrout [37], and halibut larvae [27]. *Acidovorax* has not been reported as associated with fish skin

microbiota, however it appears as a common inhabitant of the gut microbiota of the sister species to *F. grandis*, *F. heteroclitus* [44]. Species from the genus *Paracoccus* are often fed to cultured red sea bream in order to boost their color and make them more appealing to consumers [45, 46] and at least one species has been isolated from fish intestine [47]. Thus most of the major genera found associated with *F. grandis* skin have been previously observed on or in fish with the exception of *Rhodanobacter*. Most of the sequences assigned to *Rhodanobacter* were most similar to *R. lindaniclasticus*, one of a few bacterial species capable of degrading lindane, a commonly used agricultural insecticide [48]. Despite the lack of reports of this genus in fish, it was the third most abundant on *F. grandis* after taxa in the genus *Prochlorococcus* (discussed in the next paragraph).

As fish skin is in constant contact with the environment, it is expected that external bacterial communities reflect those present in the surrounding water [38, 42, 49]. However, recent studies have demonstrated distinct bacterial community structure on fish skin as compared to that of water [26-30]. In this study, large differences were seen between sample types, particularly in the phyla Cyanobacteria and Proteobacteria. Despite large decreases in Cyanobacteria associated with fish compared to water samples, Cyanobacteria (primarily the genus *Prochlorococcus*) still made up 9% of all identified sequences in *F. grandis* skin samples. Cyanobacteria are rarely reported as inhabitants of fish skin microbiota [29, 37], but studies on bacterial communities of fishes normally focus on gut microbiota. It is possible that the Cyanobacteria in this study are contaminants from the water column. It should be noted that Cyanobacteria are often reported in association with corals [50-54] and thus it is also possible this phyla is a

normal component of the skin microbiota of some fishes. Major differences between sample types at the genus level were due to varying abundances between *Prochlorococcus* and *Pseudomonas*. In this study, *Pseudomonas* sequences associated with fish samples were most closely related to *P. fluorescens*, *P. trivialis*, *P. mendocina*, and *P. veronii*, but water samples rarely contained these species. Thus the differences in this genus were even more apparent at the species level. At an overall similarity of only 10%, it is clear that *F. grandis* hold a distinct external microbial community from the surrounding water.

Results from this study showed no significant difference in *F. grandis* skin microbiota structure between sites reported to be impacted by DHOS and sites reportedly not impacted. It is possible that the microbiota did not change in response to influx of oil associated with DHOS. Barataria Bay, including all 8 of sampling sites included in this study, is no stranger to oil exposure with at least 16 incidents recorded since 1999 [55; search criteria "Barataria Bay"]. *F. heteroclitus* populations have demonstrated the ability to evolve and survive in highly polluted areas [For a review see reference 18], and a recent study has demonstrated this ability in *F. grandis* as well [56]. Thus it is possible that killifish populations in this study area have adapted to this 'oily' environment. As this study was conducted one year after an unknown exposure rate, another possibility is that communities were altered with initial contact with the oil and have since been able to adapt and return to normal community structure. The mucus layer of fish provides a relatively stable habitat for bacteria as many bacterial species have the ability to adhere to [57-60] and grow in [61-63] fish mucus. These host-microbe interactions are likely more complex than previously believed as fish-associated microbial communities have

demonstrated species-specificity in some cases [37, 64, 65]. Although studies have shown changes in bacterial communities in oil contaminated versus non-contaminated waters [66-68], no studies have examined changes in fish-associated microbiota in these conditions. A consistent bacterial community structure between sites exposed to oil and not exposed may indicate a stable, tolerant environment on the fish surface that is fairly resilient to pollutants.

Since the DHOS, several studies have indicated sublethal genomic and physiological impacts of oil exposure in the Gulf killifish [69, 70], including immune stress [71, 72]. Some of these impacts were seen as much as one year after the DHOS [69] which overlaps with time points used in this study. Stress caused by pollutants may lead to a change in bacterial community composition, but this relationship has not been studied. However, shifts in microbiota structure can be seen prior to [23, 24, 73] and during [25, 54] disease states of aquatic organisms. Specific interactions between immune stress and bacterial communities are underexplored and thus reasons as to why no effects of oil impact were seen in this study as compared to coinciding studies in nearby habitats are unknown. It is important to note that studies reporting genomic differences due to oil exposure used control sites in different geographical locations and sometimes different states. Large variation in gene expression exists within and among populations of *Fundulus* [74-77]. Considering the high site fidelity of this species [17], it is possible that the genetic variability seen in these studies is due to the sampling of different subpopulations of *F. grandis*.

Results of this study suggest seasonal variation in the microbiota structure of *F. grandis* and indicate that fish mucus microbes are subject to changes due to

environmental conditions. Our results indicate a large change in community composition between February and May, followed by gradual changes throughout the year toward a structure more similar to February. However only one year was included in this analysis and extrapolation to a more general pattern is not possible. The average water temperatures differed by month (February 20.3°C, May 27.9°C, August 35.4°C, and December 17.7°C; [56]) and may be one of the factors dictating seasonal changes. Interestingly, changing temperatures have little effect on the bacterial assemblages associated with corals except during anomalies that result in stress and disease [79-81]. However, seasonal variation in fish microbiota seems to be a normal occurrence [37-39, 82, 83]. Wilson *et al.* [39] found evidence of two groups of populations inhabiting the surface of Atlantic cod. The first group was relatively constant year-round (residents) and the second changed dependent on season (transients). Our results show that the bacterial taxa associated with *F. grandis* are relatively stable despite sampling location (68% with >70% similarity in RISA profiles and all samples >70% similar in phyla abundances) suggesting a resident population. However sequencing and SIMPER analysis pointed to a number of genera that could be considered transients. For example *Janthinobacterium* was only present in February while *Paracoccus* was only represented in August. Also large differences in abundances such as high numbers of *Prochlorococcus* in April and August and *Pseudomonas* in February and December might indicate some transient members of these genera.

In conclusion, *F. grandis* skin and mucus microbiota is significantly different from that of the surrounding water. This study represents the first time *Prochlorococcus* was found in high numbers associated with the fish surface, as well as the first report of

Rhodanobacter. Other members of the fish-associated microbiota were similar to those found associated with other fish species. No microbiota changes were detected in fish from oiled sites versus those from non-oiled sites. Although the bacteria associated with the skin and mucus are impacted by season, there appears to be a component that is stable, indicating a core microbiota for this fish species.

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Table 5-1. Individual fish samples taken from each site and month during the course of the study.

Locality	Site	# Samples				
		February	May	August	December	Site Totals
Northern Hackberry Bay: Grand Bayou, LA	**N1**	19	19	19	20	77
	N2	20	20	20	19	79
	N3	20	20	19	20	79
	N4	20	20	19	19	78
Wilkonson Canal, LA	**O1**	18	20	20	20	78
	O2	20	20	19	20	79
Wilkonson Bayou, LA	**O3**	20	20	19	20	79
Northern Bay Jimmy, LA	**O4**	20	20	19	20	79
	Month Totals	157	159	154	158	**628**

Table 5-2. Diversity statistics associated with each pooled sample as determined by pyrosequencing and calculated by Mothur.

Sample Type	Site	Month	Number of Sequences		Good's coverage	OTUs		Shannon Evenness Index
			Initial	Subsample		Observed	Expected	
Fish	N1	February	3938	3769	0.9883	177	223	0.6473
Fish	N1	May	4792	3769	0.9881	196	278	0.7381
Fish	N1	August	3769	3769	0.9883	224	300	0.8103
Fish	N1	December	4640	3769	0.9881	186	339	0.7594
Fish	O4	February	5111	3769	0.991	175	224	0.6706
Fish	O4	May	5757	3769	0.9806	283	442	0.7939
Fish	O4	August	6824	3769	0.9875	237	305	0.7561
Water	N1	February	21254	21254	0.991	926	1224	0.7245
Water	N1	May	44649	21254	0.9871	798	1348	0.5771
Water	O4	February	34971	21254	0.9913	967	1255	0.6836
Water	O4	May	53456	21254	0.9877	832	1321	0.5799

Table 5-3. Similarity percentage (SIMPER) results by genera for fish versus water samples. Only genera accounting for at least 0.7% of dissimilarity are included. Total dissimilarity between sample types was 85.4%.

Phylum	Genus	% of Total Sequences		Average Dissimilarity	Cumulative Dissimilarity
		Fish	Water		
Cyanobacteria	*Prochlorococcus*	9.73	55.8	23.0	23.0
Proteobacteria (γ)	*Pseudomonas*	22.4	0.19	11.1	34.1
Proteobacteria (α)	*Candidatus Pelagibacter*	0.58	14.3	6.85	41.0
Proteobacteria (α)	*Anderseniella*	1.28	8.66	3.69	44.7
Proteobacteria (γ)	*Rhodanobacter*	6.23	-	3.11	47.8
Proteobacteria (β)	*Janthinobacterium*	5.02	-	2.51	50.3
Proteobacteria (β)	*Acidovorax*	4.52	0.54	2.14	52.4
Proteobacteria (γ)	*Acinetobacter*	3.52	0.02	1.75	54.2
Proteobacteria (α)	*Paracoccus*	3.29	0.08	1.66	55.8
Proteobacteria (β)	*Limnobacter*	2.91	0.02	1.46	57.3
Proteobacteria (γ)	*Vibrio*	2.64	0.01	1.32	58.6
Firmicutes	*Staphylococcus*	2.27	-	1.14	59.8
Proteobacteria (γ)	*Crenothrix*	0.11	2.06	0.98	60.7
Proteobacteria (α)	*Defluviicoccus*	-	1.89	0.95	61.7
Proteobacteria (β)	*Herbaspirillum*	1.8	-	0.90	62.6
Actinobacteria	*Propionibacterium*	1.71	-	0.85	63.4
Bacteroidetes	*Cloacibacterium*	1.41	-	0.71	64.1
Proteobacteria (γ)	*Arenimonas*	1.40	0.02	0.70	68.4

Table 5-4. Major genera making up *Fundulus grandis* skin microbiota. Only genera accounting for at least 1% of total sequences are included.

Phylum (Class)	Genus	% of Sequences
Actinobacteria (Actinobacteria)	*Propionibacterium*	1.57
Bacteroidetes (Flavobacteriia)	*Cloacibacterium*	1.36
Cyanobacteria	*Prochlorococcus*	9.92
Firmicutes (Bacilli)	*Staphylococcus*	2.26
Firmicutes (Clostridia)	*Clostridium*	1.25
Proteobacteria (α)	*Anderseniella*	1.38
	Paracoccus	4.35
Proteobacteria (β)	*Limnobacter*	3.03
	Acidovorax	4.57
	Herbaspirillum	1.79
	Janthinobacterium	4.68
	Rhodocyclus	1.58
Proteobacteria (γ)	*Acinetobacter*	3.77
	Pseudomonas	20.6
	Vibrio	3.33
	Arenimonas	1.02
	Rhodanobacter	5.76

Table 5-5. Similarity percentages (SIMPER) results by genera for fish samples based on sampling month. Genera resulting in 50% of total dissimilarity between any two months are included.

		Average Dissimilarity					
Phylum (Class)	**Genus**	**February May**	**February August**	**February December**	**May August**	**May December**	**August December**
Actinobacteria (Actinobacteria)	*Corynebacterium*	0.68	0.06	0.11	0.73	1.23	-
	Propionibacterium	1.71	0.50	0.80	1.21	2.42	0.09
Bacteroidetes (Flavobacteriia)	*Cloacibacterium*	0.75	0.85	1.70	0.24	1.87	1.72
Bacteroidetes (Sphingobacteriia)	*Sediminibacterium*	1.04	1.04	1.12	0.22	-	-
Cyanobacteria	*Prochlorococcus*	8.86	6.36	2.60	2.50	5.11	5.11
Firmicutes (Bacilli)	*Bacillus*	0.12	0.50	-	0.62	-	1.00
	Staphylococcus	2.71	0.58	0.41	2.13	2.04	0.75
Firmicutes (Clostridia)	*Clostridium*	1.42	0.35	0.04	1.07	-	-
Proteobacteria (α)	*Anderseniella*	1.41	0.63	0.16	0.78	0.64	0.52
	Blastobacter	1.5	1.34	1.62	0.17	0.03	0.37
	Bradyrhizobium	0.70	0.70	1.37	-	-	-
	Gaetbulicola	1.61	1.50	2.75	0.11	0.24	0.24
	Paracoccus	-	5.71	-	5.71	-	0.63
Proteobacteria (β)	*Acidovorax*	2.64	2.99	5.88	0.35	6.32	6.42
	Alicycliphilus	0.08	0.03	2.09	0.11	1.93	2.09
	Janthinobacterium	8.79	8.79	9.31	-	-	-
	Limnobacter	4.91	0.17	-	4.74	6.46	0.33
	Rhodocyclus	0.11	1.94	0.10	1.95	-	-

Table 5-5 continued

		Average Dissimilarity					
Phylum (Class)	**Genus**	**February May**	**February August**	**February December**	**May August**	**May December**	**August December**
Proteobacteria (γ)	*Acinetobacter*	4.10	3.13	0.75	0.97	0.97	0.75
	Aeromonas	1.01	0.34	-	0.67	0.76	0.65
	Arenimonas	0.04	1.58	1.56	1.54	1.50	1.59
	Litoricola	-	-	1.16	-	1.16	1.16
	Pseudomonas	11.4	11.5	9.40	1.21	5.83	4.71
	Rhodanobacter	4.04	4.60	4.23	0.56	1.77	2.22
	Vibrio	0.32	3.08	0.53	2.84	0.19	0.27
	Other	17.45	18.65	16.94	20.16	17.45	25.92
	Total Dissimilarity	77.38	76.89	64.63	50.59	57.92	56.54

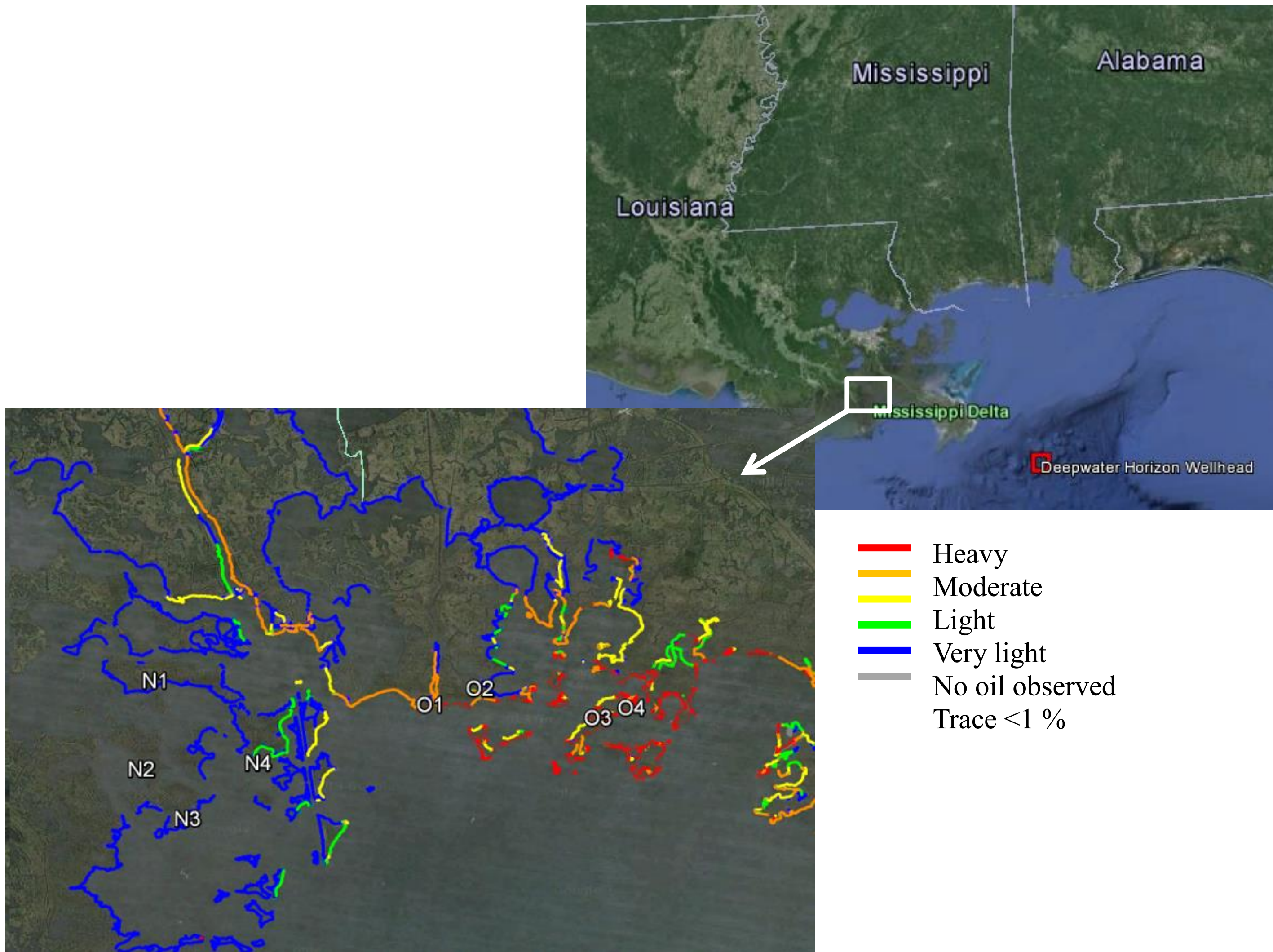
Mississippi
Alabama
Louisiana
Mississippi Delta
Deepwater Horizon Wellhead
N1
N2
N3
N4
O1
O2
O3
O4
Heavy
Moderate
Light
Very light
No oil observed
Trace <1 %

Figure 5-1. Sampling sites used in this study. Overlay is taken from National Oceanic and Atmospheric Administration's (NOAA) Environmental Response Management Application® (ERMA) Shoreline Cleanup and Assessment Technique (SCAT) data available at http://response.restoration.noaa.gov. N1-4, not impacted, O1-4, impacted.

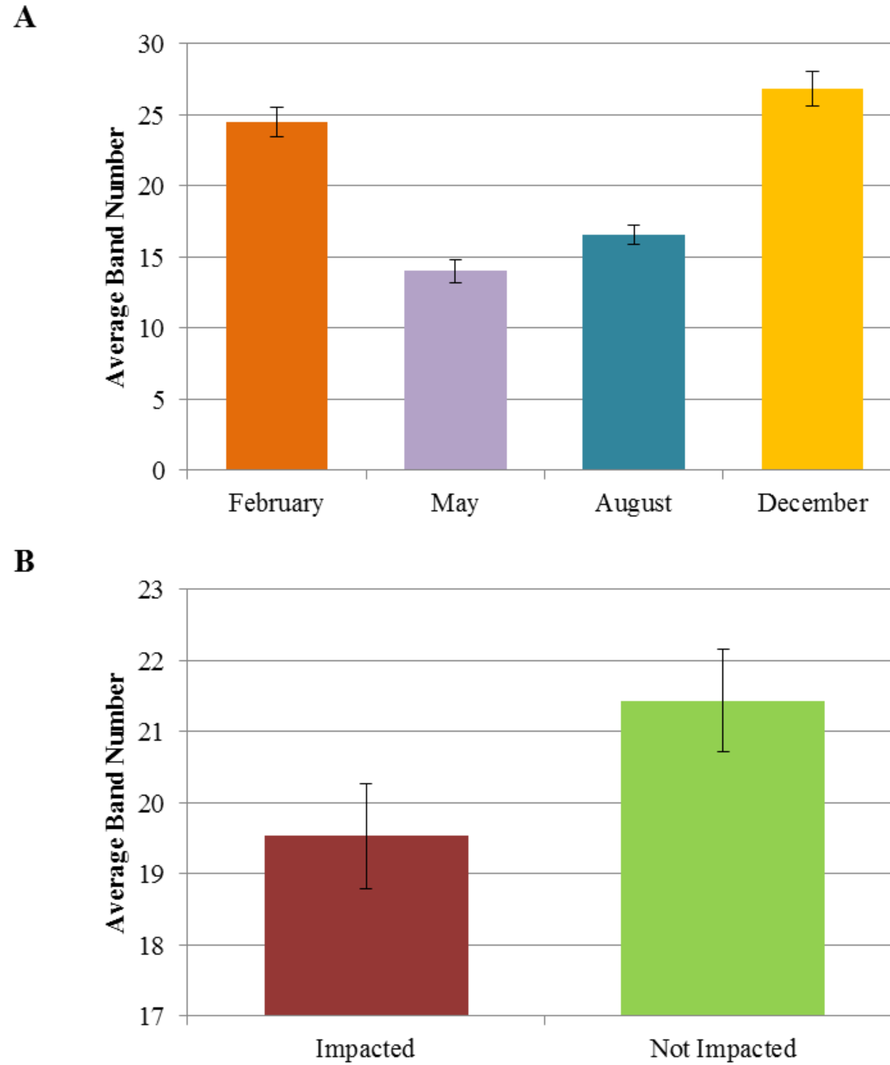

Figure 5-2. Average band number including standard error from RISA profiles by A, month and B, oil impact.

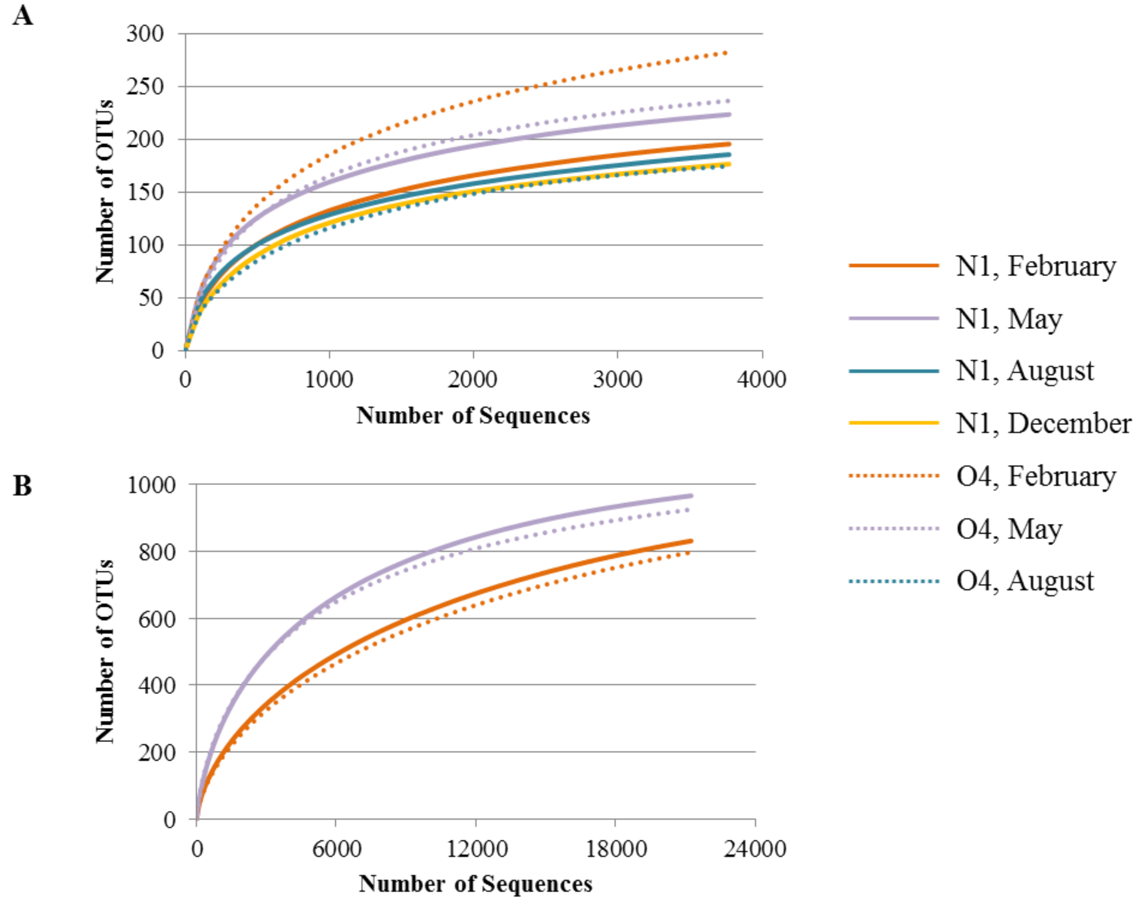

Figure 5-3. Rarefaction curves for A, fish and B, water samples. Samples were standardized by the least number of sequences for each sample type. N1, non-exposed site; O4, exposed site.

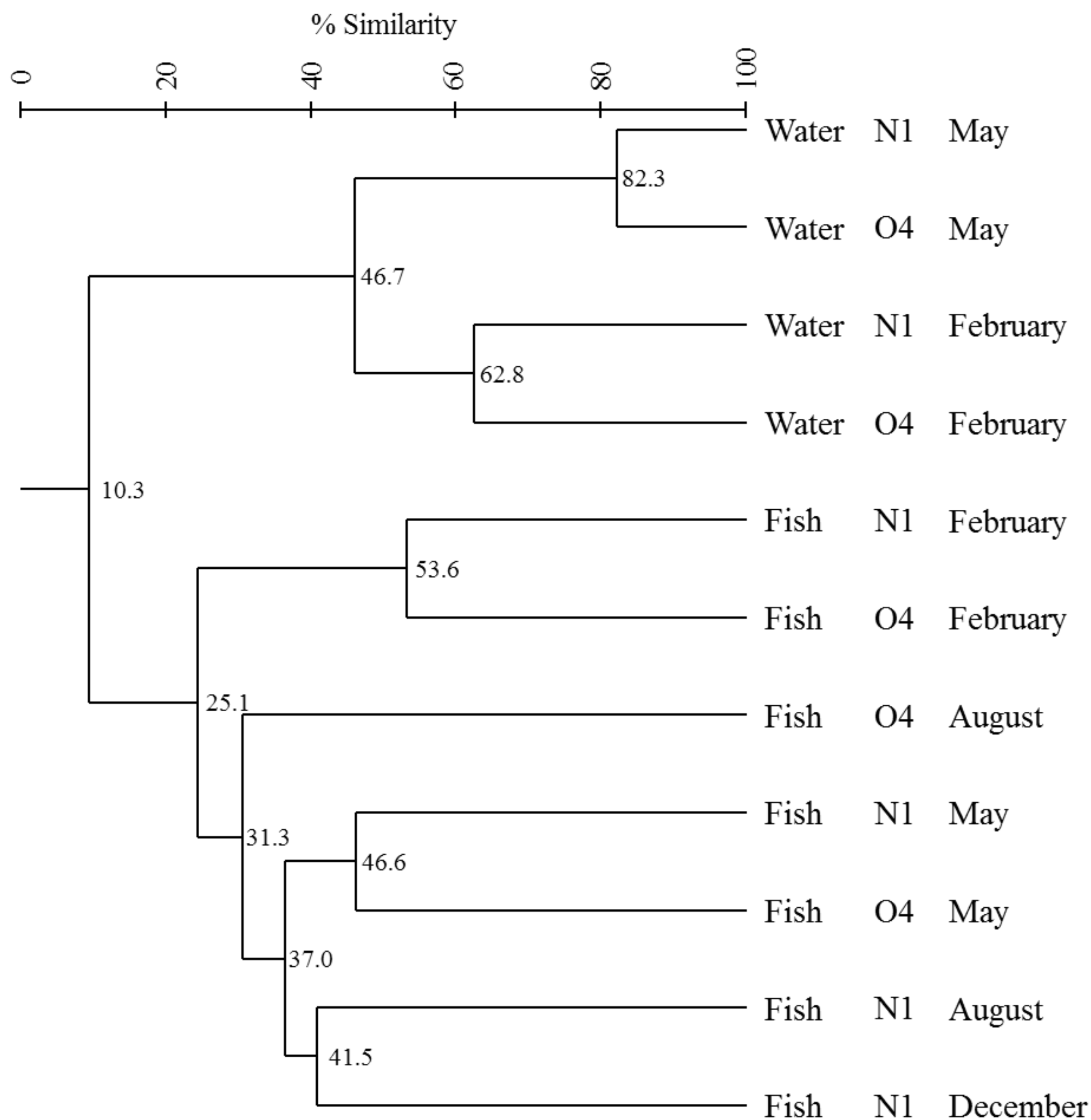

Figure 5-4. Cluster analysis based on percent similarity in operational taxonomic units (OTUs). Samples are labeled by sample type, site, and month. Exact percent similarities are given at each branching point.

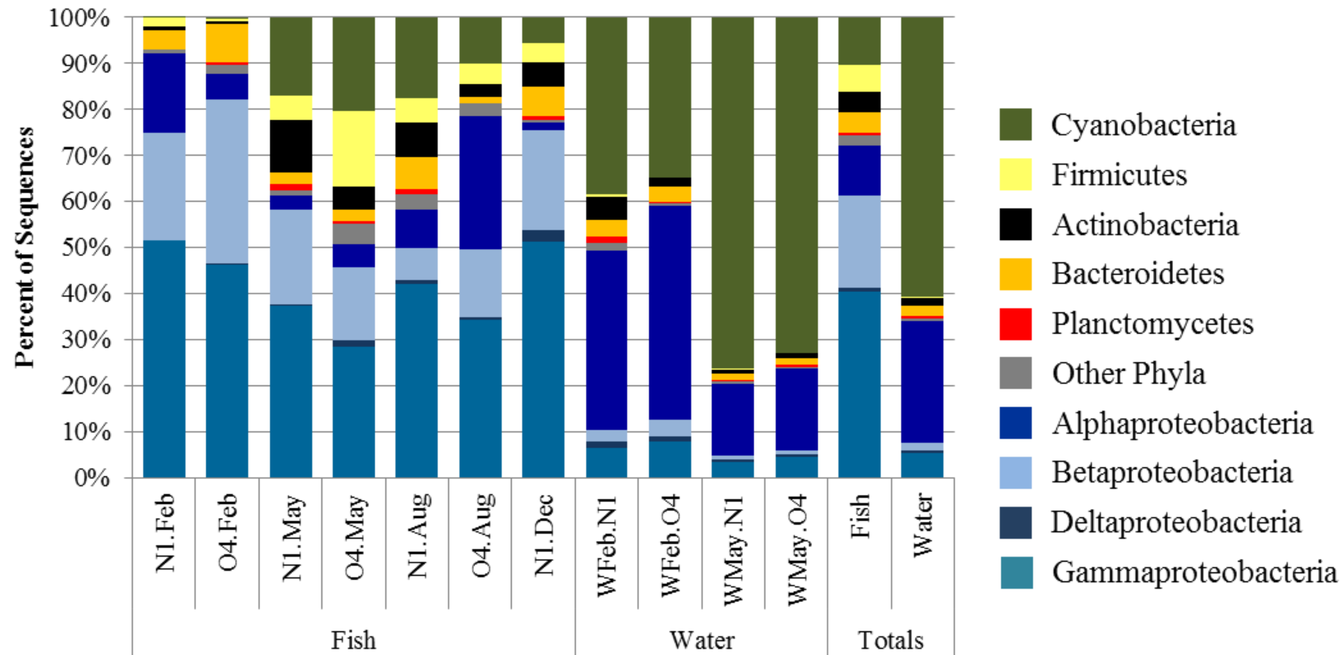

Figure 5-5. Sequencing results based on phylum composition for all samples, as well as averages for fish and water sample types. Only phyla making up >0.5% of total sequences for at least one sample were included.

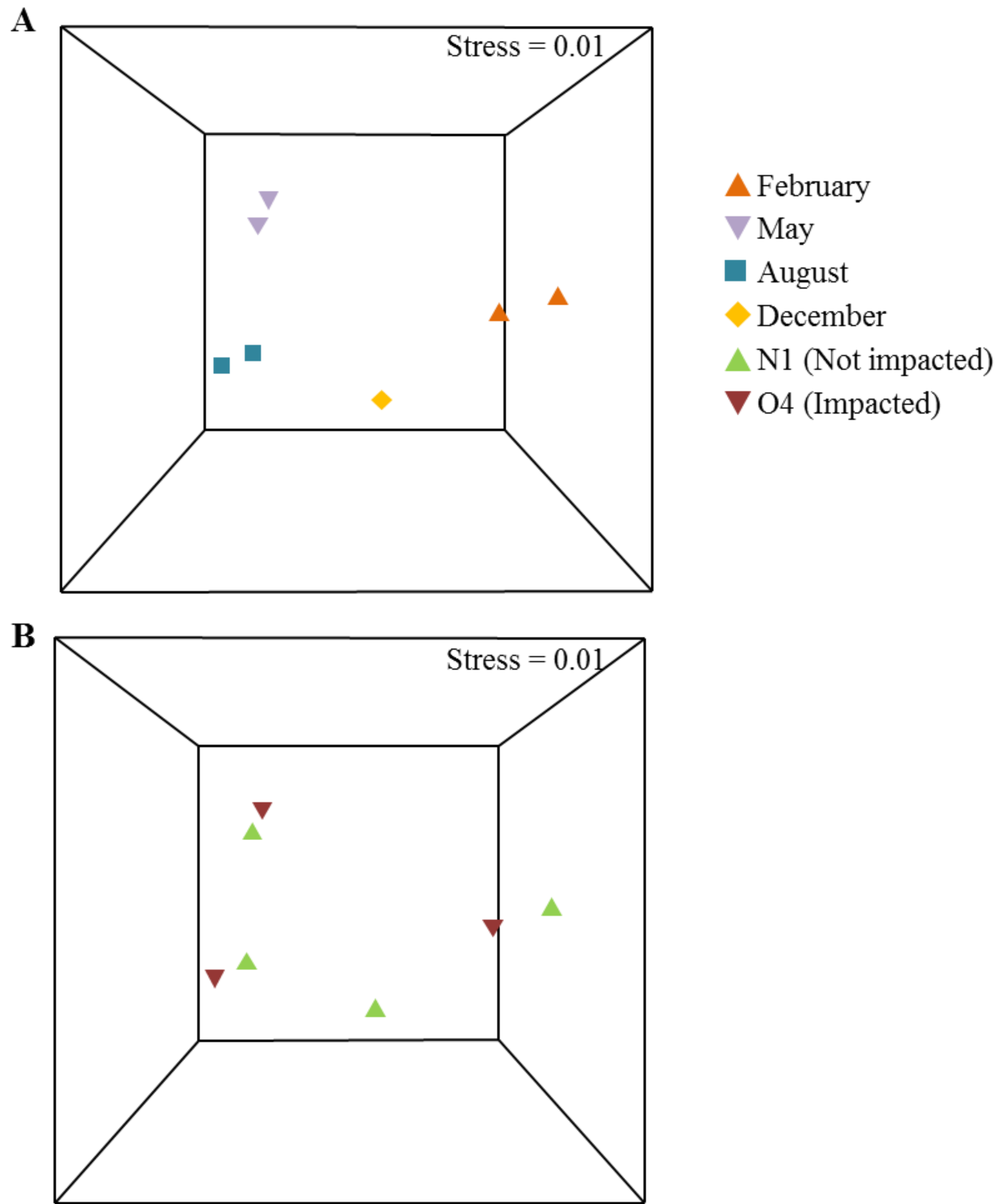

Figure 5-6. Multidimensional scaling based on percent similarity in OTU abundances for fish skin samples based on A, month, and B, oil impact.

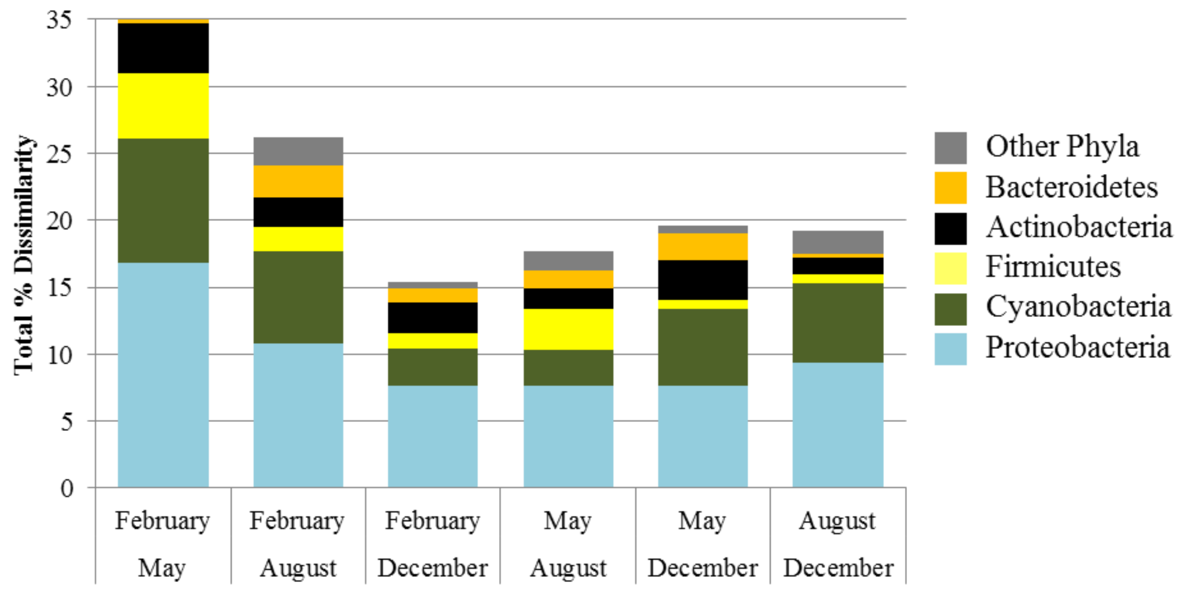

Figure 5-7. Similarity percentage (SIMPER) results indicating phyla responsible for percent dissimilarity between fish samples for each month.

CHAPTER 6. HIGH DIVERSITY OF BACTERIA IDENTIFIED FROM THE BLOOD OF APPARENTLY HEALTHY RED SNAPPER *LUTJANUS CAMPECHANUS*

Abstract

The blood of healthy fish is presumed to be sterile, but a number of studies have isolated a wide diversity of bacteria from the blood and internal organs of apparently healthy individuals. This study surveyed the microbiota of the blood, gill, and feces of 10 wild-caught red snapper from 5 artificial reef sites in the Gulf of Mexico. Sampled red snapper showed no physical signs of disease (external or behavioral). Blood was taken from the caudal vein, and gill and gut contents were also examined as they are known to be primary entry routes for pathogens. Aerobic heterotrophic counts were performed followed by pyrosequencing to identify bacteria present in each sample type. All 10 fish had positive blood cultures with counts of up to 42 CFU/mL. Aerobic counts of feces and gill samples were 10^7 and 10^4 respectively. The microbiota of all sample types was dominated by Gammaproteobacteria. However, blood and gill samples were predominately composed of the genera *Pseudomonas* and *Nevskia* whereas feces were primarily *Umboniibacter* and *Pseudoalteromonas*. However individual variation was high and one individual had a relatively large abundance of sequences from the genus *Ralstonia* present in the blood. Overall, blood microbiota was more similar to gill

bacterial communities than feces. The presence of a high diversity of bacteria in healthy fish blood suggests that isolation of bacteria from the blood and internal organs is not exclusive to diseased individuals.

Introduction

In 2010, production of food fish from aquaculture had reached a level 12 times higher than that of 1980 at nearly 60 million metric tons worth over $119 billion US dollars [1]. However disease is a significant limitation to the growth of the industry [2-4], resulting in billions of dollars of losses a year [5]. Bacterial diseases of fish are caused by organisms from over 50 genera, see [6]. Unfortunately the symptoms of bacterial infection are not unique or constant for a particular bacterial species [7, 8] and isolation by culture is necessary to diagnose bacterial disease.

The most common site for bacterial isolation is from the internal organs, particularly the kidney [9]. Often, Koch's Postulates are not performed and isolation of bacteria from a diseased fish is assumed to be sufficient for disease diagnosis [6]. The blood and internal organs of fishes are believed to be sterile [10, 11], however there are a number of reports of bacterial isolation from the blood and/or internal organs of apparently healthy individuals (see Table 6-1). Thus it is possible that a positive blood culture is not indicative of disease in fish.

The red snapper is an economically significant species in the Gulf of Mexico, supporting both a recreational and commercial fishery valued at $60 million a year in the United States [12]. Overfishing of red snapper resulted in a crash of the stocks making commercial fishing nonviable, however management strategies implemented by the Gulf

of Mexico Fishery Management Council in 1989 have the stocks on the road to recovery [13]. The economic value of the red snapper as a food and game fish and its wild stock status make this species a primary aquaculture candidate [14]. As a result disease diagnosis in this species is relevant to the aquaculture industry. No studies to date have examined the blood of apparently healthy red snapper for naturally occurring bacteria.

This study provided the first survey of the microbiota present in healthy red snapper individuals using culture-independent methods. Along with blood samples, microbiota was characterized on the gill and in feces as gill and intestine are known to be primary entry routes for opportunistic pathogens [15, 16] in fishes.

Methods

Sample collection

Five artificial reef sites were selected in the fall of 2013 approximately 15-30 km south of Orange Beach, AL and Pensacola, FL (Table 6-2). Site conditions (depth (m), salinity (psu), temperature (°C), dissolved oxygen (DO; mg/L), fluorescence (mg/m^3 and visibility (%)) were taken at each site using a 19*plus* V2 SeaCAT Profiler CTD (Sea-Bird Electronics, Inc., Bellevue, Washington, USA). Two red snapper were caught from each site on hook and line using squid as bait. Fish were measured (fork and total length, mm) and weighed (kg) and sacrificed with a sharp blow to the head. Immediately, a filet of muscle was removed from one side with a sterilized (using 70% ethanol) filet knife. Remaining exposed muscle tissue was sprayed with 70% ethanol to reduce contamination and 2 mL of blood was taken from the caudal vein using a sterile needle and syringe.

Triplicate samples of 15 μL were placed into sterile microcentrifuge tubes and the remaining sample was saved for aerobic heterotrophic counts. Following blood extraction, the outer surface of the operculum was sterilized using 70% ethanol. The operculum was pulled back to reveal the gill arches and the anterior gill arch was removed using sterile techniques. To obtain a feces sample, the ventral surface of the fish was sterilized with 70% ethanol and opened to reveal the intestine. The lower third of the intestine was removed using clamps to prevent release of fecal material. The intestinal contents were squeezed into a sterile centrifuge tube. All samples were placed on ice until arrival at the laboratory (approximately 6 hours). Samples were labeled according to species (RS = red snapper), individual sampled (1-10), and sample type (B = blood, G = gill, F = feces).

DNA extraction and sequencing

Upon arrival at the laboratory, triplicate 25 mg samples were taken from each gill clip and feces sample. These samples as well as the triplicate blood samples were subjected to DNA extraction with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to manufacturer instructions including pretreatment for Gram-positive bacteria at 37°C overnight (15 h), proteinase K digestion for one hour, and digestion of RNA using RNase A. DNA was quantified using a spectrophotometer and triplicates were combined in equimolecular amounts to obtain one sample for each sample type from each fish. Roche titanium 454 sequencing was performed using barcoding and primer 27F (5'-AGRGTTTGATCMTGGCTCAG-3') to amplify the V1-V3 variable regions of the 16S rRNA gene. PCR conditions included an initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min,

concluded with a final elongation at 72°C for 5 min. Sequences were processed using an exclusive analysis pipeline (MR DNA, Shallowater, TX). Barcodes and primers, short sequences (< 200 bp), and sequences with a base call error rate of less than 0.3% (Q<25), ambiguous base calls, and long (>6 bp) stretches of identical bases were removed. Following denoising and chimera and singleton sequence removal, operational taxonomic units (OTUs) were defined and identified using BLASTn against the Greengenes database [17] at <3% sequence agreement according to the current accepted prokaryotic species concept [18]. Rarefaction curves, diversity indices (number of OTUs, number of predicted OTUs using the catchall command, Good's coverage and Shannon evenness index), and shared OTUs were calculated using Mothur v.1.33.3 [19].

Aerobic heterotrophic counts

Remaining blood, gill, and feces were weighed and diluted 1:1 with sterile phosphate buffered saline (PBS). After homogenization, subsequent 1/10 dilutions were made and plated in six replicates onto Marine Agar 2216 (MA; Difco Laboratory, Detroit, Michigan, USA) and 5% sheep blood agar (BA; Hardy Diagnostics, Santa Maria, CA). Three of each plate was incubated at both 18°C and 30°C for one week. Colony forming units (CFUs) were counted after 2d and 7 d.

Data analysis

Sequences were randomly selected from each sample in order to standardize sampling effort to that of the sample that returned the least number of sequences (950 sequences, sample RS8B). Following standardization, ANOVAs were run on number of expected OTUs and Shannon evenness index to determine differences among sample types. Original sequence data in the form of OTU tables were uploaded in Primer v6

(Primer-E Ltd, Plymouth, UK). After standardization (transforming raw OTU abundances to percentages), multidimensional scaling (MDS) and analysis of similarities (ANOSIM) were performed between sample types (blood, gill, feces). A genera abundance table was loaded into Primer for similarity percentages (SIMPER) analysis to determine the genera responsible for differences between sample types.

Results

Sampling locations and fish

Environmental conditions were similar at all sampling sites during the course of the study, with average conditions as follows: depth – 28 m, salinity – 34 psu, temperature – 26°C, DO – 6.3 mg/L, fluorescence – 0.14 mg/m^3, visibility – 89.6% (Table 6-3). A total of 6 male and 4 female red snapper were sampled with a mean weight of 1.3 kg (SD, 1.6) and mean total length of 434 mm (SD, 114) (Table 6-4).

Aerobic heterotrophic counts.

Aerobic counts were similar between incubation temperatures except in blood samples on BA where plates incubated at 30°C yielded more CFUs than plates incubated at 18°C (Figure 6-1). Increased incubation time (2 d to 7 d) did not impact growth in gill samples, but resulted in >1 log increase in CFU counts in feces samples and on average doubled CFU counts in blood samples, regardless of incubation temperature. MA facilitated the recovery of more CFUs than BA in blood samples. After one week incubation, CFU/g of feces samples ranged from $2.00x10^5$ to $5.69x10^8$. CFU/g of gill samples ranged from $4.00x10^2$ to $2.06x10^5$. Blood samples plated on MA ranged from 0

to 42 CFU/mL and on BA from 0 to 32 CFU/mL. After 2 d, 9/10 and 5/10 individuals had culture-positive blood samples on MA and BA respectively with all blood samples showing growth on at least one media after 7 d of incubation.

Sequencing

Sequencing was successful for 19 of 30 samples, including gill samples RS1-9, feces samples RS5-10, and blood samples from RS1, RS4, RS8, and RS10 (Table 6-5). Number of sequences from individual samples ranged from 950 to 11,888 with 91 average OTUs. Following random sequence selection to standardize sampling effort across samples, average number of OTUs decreased to 66. Good's coverage indicated >96% sample coverage across all samples with lowest coverage on average occurring in feces samples. Sample types differed in terms of expected OTUs ($F_{2,16} = 4.14$, $p = 0.036$) with feces having a significantly higher number than blood samples indicating higher bacterial species richness in feces samples. Shannon evenness indices were the same between sample types.

ANOSIM analysis correlated well with shared OTU results (Table 6-6; Figure 6-2). ANOSIM analysis indicated a significant difference between sample types with 35.8% of all OTUs shared between at least two samples types. This high percentage of shared OTUs correlates with the low R value of 0.270, suggesting only slight separation overall among groups [20]. The *p* value and relatively high R value (0.464) when comparing blood and feces samples suggest the most separation between these two sample types and as verified by the low number of shared OTUs (15.8%). Gill and feces samples have slightly more overlap in terms of both R value and shared OTUs (21.4%). However blood and gill samples were not statistically separated and 64% of all OTUs

present in blood samples were also present in gill samples. Thus the bacterial species present in blood samples are more similar to those in gill than in feces.

Phylum level analysis of the microbiota indicates that Proteobacteria dominated all sample types (Figure 6-3), specifically the Gammaproteobacteria class. Feces samples contained a larger abundance of non-Proteobacteria including relatively high abundances of Cyanobacteria, Fusobacteria, and Planctomycetes which are nearly absent from the other sample types. A majority of the blood sequences that were not Proteobacteria were identified as either Actinobacteria or Bacteroidetes at an abundance of 3% total sequences each. These two phyla were also present in gill samples, but at lower abundances (1% and 2% respectively). Gill samples contained nearly 4% sequences from the Firmicutes as compared to the 1% found in blood samples.

Only one individual resulted in successful sequencing from all three sample types, sample RS8 (Figure 6-4). In this individual, feces samples were dominated by 4 genera (*Pseudoalteromonas* and *Umboniibacter* of the Gammaproteobacteria, *Prochlorococcus* of the Cyanobacteria, and *Pirellula* of the Planctomycetes). At the genus level, feces samples were highly different from blood and gill samples. Blood and gill samples resulted in a high abundance of genera of the Proteobacteria (Gamma- and Betaproteobacteria; blue and purple bars respectively) with *Pseudomonas* and *Nevskia* as predominant genera. However, samples differed in less abundant genera. Blood samples had a higher abundance of *Micrococcus* (Actinobacteria), *Staphylococcus* (Firmicutes), and *Cetobacterium* (Fusobacteria) while gill samples were dominated by *Cloacibacterium* (Bacteroidetes) and other genera.

Although sample RS8 had high similarity between blood and gill samples, RS1 and RS4 did not (Figure 6-5). Gill from RS1 was dominated by *Acinetobacter* and *Clostridium* whereas blood samples were primarily *Pseudomonas*, *Nevskia*, and *Ralstonia*. RS4 gill samples were predominately *Acidithiobacillus*, whereas blood samples had a large abundance of *Pseudomonas* followed by a wide variety of other Gamma- and Betaproteobacteria. Thus the pattern of the microbiota of blood being similar in composition to that found on the gill is not seen in all fish individuals, and individual variability is high in both blood and gill microbiota composition. However overall similarity between blood and gill samples (22.5%) is higher than that between blood and feces samples (12.3%) largely due to greater abundances of *Photobacterium*, *Vibrio*, and *Pseudoalteromonas* in feces samples and *Pseudomonas* and *Nevskia* in blood samples (SIMPER; Table 6-7). Between individual red snapper, the most variability occurred between gill samples followed closely by feces samples with only 21.3% and 22.4% similarity respectively (Table 6-7; Figures 6-6 through 6-8). Blood samples were much more similar across individuals with a similarity of 58%. One interesting discrepancy between red snapper individuals was the presence of a large abundance of the genus *Ralstonia* in the blood of RS1 only (29.5% of RS1B sequences).

Discussion

The results of this study indicate that fish blood may not be sterile as previously believed. All of our fish showed positive blood cultures with up to 32 CFU/mL or 42 CFU/mL on BA and MA, respectively. A majority of aerobic growth occurred after 48 h

incubation, regardless of incubation temperature. The microbiota of blood samples as determined by sequencing was more similar to that of the gill than the feces, due largely to differences in abundances of genera from the Gammaproteobacteria. Blood samples were predominately Proteobacteria, specifically Gamma- and Betaproteobacteria, with minor abundances of Alphaproteobacteria, Actinobacteria, and Bacteroidetes. The majority of sequences from blood samples belonged to the genera *Pseudomonas* and *Nevskia*. However genera abundances differed greatly between individual red snapper with only one fish containing *Ralstonia* and in high abundances.

In terms of sequencing, only 19 of 30 samples were successful. Compounds from feces including bile salts [21] and complex polysaccharides [22] and hemoglobin [23] in blood are known to inhibit PCR reactions. Thus the loss of these samples may be due to the presence of PCR inhibitors in the samples, although inhibitor removal did not increase success. In blood samples, small sample size (45 µL total) for DNA extraction might have also failed to detect bacteria, resulting in unsuccessful PCR amplification. To our knowledge, there is no method optimized for extraction of bacterial DNA from fish blood as it is believed to be sterile. The procedures used in this study were optimized for extraction of fish DNA from nucleated blood cells. For future studies, the removal of blood cells from serum could reduce the presence of inhibitors and fish DNA and allow for use of larger sample volumes.

Feces aerobic heterotrophic counts (average 8.32×10^7) were similar to those seen in other studies on fish gut microbiota as indicated by Austin [24] and other studies on wild marine fish species including Atlantic cod *Gadus morhua* [25], daisy parrotfish *Chlorurus sordidus*, whitecheek surgeonfish *Acanthurus nigricans* and two-spot red

snapper *Lutjanus bohar* [26]. Aerobic counts from gill (average 6.46×10^5) were also within normal range for fishes [24]. Previous studies reporting bacterial isolation from fish blood did not report aerobic counts. Therefore, only composition of microbiota can be compared with these studies, not abundance of bacteria.

All of the individuals sampled in this study showed positive blood culture growth. Although contamination cannot be completely ruled out, this high percentage of culture-positive individuals may be due to the use of a larger sample volume than previous studies. Cultures in this study were made from 2 mL samples whereas the maximum volume used in other bony fish studies is 0.5 mL [27]. All other studies used 10-100 µL [28-30]. In addition, longer incubation times seem to be necessary to grow bacteria present in blood. Mylniczenko et al. [31] determined most growth in elasmobranch blood samples occurred after 72 hours. This study showed similar results in bony fish by counting more isolates after 7d as opposed to 2d. Studies on the blood and internal organs of freshwater bony fish stopped incubation after 5 d at most [27-29, 32], whereas previous studies on marine fish stopped incubation after 2-3 d [30, 33]. Another factor leading to our high percentage of positive results may be our low sample size, as other studies on marine fish have seen percentages ranging from 25-42% with much larger sampling efforts [30, 31, 33]. This discrepancy warrants further investigation with surveys of a larger number of marine fish species.

Many similarities exist between the microbiota found in the blood and internal organs of apparently healthy fish species. All studies that characterized isolates to the genus level in both marine and freshwater species have found isolates of the genera *Pseudomonas* [28-31, 33, 34]. This study also found a large abundance of *Pseudomonas*

from pyrosequencing. Members of this genus 1) may be permanent residents of the blood microbiota or 2) may be better equipped to penetrate the epithelium of the fish to enter the bloodstream. The presence of *Pseudomonas* in a number of fish species makes this genus an interesting target for future studies. Other genera that seem to be commonly located in the blood and internal organs of marine and freshwater fish include *Aeromonas* [28, 29, 31, 33, 34], *Enterobacter* [29, 30], *Micrococcus* [33, 34], *Streptococcus* [31, 33, 34], and *Bacillus* [29, 30, 34]. *Achromobacter* [29, 34] have only been identified in freshwater fish, whereas *Vibrio*, *Staphylococcus*, [30, 31, 33], *Photobacterium*, and *Stenotrophomonas* [30, 31] have only been isolated from marine fish. In this study, sequences from all of these genera were identified in red snapper blood with the exception of *Streptococcus*. It is possible that fish blood contains a wide diversity of bacteria that cannot be identified using culture-based techniques. As this is the first study to use sequencing to survey bacteria in fish blood, more studies should be done to expand this dataset.

The presence of a high abundance of sequences from the genus *Ralstonia* in one red snapper individual is unexpected, as sequences from this genus were not identified from the other 3 individuals sequenced. In our sample, all sequences from *Ralstonia* were closely related to sequences identified as *R. pickettii*. This species has been identified in the feces [35-37] and gill [36] of pinfish, pipefish, sea bass, and yellow catfish. In this study, it was present in feces but not gill samples. It can occur in the blood of humans and has been identified as a pathogen that may cause serious infections in immunocompromised individuals or those suffering from cystic fibrosis [38, 39]. Its

presence in the red snapper is therefore curious although more thorough sampling may indicate its occurrence as common.

The similarity in RS8 between blood and gill samples as well as results from MDS and ANOSIM suggest that the blood microbiota is similar in composition to that of the gill and much less so to that of the feces. However, RS1 and RS4 showed much higher variability between blood and gill microbiota. It is important to note that, among the 4 red snapper with successful amplification from blood samples, RS1 was much different in blood bacterial community composition due to its large abundance of *Ralstonia*. Also, RS4 had a much higher abundance of Betaproteobacteria than RS8 and RS10. Thus large individual variation in blood microbiota between individuals makes interpretation of similarities between blood and gill difficult. Larger sample sizes will be necessary to determine the true pattern and make hypotheses as to the entry route of these bacteria into the bloodstream.

In conclusion, 100% of apparently healthy red snapper surveyed in this study had positive blood cultures. These results challenge the current paradigm that fish blood is sterile. A majority of the bacteria in fish blood were identified as *Pseudomonas* which have been isolated from blood and internal organs of a number of other fish species. Fish blood has the potential to contain a high diversity of bacteria without showing external signs of disease. Thus a positive culture from blood or kidney of diseased fish cannot be considered a definitive method for identifying the causative agent of infection.

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Table 6-1. Studies that have identified bacteria in the blood and/or internal organs of apparently healthy fishes.

Species	# Fish	Sample Type	Sample volume	Method	Time of Incubation	Bacteria Identified	% Fish Positive	Source
Silver hake (*Merluccius bilinearis*) squirrel hake (*Urophycis chuss*)	3	Blood (heart)	0.5 mL	Culture	96h	NS	33.30%	[27]
Perch (*Perca fluviatilis*)	NS	Blood (heart)	NS	Culture	2d	Gram-negative bacilli; Gram-positive cocci; non-sporing Gram-positive bacilli	NS	[32]
Brook trout (*Salvelinus fontinalis*) Rainbow trout (Oncorhynchus mykiss) Brown trout (Salmo trutta) Lake trout (Salvelinus namaycush)	350	Heart, liver, kidney	NS	Culture	NS	*Pseudomonas*, *Aeromonas*, *Micrococcus*, *Lactobacillus*, *Escherichia*, *Brevibacterium*, *Paracolobactrum*, *Aerobacter*, *Proteus*, *Alcaligenes*, *Bacillus*, *Achromobacter*, *Flavobacterium*, *Streptococcus*	Up to 59.5%	[34]

Table 6-1 continued

Species	# Fish	Sample Type	Sample volume	Method	Time of Incubation	Bacteria Identified	% Fish Positive	Source
Brook trout (*Salvelinus fontinalis*) Rainbow trout (*Oncorhynchus mykiss*) Brown trout (*Salmo trutta*)	244	Blood (heart), kidney	0.1 mL - 0.2 mL	Culture; Enrichment	5d	Oxidative fluorescent pseudomonads; non-oxidative, non-fluorescent pseudomonads; Flavobacteria; *Aeromonas salmonicida; A. liquifaciens*, Gram-positive rods; lactose fermenting enteric bacteria; unidentified Gram-negative rods	12.5-26.1% (kidney) 27.5% (blood)	[28]
White perch (*Roccus americanus*)	52	Blood (heart), liver, spleen, kidney	inoculati on loop	Culture; Enrichment	3-5d	*Bacillus*, *Achromobacter*, *Pseudomona*, *Aeromonas*, *Enterobacter*, *Micrococcus*, *Pseudomonas*-like	NS	[29]
Turbot (*Scophthalmus maximus*)	900	Kidney, liver	NS	Culture	48-72h	*Vibrio*, *Pseudomonas*, *Aeromonas*, *Moraxella*-*Acinetobacter*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, *Sarcina*, *Corynebacterium*	27-42%	[33]

Table 6-1 continued

Species	# Fish	Sample Type	Sample volume	Method	Time of Incubation	Bacteria Identified	% Fish Positive	Source
Many species	195	Blood (caudal vein)	1 mL	Culture; Enrichment	7d	*Photobacterium* , *Staphylococcus* , *Vibrio* , *Pseudomonas, Pasteurella* , *Shewanella* , *Citrobacter* , *Stenotrophomonas* , *Aeromonas, Alcaligenes* , *Chryseomonas* , *Moraxella* , *Morganella* , Plesiomonas, Proteus, *Sphingomonas* , *Streptococcus*	26.7% total 21% shark; 50% rays 38.7% pelagic; 18.3% benthic	[31]
Red snapper (*Lutjanus campechanus*)	60	Kidney	inoculati on loop	Culture	2d	*Photobacterium* , *Vibrio* , *Stenotrophomonas* , *Enterobacter* , *Bacillus* , *Exiguobacterium* , *Shewanella* , *Pseudoalteromonas* , *Pseudomonas* , *Staphylococcus* , *Microbacterium*	25%	[30]

Table 6-1 continued

Species	# Fish	Sample Type	Sample volume	Method	Time of Incubation	Bacteria Identified	% Fish Positive	Source
Red snapper (*Lutjanus campechanus*)	10	Blood (caudal vein)	2 mL	Culture	7d	*Pseudomonas, Nevskia, Ralstonia, Herbaspirillum, Aquabacterium, Alicycliphilus, Acidovorax, Methylobacterium, Methylibium, Stenotrophomonas, Corynebacterium, Micrococcus, Staphylococcus, Cetobacterium*	100%	This study

Table 6-2. Coordinates and sampling date for each artificial reef site. Two red snapper were caught from each site.

Sampling date	Site number	Coordinates
26 September 2013	1	30° 00'00.00" N 87° 42'00.00" W
30 September 2013	2	30° 09'48.00" N 87° 09'24.00" W
17 October 2013	3	30° 02'30.00" N 87° 39'12.00" W
13 November 2013	4	30° 02'24.00" N 87° 34'48.00" W
	5	30° 02'06.00" N 87° 34'54.00" W

Table 6-3. Conditions at each sampling site as determined by CTD sampling.

Site	Depth (m)	Salinity (psu)	Temperature (°C)	DO (mg/L)	Fluorescence (mg/m^3)	Visibility (%)
1	30.9	33.6	28.6	4.77	0.134	88.2
2	26.5	33.1	28.8	6.02	0.135	91.5
3	26.9	34.1	27.2	6.18	0.089	90.1
4	27.9	34.5	22.7	6.68	0.187	89.4
5	28.5	34.5	22.7	7.71	0.178	88.7
Average	28.1	34	26	6.27	0.145	89.6

Table 6-4. Sex, mass, and lengths of each red snapper included in this study.

Fish ID	Sex	Mass (kg)	Fork length (mm)	Total length (mm)
RS1	F	1.13	406	440
RS2	M	0.56	373	405
RS3	F	0.83	380	405
RS4	F	6.1	687	752
RS5	M	0.46	312	335
RS6	M	0.46	315	338
RS7	M	1.52	446	487
RS8	F	0.64	360	389
RS9	M	0.74	360	395
RS10	M	0.86	365	395
Average	-	1.33	400	434

Table 6-5. Sequence results and diversity indices for each individual red snapper and sample type, and average for each sample type.

Fish ID	Sample Type	Original # sequences	Original # OTUs	Final # sequences	Final # OTUs	Expected # OTUs	Good's coverage	Shannon evennness index
1B	Blood	2263	48	950	43	56	0.991	0.724
4B	Blood	3019	83	950	64	103	0.98	0.735
8B	Blood	950	49	950	49	99	0.991	0.712
10B	Blood	2495	52	950	46	62	0.988	0.665
1G	Gill	3055	74	950	59	103	0.984	0.599
2G	Gill	2581	80	950	68	104	0.981	0.602
3G	Gill	9276	91	950	51	102	0.984	0.533
4G	Gill	3045	81	950	61	101	0.982	0.455
5G	Gill	11888	153	950	90	245	0.963	0.689
6G	Gill	4000	134	950	90	170	0.973	0.762
7G	Gill	4170	109	950	75	247	0.983	0.816
8G	Gill	3140	77	950	64	107	0.986	0.706
9G	Gill	4355	76	950	54	121	0.986	0.649
5F	Feces	3859	109	950	74	147	0.973	0.545
6F	Feces	3669	141	950	99	195	0.967	0.704
7F	Feces	4156	92	950	66	141	0.987	0.8
8F	Feces	4626	116	950	72	354	0.966	0.512
9F	Feces	2595	66	950	53	126	0.986	0.727
10F	Feces	3154	107	950	80	251	0.968	0.591
Averages	Blood	2182	58	950	51	80	0.988	0.709
	Gill	5057	97	950	68	144	0.98	0.646
	Feces	3677	105	950	74	202	0.975	0.647

Table 6-6. Analysis of similarities (ANOSIM) results for the global test and pairwise comparisons. Shared operational taxonomic units (OTUs) are also included as calculated with the Mothur program.

ANOSIM	***p* value**	**R value**	**Shared OTUs**
Global Test	0.01	0.270	68
Blood *vs* gill	0.178	-	174
Blood *vs* feces	0.019	0.464	80
Gill *vs* feces	0.011	0.341	131

Table 6-7. Similarity percentages (SIMPER) results indicating genera responsible for differences between sample types. Only genera accounting for at least 2% of dissimilarity between any combination of sample types are included. Total dissimilarity is given for each sample type and each comparison.

			Average Abundance			% Dissimilarity		
						Blood	Blood	Gill
Phylum	**Order**	**Genus**	**Blood**	**Gill**	**Feces**	**Gill**	**Feces**	**Feces**
	Acidithiobacillales	*Acidithiobacillus*	-	8.43	-	4.21	-	4.93
	Aeromonadales	*Aeromonas*	0.13	3.73	0.13	1.85	0.11	2.15
	Alteromonadales	*Pseudoalteromonas*	0.12	1.42	15.9	0.7	7.95	9.49
	Pseudomonadales	*Acinetobacter*	1.24	6.23	1.72	3.18	1.13	4.00
Proteobacteria (Class	Pseudomonadales	*Pseudomonas*	43.8	13.0	13.1	15.4	15.9	8.13
Gammaproteobacteria)	Pseudomonadales	*Psychrobacter*	0.01	3.71	0.01	1.86	0.01	2.17
	Vibrionales	*Photobacterium*	0.05	0.67	20.3	0.32	10.1	11.8
	Vibrionales	*Vibrio*	0.06	17.3	4.11	8.64	2.06	10.7
	Xanthomonadales	*Nevskia*	12.3	10.7	0.55	6.82	5.85	6.16
	Unassigned	*Umboniibacter*	-	0.15	7.78	0.1	3.89	4.56
	Burkholderiales	*Herbaspirillum*	4.73	1.21	2.07	1.83	2.00	1.35
Proteobacteria (Class Betaproteobacteria)								
	Burkholderiales	*Ralstonia*	7.37	-	0.32	3.68	3.76	0.19
	Rhodocyclales	*Rhodocyclus*	-	7.22	-	3.61	-	4.22
Cyanobacteria	Prochlorococcaceae	*Prochlorococcus*	0.01	0.24	5.98	0.16	2.99	3.38
Fusobacteria	Fusobacteriales	*Cetobacterium*	0.82	1.16	4.47	0.78	2.48	3.01
Firmicutes	Clostridiales	*Clostridium*	0.01	4.30	0.58	2.15	0.36	2.63
		Other genera	29.4	20.5	23.0	20.0	21.0	6.69
		Total Dissimilarity	42.01	78.7	77.6	75.33	79.62	85.56

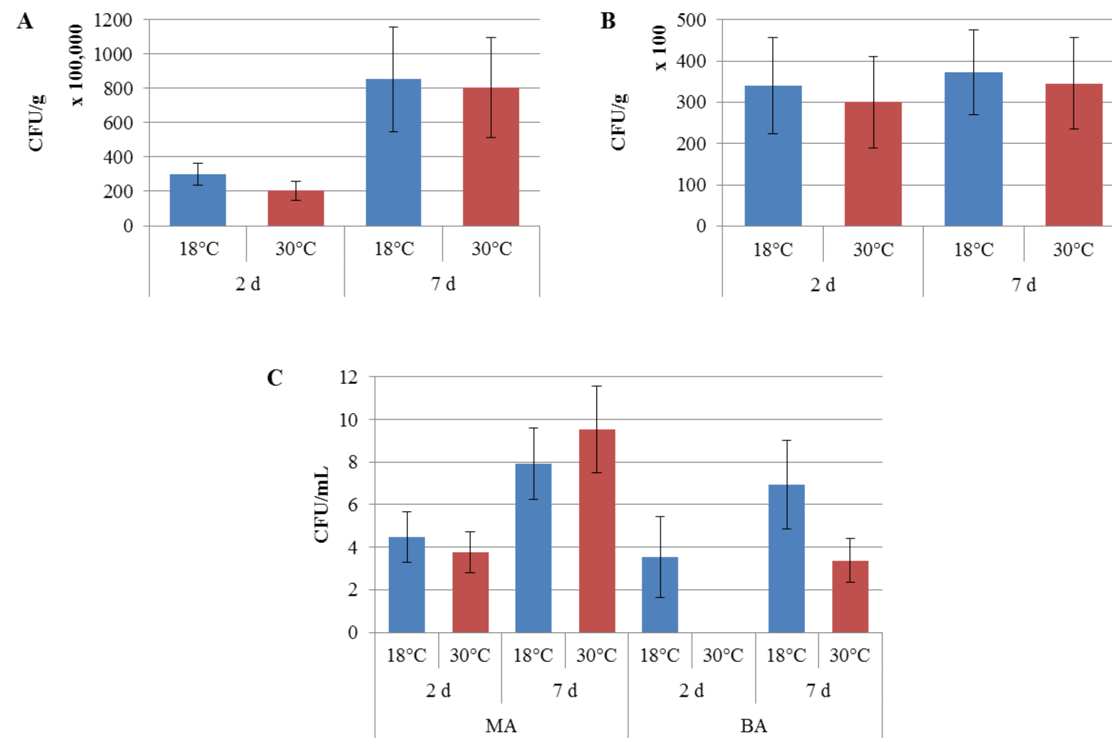

Figure 6-1. Average colony forming units (CFU) including standard error for each sample type. Counts were taken after 2 d and 7 d at two temperatures. Blood counts are taken from both marine agar and blood agar. A, feces; B, gill; C, blood.

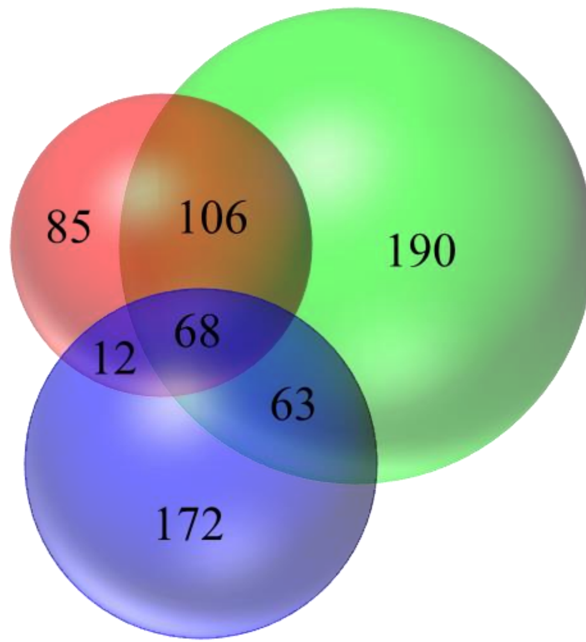

Figure 6-2. Venn diagram representing shared operational taxonomic units (OTUs) between sample types. Red, blood (total OTUs = 271); green, gill (total OTUs = 427); blue, feces (total OTUs = 315).

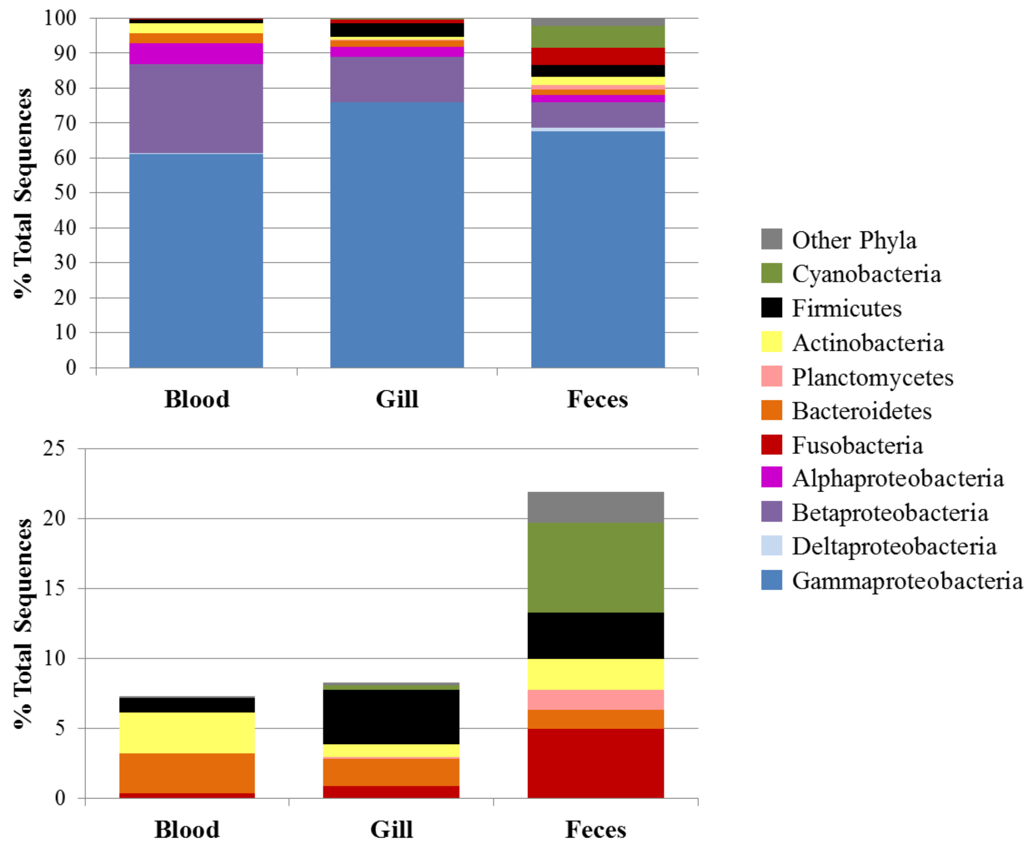

Figure 6-3. Average phylum level composition of the microbiota of all three sample types. A, including Proteobacteria; B, Proteobacteria removed for better visualization of differences between minor phyla.

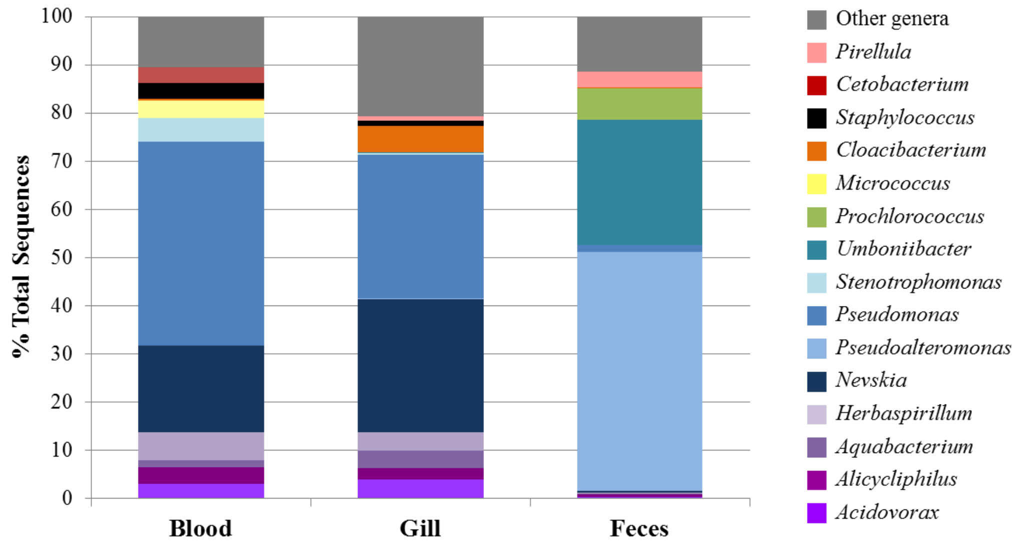

Figure 6-4. Bacterial genera from sequences recovered from sample RS8. Genera accounting for at least 3% of all sequences are included in the graph which all other genera grouped into "Other genera."

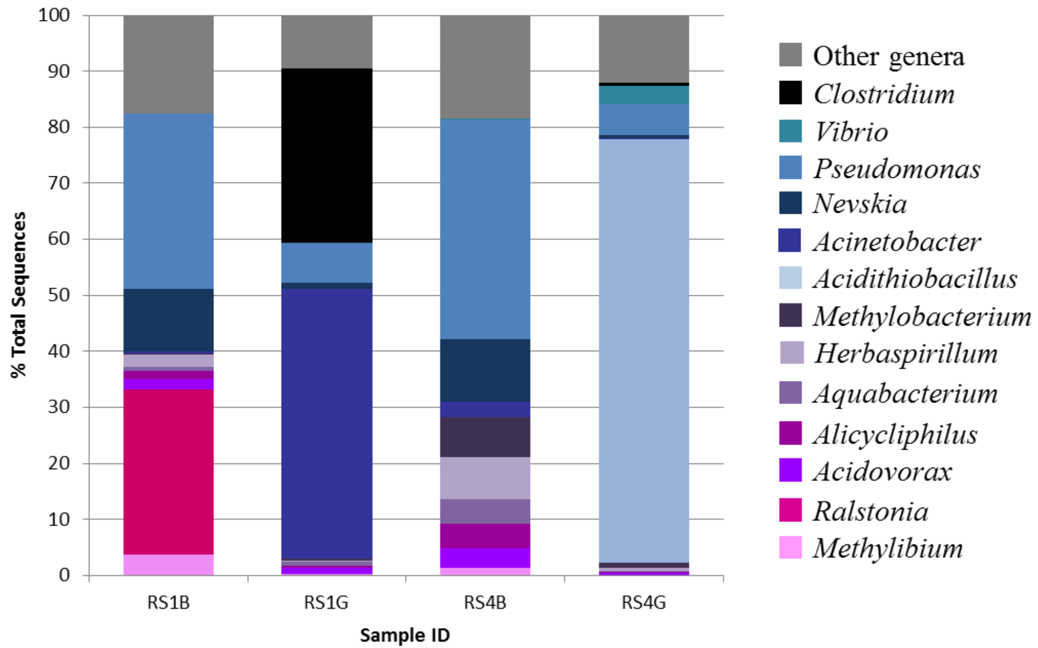

Figure 6-5. Bacterial genera from sequences recovered from samples RS1 and RS4. Genera accounting for at least 3% of all sequences are included in the graph which all other genera grouped into "Other genera." B, blood; G, gill.

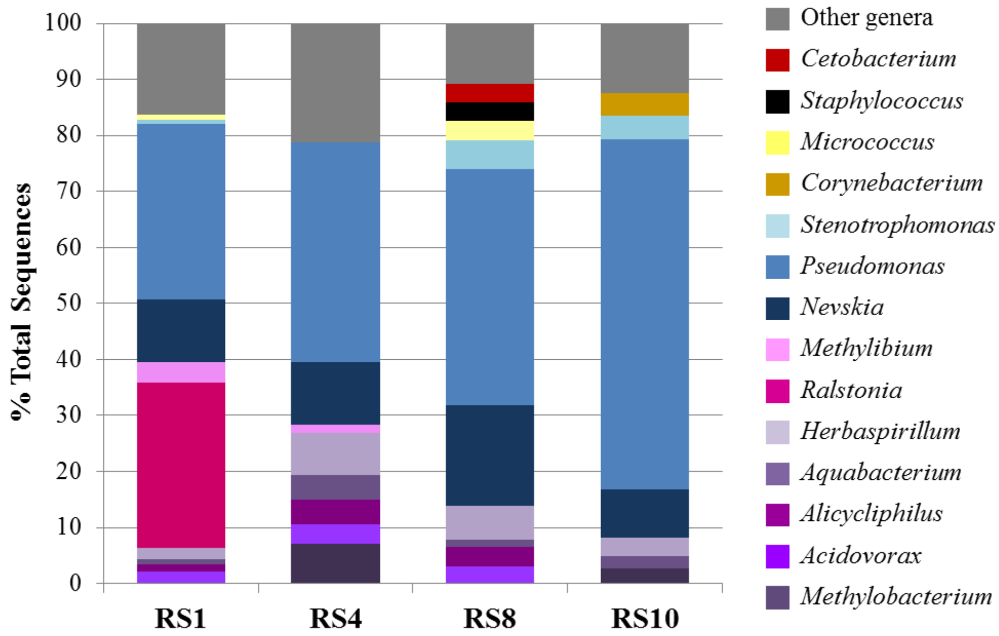

Figure 6-6. Genera results for each blood sample. Genera accounting for at least 3% of all sequences are included in the graph which all other genera grouped into "Other genera."

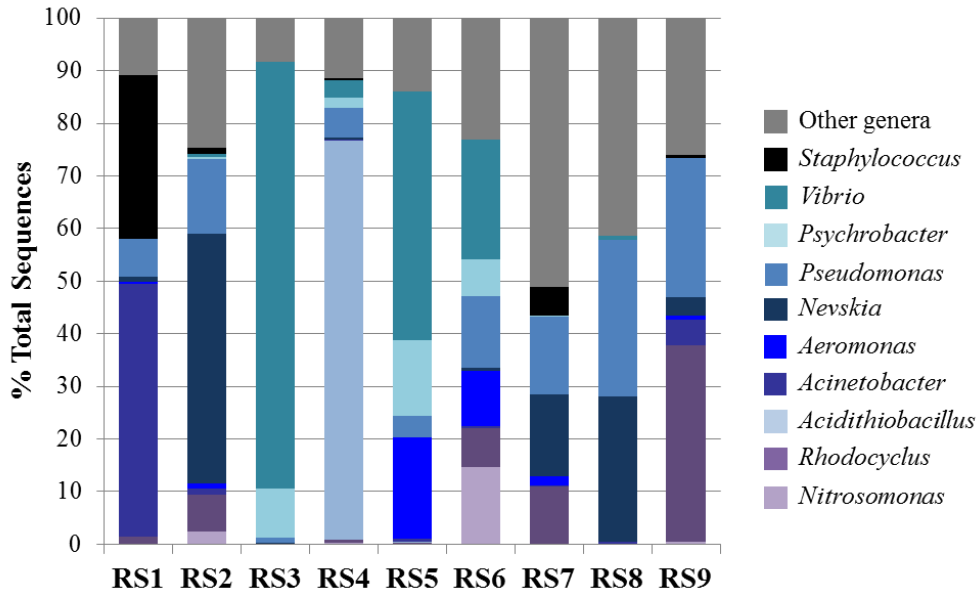

Figure 6-7. Genera results for each gill sample. Genera accounting for at least 10% of all sequences are included in the graph which all other genera grouped into "Other genera."

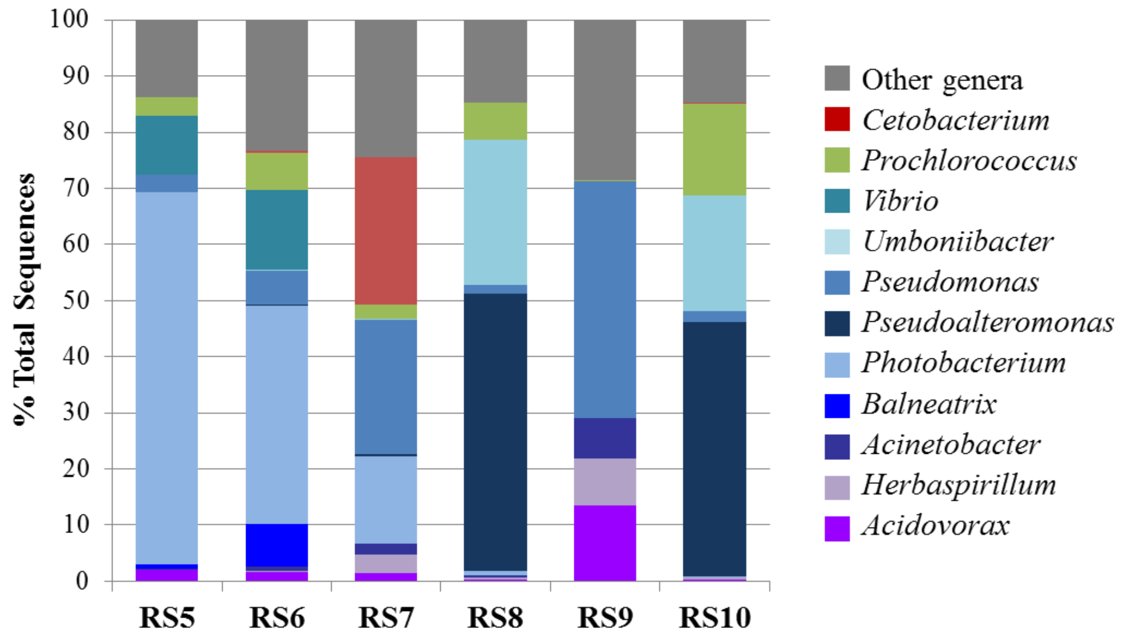

Figure 6-8. Genera results for each feces sample. Genera accounting for at least 3% of all sequences are included in the graph which all other genera grouped into "Other genera".

CHAPTER 7. SUMMARY AND CONCLUSIONS

In this dissertation, Proteobacteria dominated the microbiota of marine fish skin, gill, and blood. More specifically, the class Gammaproteobacteria comprises the bacterial communities of most fish species (pinfish, Atlantic croaker, sand seatrout, spotted seatrout, red snapper, Gulf killifish; exception: striped mullet). However, Fusobacteria dominated freshwater fish gut microbiota (channel catfish, bluegill, largemouth bass).

DNA extraction method significantly influences downstream gut microbial community analysis. Freezing samples results in reduced detected bacterial diversity. Fresh samples should be used when possible, but RNA*later*® is an acceptable substitute. The stool-formulated kit performed better for feces samples whereas the animal tissue kit performed better for intestinal tissue samples.

I detected no significant influence of oil exposure on the fish skin microbiota, indicating a level of stability in these communities. The differences between fish skin microbiota and that of the surrounding water supports the theory that these communities are resistant to environmental influences. However, season impacted the bacterial assemblages of both the fish skin and water.

I identified diverse bacterial assemblages in the blood of red snapper dominated by members of the genus *Pseudomonas*. These communities were more similar to those on gill than those in feces. Thus fish blood may not be sterile as previously believed.

Unidentified factors associated with different fish species strongly influence the microbiota structure of fish and explain more variation between the microbial communities of individual fish than either geographic location or season. These bacterial assemblages are distinguishable from species to species. Studies detected species-specificity in both the skin and gut of fishes.

The studies in this dissertation demonstrated microbiota diversity at three levels: alpha, beta, and gamma. Alpha diversity is that within an individual fish. Red snapper samples exhibited alpha diversity through differences in gill, feces, and blood microbiota. MDS plots in Chapters 4 and 5 illustrate beta diversity, that between individuals within a fish species. Varying distances between individual fish depict differences in microbial community structure at this level. Finally, the differences seen between fish species represent gamma diversity.

The results of this dissertation support the hypothesis that microbiota of fishes contain both core and transient members. Core members are permanent members of the community shared at a particular level of interest. For example, these are the bacterial species that are present in all sample types in red snapper, in all individuals of a fish species, or in all fishes. This core microbiota is stable despite environmental influences. Transient members are temporary and their presence varies over time. These are the members impacted by environmental factors such as location and season.

In conclusion, fish microbiota are complex communities influenced by many factors. The interactions between fish and microbes leading to species-specific microbiota are of great interest for understanding fish health and future development of successful probiotics.