A FISH TALE: COMPARISON OF THE GUT MICROBIOME OF 15 FISH SPECIES AND THE INFLUENCE OF DIET AND TEMPERATURE ON ITS COMPOSITION

by

Carrie Elizabeth Givens

(Under the Direction of James T. Hollibaugh)

ABSTRACT

This dissertation addresses four aspects of the biology of the fish gut. 1) What bacteria constitute the fish gut microbiome, how variable is the composition within a species; how different are the gut microflora of different fish species; and how do fish gut microbiomes different from those of other organisms that have been studied? 2) How do food quality and diet-associated bacteria affect the composition of the gut microbiome? 3) Ocean temperatures are expected to rise in the future in response to increased atmospheric CO_2 concentrations, we know that the incidence of marine pathogenic Vibrios is higher during warm summer months and we know that Vibrios are common, and often dominant, taxa in the gut microbiome. Does increased habitat temperature influence the composition of the gut microbiome and specifically does the abundance of potentially pathogenic Vibrios increase when fish are held at higher water temperatures? 4) Conversely, can fish serve

as refuges for these Vibrios when growth conditions are less favorable and as vectors for their distribution?

We used 454-pyrosequencing to survey the 16S rRNA ribotypes in the gut microbiomes of 12 finfish and 3 shark species. Fish were selected to encompass herbivorous and carnivorous lifestyles, to have varied digestive physiologies, to represent pelagic and demersal species, and as representatives of a range of habitats from estuarine to marine. Proteobacteria ribotypes were present in all fish and often dominated the gut microflora community of many fish species. Firmicutes were also prevalent within the fish gut community, but at a lower relative abundance. Each species had a core gut microflora; however, no individual ribotype was present among all species suggesting that the gut microflora community is adapted to the autecological properties and physiological conditions of each fish species.

We determined the effects of both diet quality and food-associated bacteria on gut microflora using mummichogs (*F. heteroclitus*) and pinfish (*L. rhomboides*) as model organisms. We identified a core gut microflora for these species and determined that food-associated microbiota strongly influenced the composition of the gut microflora in mummichogs, but not pinfish. We also tested the effect of temperature on the composition of gut microflora and on the occurrence of *Vibrio* spp. 16S rRNA and *V. vulnificus vvh* genes in the two model fish (mummichogs and pinfish) using clone libraries and quantitative PCR (qPCR). In a related set of experiments, we asked whether fish guts might serve as a refuge for *Vibrio parahaemolyticus* and *Vibrio vulnificus* during periods of sub-optimal environmental conditions. We found that both of these Vibrio species were present in the gut microbiome and that they could be transferred to

other environmental reservoirs, implicating fish in the persistence and dispersal of these potential pathogens. Lastly, we examined the microbiome of the Atlantic blue crab (*Callinectes sapidus*) to address how the crab-associated bacterial community may affect crab, fish, and human health.

INDEX WORDS: Fish gut, Gut microbiome, 16S rRNA, Gut microflora, 454-pyrosequencing, Shark gut, Blue crab microbiome, core gut microbiome, Proteobacteria, Firmicutes, Tenericutes, *Vibrio* sp., *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Trinectes maculatus*, *Bairdiella chrysoura*, *Lagodon rhomboides*, *Paralichthys lethostigma*, *Fundulus heteroclitus*, *Centropristis striata*, *Sciaeops ocellatus*, *Caranx hippos*, *Scomberomorus maculatus*, *Scomberomorus cavalla*, *Coryphaena hippurus*, *Sphyraena barracuda*, *Carcharhinus brevipinna*, *Rhizoprionodon terraenovae*, *Carcharhinus plumbeus*, Quantitative PCR (qPCR), 16S rRNA clone library, fish health, crab health, human health

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DEDICATION

To my parents Mike and Vickie Givens who have always believed in me.

In memory of my grandfather Harold A. Rutland, Sr. who introduced me to fishing. He tied the first knot and helped me unhook my first “flopping” fish.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The Gut Microflora Community: Knowns and Unknowns

Bacteria are abundant in the guts of fishes and are expected to influence fish physiology and health (MacFarlane et al. 1986; Cahill 1990). Elevated abundances in the intestine of certain bacteria when compared to the composition of the microbial assemblage in the surrounding water suggest that the intestine provides a unique niche for a selected, but diverse, group of bacteria (Austin and Austin 1987; Cahill 1990; Ringø et al. 1995). Some of the species found in the gut appear transiently while others seem to be resident flora (Kim et al. 2007). The permanent or resident microbes are often attached to the intestinal wall (Ringø et al. 2001). Fish guts receive inocula of bacteria from a variety of sources in nature. Bacteria are ingested with water at the larval stage, and this microflora may colonize the gut tract to become the resident microflora in juvenile fish (Hansen and Olafsen 1999). Microbes associated with the chorion of fish eggs and present in their early diet may also influence the development of the gut microflora (Hansen and Olafsen 1999; Ringø and Birkbeck 1999; Romero and Navarrete 2006). For these bacteria to proliferate and persist as "resident" microflora, they must be retained within the gut, which requires that they are adapted to gut environmental conditions including nutrient availability, pH and digestive enzymes (Hansen and Olafsen 1999). Previous studies have shown that gut microflora respond to a variety of

factors affecting the host, including changing environmental conditions (Yoshimizu and Kimura 1976; MacFarlane et al. 1986), developmental stage (Verner-Jeffreys et al. 2003; Romero and Navarrete 2006), digestive physiology (Cahill 1990), and feeding strategy (Uchii et al. 2006).

Once established, the gut microbial communities interact with the host in a number of ways. This community aids in digestion and can affect nutrition, growth, reproduction, overall population dynamics, and vulnerability of the host to disease (MacFarlane et al. 1986). Ringø et al. (1995) suggested that *Bacteroides* spp. and *Clostridium* spp. enhance nutrition by providing essential fatty acids and vitamins. Lactic acid bacteria (often *Lactobacillus* sp.) have been found to be a minor component of the gut microflora (Izvekova et al. 2007), but they may be crucial in promoting fish health and blocking the establishment and growth of potential pathogens (Strøm 1988).

The composition of gut microflora appears to vary among fish species; however direct comparisons between species are hampered by inconsistencies in the methods used. Studies conducted prior to ~2005 have relied on culture-based techniques to enumerate and identify bacteria (Newman et al. 1972; MacFarlane et al. 1986; Spanggaard et al. 2000; Aschfalk and Müller 2002; Verner-Jeffreys et al. 2003; Al-Harbi and Naim Uddin 2004; Martin-Antonio et al. 2007; Skrodenytė-Arbaĉiauskienė 2007). These studies have provided valuable insights into the composition of microbial communities and have yielded isolates for detailed physiological investigation; however, they are known to provide biased assessments of the microbial community composition as typically <1% of the cells known to be present by direct microscopic enumeration produce colonies on solid media (Ferguson et al. 1984a; Head et al. 1998b). With that

caveat, Table 1.1 lists the dominant gut microflora reported in published studies of a variety of fresh- and saltwater fish species from wild and cultured populations. Most of these studies only examined a single fish species and a variety of culture-dependent and culture-independent methodologies were used to assess microflora community composition.

Based on this review of the literature (Table 1.1), the gut microbiome of most fish seem to be dominated by γ-Proteobacteria such as *Aeromonas* sp., *Escherichia coli, Photobacterium* sp., *Pseudomonas* sp., and *Vibrio* sp. (Newman et al. 1972; MacFarlane et al. 1986; Ringø 1993a; Ringø 1993b; Ringø and Strøm 1994; Spanggaard et al. 2000; Verner-Jeffreys et al. 2003; Al-Harbi and Naim Uddin 2004; Bates et al. 2006; Romero and Navarrete 2006; Skrodenyte-Arbaciauskiene et al. 2006; Martin-Antonio et al. 2007; Skrodenytė-Arbaĉiauskienė 2007; Ransom 2008; Ward et al. 2009a). However, some fish such as Atlantic salmon (*Salmo salar*) (Holben et al. 2002b) and long-jawed mudsucker (*Gillichythys mirabilis*) (Bano et al. 2007) have intestinal microflora dominated by Tenericutes (*Mycoplasma* sp.). Unlike finfish, there has been little research on the gut microbiome of sharks. One study found that *Photobacterium damselae* was a normal member of the gut microflora of sharks (Grimes et al. 1985).

The microbial communities of mammals (humans and other terrestrial mammals) are much different from that of either finfish or shark species (Turnbaugh et al. 2006; Ley et al. 2008a). In general, the fish gut microbiome has been thought to be less diverse than that of mammals (Trust et al. 1979; Sakata 1990; Holben et al. 2002b) and gut microflora appear to contribute less to the volume of material in fish guts, with an estimated 10^6 to 10^8 CFU/gram within the fish intestine (Kim et al. 2007) compared to

~10^{11} CFU/gram reported for intestines of terrestrial mammals, including humans (Mead 1997). A recent 16S rRNA gene sequence analysis of gut (fecal) microflora from humans and 59 other mammals found that gut physiology was a strong indicator of the fecal microflora community composition (Ley et al. 2008a). Mammal gut microbiomes grouped by digestive physiologies, with hindgut fermenters, foregut fermenters, and those with simple guts with minimal differentiation hosting different gut microbial communities (Muegge et al. 2011). Bacteria from the 60 mammals surveyed by Ley et al. (2008) binned into 17 phyla and were dominated by Firmicutes (65.7%) and Bacteriodetes (16.3%). This is slightly different from previous studies (Ley et al. 2005; Turnbaugh et al. 2006) that categorized human and mouse gut communities as being dominated by Bacteroidetes followed by Firmicutes. Finally a study characterizing the gut microflora of Burmese pythons, *Python molurus*, found that it too was dominated by Firmicutes (61.8%) and Bacteroidetes (20.6%) (Costello et al. 2010).

Gut Microflora and The Environment: Altered States and Changing Communities

The composition of the transient (versus core) microflora of fish guts is reported to be affected by diet, by bacteria in the water column, and by environmental factors (Hansen and Olafsen 1999). Nayak (2010) suggested that microbes from water, sediment, and food items colonize the fish gastrointestinal tract. Thus, microbes from the surrounding environment influence the resident gut microflora community. This may affect fish health if pathogens are present in the environment that are able to colonize the gut. In fact, the majority of bacteria that cause fish diseases are opportunistic pathogens frequently found in the water column (Hansen and Olafsen 1999). Environmental

stressors such as temperature, oxygen concentration, and pollutants can weaken the host's immune system and allow these pathogens to colonize the intestinal tract (Hansen and Olafsen 1999).

Variations in water temperature and salinity can affect gut microflora communities. DePaola et al. (1994; 1997) documented the prevalence of the potentially pathogenic *Vibrio vulnificus* in sheepshead (*Archosargus probatocephalus*) sampled from the Gulf of Mexico. Presence and abundance of this bacterium is closely linked to increased water temperatures, with highest densities of these cells occurring when the water temperatures range between 20 and 30 °C (Kelly 1982; DePaola et al. 2003; Tantillo et al. 2004). *V. vulnificus* densities in sheepshead intestines were 2-3 orders of magnitude lower in March and December when water temperatures were 21.9 °C and 17.5 °C compared to those recorded in May and September when water temperatures were 24.7 °C and 30.6 °C (DePaola et al. 1997). This suggests that although this bacterium is naturally present within the sheepshead gut, increased abundance correlates with warmer water temperatures.

DePaola et al. (1994) also found both a higher prevalence and density of *V. vulnificus* in the guts of inshore bottom fish when compared to offshore fish suggesting that the presence and abundance of *V. vulnificus* within the fish gut is correlated to this bacterium's optimal salinity range (5-15 psu) (Kelly 1982; Wright et al. 1996; Motes et al. 1998; Lipp et al. 2001; Randa et al. 2004). Several other studies have documented shifts in the composition of fish gut microflora coinciding with salinity variations encountered in estuarine environments. (Yoshimizu and Kimura 1976; MacFarlane et al. 1986). For example, many freshwater fish have *Aeromonas* sp. within their guts;

whereas, *Vibrio* sp. is more frequently documented in estuarine and marine species (Cahill 1990; Ringø et al. 1995; Ringø and Birkbeck 1999). Changes in environmental conditions like global warming or changes in rainfall pattern may thus affect fish indirectly by driving potentially detrimental changes in the composition of their gut

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Vibrio species are often found to be the dominant bacteria in and on marine fish and are common members of the gut microflora in both farmed and wild fish (MacFarlane et al. 1986; Cahill 1990; Sakata 1990; Blanch et al. 1997; Martin-Antonio et al. 2007; Ward et al. 2009a). Baross and Liston (1970) observed that 32% of fish gut samples collected from Puget Sound contained hemolytic vibrios based on activity detected on Kanagawa blood agar. Additionally, Liston (1990) isolated *V. parahaemolyticus* from various commercial finfish including cod, sardines, mackerel, and flounder. DePaola et al. (1994; 1997) reported high densities of *V. vulnificus* in several finfish species collected from the Gulf of Mexico.

Although several members of the *Vibrio* genus are pathogenic to humans and marine animals, *V. parahaemolyticus* and *V. vulnificus* in particular are leading causes of seafood-associated bacterial illness and mortality (Iwamoto et al. 2010). Infections involving these two bacteria can cause gastroenteritis and septicemia through consumption of raw or undercooked seafood, and wound infections can result in *V. vulnificus* septicemia (Constantin de Magny et al. 2009). Although *V. vulnificus* infections are rare, they are the leading cause of seafood-related deaths domestically and have one of the highest hospitalization (91.3%) and mortality (34.8%) rates of all

foodborne pathogens (Iwamoto et al. 2010; Scallan et al. 2011). In the United States, *V. parahaemolyticus* is the leading cause of bacterial illness from seafood consumption (Iwamoto et al. 2010), but has a lower hospitalization (22.5%) and mortality (0.9%) rate than *V. vulnificus* (Scallan et al. 2011).

V. parahaemolyticus and *V. vulnificus* are not the only microbes of concern from a public health standpoint. There are virulent strains of *Photobacterium damselae* that can adversely impact fish and humans, causing septicemia in fish (Fouz et al. 2000b), and septicemia or wound infections in humans (Shin et al. 1996). Although *P. damselae* subsp. *piscida* is not a human pathogen (Fouz et al. 2000b), it is a serious fish pathogen (Thyssen et al. 1998a), frequently resulting in disease and mortality. Human diseases associated with handling fish have also been attributed to *Streptococcus inae* (Zlotkin et al. 1998; Colorni et al. 2002), *Aeromonas hydrophilia*, *Edwardsiella tarda*, *E. rhusopathiae*, *Mycobacterium marinum*, and additional *Vibrio* spp. (Lehane and Rawlin 2000).

If gut microflora can persist in seawater, there is a risk of increased transmission of pathogens via infections and open wounds. The population of *Aeromonas* spp. in a seawater aquarium increased when fish were held for an extended period (181 days) (Cahill 1990). Since *Aeromonas* spp. is not typically isolated from seawater, (Cahill 1990) suggested that this population may have accumulated from fish feces . Gut microflora capable of persisting in seawater may affect water quality, especially in areas with dense populations of fish (aquaculture), in shallow water, and in areas of decreased tidal flushing and increased residence time. Additionally, Janssen and Meyers (1968) suggested that fish inhabiting water polluted by sewage may become infected with

human pathogens and thus represent further public health threats as either "carriers" or "vectors" of human disease.

There is also a potential for fish to participate in the transfer of pathogenic bacteria to new hosts within the same environment (i.e. humans, other marine animals). The public health risk from pathogenic bacteria originating from fish may be rare; however, this risk depends on the interaction between the organism, the physiology of the infected person, and environmental factors (that can influence virulence) (Strom and Paranjpye 2000; Oliver 2006). Consumption of raw and undercooked shellfish and fish is increasingly popular(DePaola et al. 1994) with the attendant risk of infection by pathogenic bacteria. Gram-negative bacteria such as *V. vulnificus* can move directly from the intestine into edible portions of the fish (DePaola et al. 1994). Buras et al. (1985) found *E. coli* and *Salmonella* spp. in fish blood and muscle two hours after injecting these bacteria into the stomach. Edible portions of the fish may also be contaminated if the intestine is cut during filleting. There is also a possibility of infection by pathogenic bacteria during direct contact and handling of some species (i.e. contact with fecal matter, puncture wounds from spines). Increased occurrences of certain bacteria (i.e. *Vibrio* spp., *Photobacterium* sp., *Mycoplasma* sp.) within the intestine may thus affect public health both through food transmissions and wound infections.

The pathogenesis of some *Vibrio*-spp. infections in mammals is initiated as a gut infection (Ringø et al. 2003). Although unproven, this may also be true for fish species. Some pathogens require nutrient rich environments such as the gut for growth and survival (Thompson-Chagoyán et al. 2005). They may be expelled with fecal matter, and

thus in theory may represent "seed populations" that can colonize the surrounding environment (Ruby and Nealson 1978). As a consequence, fish may be a key link in pathogen, or *Vibrio*, cycling between fish, the water column, sediments, and other marine organisms. Thus although most of the documented *Vibrio* spp. illnesses and deaths stem from oyster or shellfish consumption, fish may be integral to the epidemiology because they harbor potential pathogenic bacteria within their intestines and affect fish, oceans, and human health.

OBJECTIVES

In Chapter Two, we use massively parallel sequencing (pyrosequencing) to survey the 16S rRNA ribotypes of 12 finfish and 3 shark species from a wide range of lifestyles to assess the extent to which gut microflora varies among species. Target fish species reside in estuarine to marine environments with varied habitats (demersal and pelagic), have varied feeding strategies (herbivore and carnivore), and differing digestive physiologies (i.e. pyloric caeca, varied intestinal length and morphologies, stomachless). Finfish gut microflora assemblages are also compared to the microflora of three shark species to determine whether microflora differs with physiology (Class Actinopterygii vs. Chondrichthyes) and spiraled valve digestive system. In addition to the 15 species used in the cross-species analysis, we further compare the gut microflora of wild and cultured *Fundulus heteroclitus* and juvenile and adult *Lagodon rhomboides*.

In Chapter Three, we analyze 16S rRNA ribotypes in pyrosequenced libraries to determine the effects of both diet and diet-associated bacteria on the gut microflora and to investigate the effects of forced dietary change on the species' intestinal microflora.

In the first feeding study, cultured *F. heteroclitus* were fed different diets including sterilized and unsterilized food with differing protein contents. This study aimed to determine if there was a baseline or core gut microbiome for the fish and then to follow changes in the core microbiome among fish fed four different diets over a 2-month time-series. The second feeding experiment had similar goals but used juvenile and adult pinfish (*L. rhomboides).* Pinfish were chosen for this study because they undergo an ontogenetic diet shift with the transition from juvenile (primarily carnivorous) to adult (primarily herbivorous) life stages.

Chapter Four describes studies designed to determine the relative abundance of *Vibrio* spp. within the gut microflora community and to assess the response of the Vibrio assemblage to elevated temperature in the fish's habitat and the occurrence of *Vibrio* spp. within the fish gut. Other studies indicated that the gut microflora of the mummichog (*F. heteroclitus*) and pinfish (*L. rhomboides*) are dominated by *gamma*-Proteobacteria ribotypes (57% and 41% respectively), mainly members of the family Vibrionaceae (34% and 41%), including some that are closely related to potential pathogens. Thus, we used the distribution of 16S rRNA genes in clone libraries and quantitative PCR (qPCR) with primer sets for Bacteria 16S rRNA, *Vibrio* spp. 16S rRNA, and *V. vulnificus vvh* genes to assess the response of these potentially pathogenic bacteria species to elevated temperature.

In Chapter Five, we determine whether fish guts and sediments served as reservoirs of *V. vulnificus* or *Vibrio parahaemolyticus* during periods of sub-optimal environmental conditions. We quantified the abundance of both of these bacteria in fish, sediment, oysters, and water from coastal sites in Alabama using both culture-

independent (DNA extractions and qPCR) and culture-dependent (plating and hybridization) techniques. These samples were collected from the field during the spring (mid-March to May) when a predictable increase in water temperature was expected to trigger an increase in *V. parahaemolyticus* and *V. vulnificus* abundance.

Chapter Six analyzes Bacteria 16S rRNA genes in clone libraries and uses quantitative PCR (qPCR) to characterize and quantify the microflora community of the blue crab *Callinectes sapidus*. We sampled bacteria associated with crab carapaces (swabs and clips), guts, and hemolymphs. This analysis describes the bacteria commonly associated with different parts of components of the *C. sapidus* anatomy and allowed us to compare microbial assemblages of the carapace, gut, and hemolymph; and to assess the potential for other parts of the crab to serve as potential sources of bacteria for hemolymph infections.

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Table 1.1: Dominant Gut Microflora of Freshwater and Saltwater Fish Species

Species	Dominant Gut Microflora	Methodology[1]	Reference
Bluefish[W, SW] (*Pomatomus saltatrix*)	*Vibrio* sp., *Pseudomonas* sp., Enterobacteraceae	CD	Newman et al., 1972
Striped Bass[W, SW] (*Morone saxatilis*)	*Aeromonas* sp., *Pseudomonas* sp., *Vibrio* sp.	CD Plating	MacFarlane et al., 1986
Arctic Charr[C/W,FW] (*Salvelinus alpinus*)	*Aeromonas* sp., *Pseudomonas* sp.	CD	Ringø 1993a; Ringø 1993b; Ringø & Strøm, 1994
Rainbow Trout[C, FW] (*Oncorhynchus mykiss*)	*γ*-Proteobacteria; *Citrobacter* sp., *Aeromonas* sp., *Pseudomonas* sp., *Carnobacterium* sp.	CD Plating, Isolates 16S rRNA	Spanggarrd et al., 2000
Atlantic Cod[W, SW] (*Gadus morhua*)	*Clostridium perfringens*	CD Fecal samples isolates	Aschfalk & Miller, 2002
Atlantic Salmon[C/W, FW/SW] (*Salmo salar*)	*Acinetobacter junii, Mycoplasma* sp.	CI 16S rRNA	Holben et al., 2002
Atlantic Halibut[C, SW] (*Hippoglossus hippoglossus*)	Vibrionaceae (larvae, juveniles) *Photobacterium phosphoreum* (adults)	CD Biochemical/Biolog plates 16S rRNA isolates/RFLP	Verner-Jeffreys et al., 2003
Hybrid Tilipia[C, FW] (*Oreochromis niloticus X O. aureus*)	*Aeromonas hydrophila, Shewanella putrefaciens, Corynebacterium urealyticum, Escherichia coli, Vibrio cholerae*	CD Plating/ API, Biolog	Al-Harbi & Uddin, 2004
Zebrafish[C, FW] (*Danio rerio*)	*Aeromonas* sp., *Pseudomonas* sp., *Vibrio* sp., *Lactococcus* sp.	CI 16S rRNA library	Rawls et al., 2004
Silver Drummer[W, SW] (*Kyphosus sydneyanus)*	*Clostridium* sp.	CI 16S rRNA clone library	Moran et al., 2005
River trout[W, FW] (*Salmo trutta*)	*Citrobacter* sp., *Aeromonas* sp., *Pseudomonas* sp.	CD 16S rRNA isolates	Skrodenyte-Arbačiauskiene, 2006

Coho Salmon[C, FW] (*Oncorhynchus kisutch*)	*Aeromonas* sp., *Pseudomonas* sp. (juveniles)	CD/CI 16S rRNA isolates/DGGE	Romero & Navarrete, 2006
Rainbow Trout[C, FW] (*Oncorhynchus mykiss*)	*Aeromonas* sp., *Carnobacterium piscicola*, *Clostridium gasigenes*	CD/CI Isolates – BIOLOG, 16S rRNA 16S rRNA clones, RFLP	Pond et al., 2006
Zebraperch[W, SW] (*Hermosilla azurea*)	*Enterovibrio* sp.	CI 16S rRNA clone library	Fidiopiastis et al., 2006
Zebrafish[C, FW] (*Danio* rerio)	*Aeromonas* sp., *Pseudomonas* sp. (embryos & larvae)	CI 16S rRNA clone library	Bates et al., 2006
Roach[W, FW] (*Rutilus rutilus*)	*Aeromonas* sp.	CD Plating/Isolates	Skrodenyte-Arbačiauskiene, 2007
Senegalese Sole[C] (*Solea senegalensis*)	*Vibrio* sp. (*Vibrio ichthyoenteri*)	CD 16S rRNA isolates	Martin-Antonio et al., 2007
Rainbow Trout[C] (*Oncorhynchus mykiss*)	Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae (CD); Proteobacteria (CI)	CD/CI 16S rRNA isolates/clone library	Kim et al., 2007
Long-Jawed-Mudsucker[W, SW] (*Gillichthys mirabilis*)	*Mycoplasma* sp.	CI 16S rRNA PCR/DGGE	Bano et al., 2007
Silver Perch[W, SW] (*Bairdiella chrysoura*)	*Escherichia coli*	CI 16S rRNA clone library	Ransom, 2008
Red Drum[W, SW] (*Sciaenops ocellatus*)	Mycoplasmataceae	CI 16S rRNA clone library	Ransom, 2008
Speckled Trout[W, SW] (*Cynoscion nebulosus*)	*Escherichia coli*	CI 16S rRNA clone library	Ransom, 2008
Southern Flounder[W, SW] (*Paralichthys lethostigma*)	*Clostridium* sp.	CI 16S rRNA clone library	Ransom, 2008

Pinfish[W, SW] (*Lagodon rhomboides*)	Mycoplasmataceae	CI 16S rRNA clone library	Ransom, 2008
Pipefish[W, SW] (*Syngnathus scovelli*)	*γ*-Proteobacteria	CI 16S rRNA clone library	Ransom, 2008
Black Rockcod[W, SW] *Notothenia coriiceps*	*Photobacterium* sp., *Vibrio* sp.	CI 16S rRNA clone library	Ward et al., 2009
Blackfin Icefish [W, SW] (*Chaenocephalus* aceratus)	*Photobacterium* sp.	CI 16S rRNA clone library	Ward et al., 2009
Zebrafish[C/W, FW] (*Danio rerio*)	*γ*-Proteobacteria and Fusobacteria	CI Pyrosequencing	Roeselers et al., 2011
Carp[C, FW] (*Cyprinus carpio*)	Fusobacteria (mostly *Cetobacterium* sp.)	CI Pyrosequencing	Kessel et al., 2011

[1]CD=culture-dependent, CI-culture-independent; C=cultured, W=wild; FW=freshwater, SW=saltwater (estuarine and marine)
This table includes studies where authors indicated a dominant gut microflora. Studies that just made assessments of presence/absence were not included.

CHAPTER 2

COMPARSION OF THE GUT MICROFLORA FROM 12 FINFISH AND 3 SHARK SPECIES[1]

[1] Givens, C.E. and J.T. Hollibaugh. To be submitted to The International Society for Microbial Ecology Journal.

ABSTRACT

We used massively parallel sequencing (pyrosequencing) to survey the 16S rRNA ribotypes in 12 finfish and three shark species from a wide range of lifestyles. Targeted species encompass herbivores and carnivores with varied digestive physiologies, are classified as pelagic and demersal, and reside in estuarine to marine environments. We also compared the gut microbial assemblage of finfish vs. shark species, wild vs. cultured *Fundulus heteroclitus* and juvenile vs. adult *Lagodon rhomboides*. We sampled 4 *F. heteroclitus* each from the wild and cultured populations, 4 *L. rhomboides* at each developmental stage, and 2-3 fish for all other species. A total of 1,214,355 sequences were filtered, denoised, trimmed, and then sorted into OTUs based on 97% sequence similarity using the Qiime software pipeline. Bacteria representing 17 phyla were found among the sampled fish with most fish hosting between 7 and 15 phyla. Proteobacteria ribotypes were present in all fish and often dominated the libraries (3.0-98%; average 61%). Firmicutes were also prevalent, but at a lower relative abundance, ranging between 1.3-45% (average 17%). In most cases, the gut microflora of individual fish of a given species contained many of the same OTUs; however, some species (i.e. great barracuda) shared few OTUs among the individuals sampled.

INTRODUCTION

Skin, gills, eggs, and intestinal tracts of fish all harbor abundant populations of bacteria (MacFarlane et al. 1986; Cahill 1990) that impact their overall health and

physiology. Fish intestines in particular harbor large and diverse populations of bacteria (Austin and Austin 1987; Cahill 1990; Ringø et al. 1995). Most studies have shown that this gut microflora varies among fish species, and that dominant bacteria are typically either aerobes or facultative anaerobes (Ringø et al. 1995). However, some studies have documented obligate anaerobes as part of the gut microbial assemblage (Trust et al. 1979; Ringø et al. 1995). Izvekova et al. (2007) reviewed studies of fish gut microflora published between 1929 and 2006 and found that of the 73 bacteria taxa documented, 53% were Gram-negative aerobes, 34% were Gram-positive aerobes, 8.2% were Gram-negative anaerobes , and 4.1% were Gram-positive anaerobes.

Many fish species have gut microflora dominated by γ-Proteobacteria (Ohwada et al. 1980; MacFarlane et al. 1986; Spanggaard et al. 2000; Rawls et al. 2004; Romero and Navarrete 2006; Kim et al. 2007; Ward et al. 2009a). Populations of anaerobes found in some fish were dominated by Firmicutes and Tenericutes such as *Mycoplasma* sp. (Holben et al. 2002b; Bano et al. 2007) and *Clostridium* sp. (Trust et al. 1979; Sugita et al. 1988; Moran et al. 2005). The gut microflora of freshwater fish species is generally comprised of *Aeromonas* sp., *Pseudomonas* sp., Flavobacterium/Cytophaga species, *Enterobacter* sp., and/or *Acinetobacter* sp. (Trust et al. 1979; Cahill 1990; Ringø et al. 1995; Ringø and Birkbeck 1999). Marine species harbor a different assemblage featuring *Vibrio* spp., *Pseudomonas* sp., *Acinetobacter* sp., *Achromobacter* sp., Enterobacteraceae, Flavobacterum, and/or *Micrococcus* sp. (Liston 1957; Colwell 1962; Newman et al. 1972; Sera and Ishida 1972a; Sugita et al. 1988; Cahill 1990; Onarheim et al. 1994; Ringø et al. 1995; Ringø and Birkbeck 1999; Izvekova et al. 2007). Lactic acid

bacteria (mainly *Lactobacillus* sp.) have also been found to be minor components of the gut microflora of both freshwater and marine fish (Izvekova et al. 2007).

The gut microbial community can respond to a variety of factors affecting the host, including changing environmental conditions such as temperature and salinity (Yoshimizu and Kimura 1976; MacFarlane et al. 1986), developmental stage (Verner-Jeffreys et al. 2003; Romero and Navarrete 2006), digestive physiology (Cahill 1990), and feeding strategy (Uchii et al. 2006). Some of the gut microflora appear to be transient while other bacteria seem to be resident flora (Kim et al. 2007). Resident gut microflora are those bacteria from the diet or environment that are able to colonize, persist, and proliferate within the gut (Sugita et al. 1988; Cahill 1990). Within a species' natural habitat, stable environmental conditions may lead to a stable gut microflora community that is representative of the "natural flora" (Lynch and Hobbie 1988; Oxley et al. 2002). However in culture systems, conditions of diet, water quality, and population density may be very different from those of the natural habitat. This may result in differences between the gut microflora of wild and cultured populations of the same species and indeed, MacFarlane et al. (1986) observed that farm-raised fish had a simpler gut flora than their wild counterparts.

Several studies have shown that many herbivorous fish such as pinfish (*Lagodon rhomboides*) undergo an ontogenetic diet shift, transitioning from carnivorous juveniles to either omnivorous or herbivorous adults (Benavides et al. 1994; Muñoz and Ojeda 2000; Gallagher et al. 2001). Luczkovich and Stellwag (1993) indicated that this ontogenetic shift in diet resulted in both qualitative and quantitative variability within the

L. rhomboides gut microbiome. Considering the importance of gut microflora with regard to digestive capability and nutrient acquisition, it is likely that fish adapted to a piscivorous lifestyle have gut microbial assemblages that are different than those that feed on invertebrates or plant material.

We used massively parallel sequencing (pyrosequencing) to survey the 16S rRNA ribotypes in the gut microbiomes of 12 finfish and 3 shark species, selected to encompass a wide range of lifestyles. The fish species sampled include both herbivores and carnivores, represent varied digestive physiologies, are classified as pelagic and demersal species, and reside in estuarine to marine environments. We also included three species of sharks as there is little additional information about the composition of shark gut microbiomes beyond one study suggesting that *Photobacterium damselae* is a normal member (Grimes et al. 1985). Unlike finfish, sharks have a short intestine that incorporates a spiraled valve (Budker and Whitehead 1971), which increases the intestinal surface area and allows for increased absorption (Castro and Huber 2003). We hypothesized that the difference in gut physiology between sharks and finfish may lead to differences between the natural microflora of sharks as compared to that of finfish.

METHODS

Fish Collection

Table 2.1 lists species used in this study, along with their phylogenetic classification, feeding strategies, common habitats, and digestive physiologies. In

addition to the 15 species used to compare across species, we also compare wild and cultured mummichogs, *F. heteroclitus* and juvenile and adult pinfish, *L. rhomboides*. Finfish and sharks were caught by trap, trawl, or hook and line. All were kept in recirculating tanks or on ice until dissections were completed.

Wild mummichog specimens were collected from Sapelo Island, GA, and cultured fish were acquired from a population that has been reared in captivity for 11 generations at the Aquatic Biotechnology and Environmental Lab, University of Georgia (courtesy of Dr. R. Winn). Cultured fish were reared in recirculating seawater culture tanks and were fed a diet of brine shrimp (San Francisco Bay Brand), freeze-dried plankton (San Francisco Bay Brand), and Otohime EP1 (Aquatic Ecosystems).

Pinfish were collected by trawl from the Gulf of Mexico (29° 52' N 84° 29' W) with logistic support from the Florida State University Coastal & Marine Laboratory (St. Teresa, FL). Juveniles and adults were differentiated by size: Juveniles were defined as fish <100 mm body length and adults were defined as fish >101 mm in length. All fish were kept in recirculating tanks for no longer than four hours prior to dissection.

Dissections and DNA extractions

Pinfish and mummichogs were euthanized with tricaine methanesulfonate (MS-222; Sigma). The exterior of each fish was cleaned with 95% ethanol prior to dissection. Microbes attached to the intestinal wall were considered to be part of the natural gut microflora (Ringø et al. 2001), and thus, the whole intestine and not just gut contents were used for all extractions. Lengths of pinfish were measured to assess developmental

stage. The mid- to hind-gut region of the intestine was removed, sliced open, and placed into a PowerBead tube (MoBio; Solana Beach, CA). The intestines of several species including southern flounder, black sea bass, red drum, crevalle jack, Spanish mackerel, king mackerel, mahi-mahi, great barracuda, spinner shark, Atlantic sharpnose shark, and sandbar shark were too large to fit directly into PowerBead tubes. These intestines were placed in 50 or 250-mL tubes with phosphate buffered saline buffer (PBS) and sonicated for 30 minutes. The supernatant was then centrifuged at 10,000 rpm for five minutes. The bacterial pellet was transferred directly into a PowerBead tube using a sterile spatula. DNA extractions were then completed using the MoBio Power Soil DNA Extraction Kit according to manufacturer's instructions.

16S rRNA pyrosequencing and analysis

We analyzed the distribution of 16S rRNA ribotypes with massively parallel sequencing (pyrosequencing) using a Roche 454/FLX instrument running Titanium chemistry. Bacterial DNA was amplified using universal 16S rRNA primers 27F and 338R-I and II (Roeselers et al. 2011), which were modified with Titanium (Lib-L) adaptors and sample-specific barcodes. PCR assays were performed in triplicate using Phusion Hot Start II High Fidelity Polymerase (Thermo Scientific) and 1 μM forward and reverse (pooled 338R I & II) primers with the following conditions: initial denaturation at 95 °C for 10 minutes; 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 10 minutes.

PCR products were pooled following amplification and purified using Agencourt Ampure XP (Beckman Coulter) with a modified 1:1 volume of PCR product to Ampure XP beads. Purified amplicons were quantified (Quant-iT PicoGreen; Invitrogen), pooled in equal concentration and submitted to the Georgia Genomics Facility (University of Georgia) for sequencing. A total of 1,214,355 sequences were obtained. These were filtered, denoised, checked for chimeras, and then sorted into OTUs based on 97% sequence similarity using the Greengenes classifier through the Qiime software pipeline (Caporaso et al. 2010; Caporaso et al. 2011). All chloroplasts and unassigned species (defined as those not binned to the kingdom level) were removed from the data set before further analysis. Rarefaction curves were determined using the alpha_rarefaction.py script in Qiime for the Chao1, Shannon, Phylogenetic Diversity (PD) Whole Tree, and Observed Species metrics. The Chao1 metric was incorporated to assess species richness, the Shannon index estimated alpha-diversity, Phylogenetic Diversity is a phylogenetic measure that incorporated branch lengths of taxa from a phylogenetic tree (Faith and Baker 2006), and Observed Species counted the number of unique OTUs found within a sample (Caporaso et al. 2010; Caporaso et al. 2011).

We used the jackknifed_beta_diversity.py workflow script in Qiime (Caporaso et al. 2010; Caporaso et al. 2011) to compare the gut microbiomes of individual fish. This analysis assesses the robustness of our sequencing effort (Caporaso et al. 2010; Caporaso et al. 2011) and determines how often individual microbiomes are clustered randomly (Lozupone et al. 2011). The analysis used weighted UniFrac (based on normalized abundance data) distances from our complete OTU table at an even sampling depth for all

samples. A consensus tree was constructed from 999 jackknifed iterations using UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering. We also used the software package PRIMER (v.6; (Clarke and Gorley 2006a)) for non-metric multidimensional scaling visualization of core gut OTUs from each species. Core gut OTUs were transformed as Presence/Absence data of individual OTUs. The Multiresponse permutation procedure (MRPP) performed in R (R Core Team 2009) with the vegan statistical package (Oksanen et al. 2009) was used to test whether there were significant differences between clustered groups of samples. MRPP was run with the Bray-Curtis distance matrix with 999 permutations. Additional statistical analyses including t-test, Kruskal-Wallis one way analysis of variance, and pairwise Wilcoxon rank sum tests were performed in R (R Core Team 2009) using the vegan statistical package (Oksanen et al. 2009).

16s rRNA Sanger Sequencing and Analysis of Sequences from Clone Libraries

DNA from mummichogs (n=5), pinfish (n=11), silver perch (n=3), black sea bass (n=4), striped burrfish (n=4), spinner shark (n=2), and sharpnose shark (n=2) was also amplified using Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare) with the Bacteria-specific 16S rRNA primers 27F and 1492R (Lane 1991a) with the following PCR conditions: initial denaturation at 95 °C for 5 minutes; 35 cycles of: denaturation at 95 °C for 45 seconds, annealing at 62 °C for 30 seconds, and extension at 72 °C for 1 minute; finishing with a final extension at 72 °C for 45 minutes. Amplified DNA was electrophoresed on a 1% agarose gel, bands of the expected product size were excised,

and then the DNA was extracted and purified using QIAGEN QIAquick gel extraction kits. DNA extracted from the gel was cloned with TOPO TA cloning kits (Invitrogen) using the pCR 4.0-TOPO TA vector and competent *E. coli* cells. Clones were selected randomly and sequenced using the 27F primer by Genewiz (South Plainfield, NJ). All sequences were checked for chimeras using the Bellerophon server (Huber et al. 2004). Sequences were identified by both RDP SeqMatch (Cole et al. 2007a; Cole et al. 2009a) and by BLAST (Johnson et al. 2008) against the non-redundant nucleotide database (NCBI GenBank) and aligned using ClustalW (Larkin et al. 2007). Phylogenetic trees were constructed using MEGA 5.05 (Tamura et al. 2011).

RESULTS

Cross Species Comparison

We obtained a total of 1,038,277 sequences from the 15 target species. Most samples contained 0-10% (average 2.3%) chloroplast sequences; however, some libraries (cultured mummichogs 2-4) contained more (59%, 96%, and 67% respectively). A total of 719,216 sequences remained after removing chloroplasts and unassigned OTUs (Supplementary Table 2.1), and these were assigned to 2, 226 OTUs binned to 17 phyla (Acidobacteria, Actinobacteria, Bacteroidetes, Caldithrix, Chlorobi, Chloroflexi, Firmicutes, Fusobacteria, Gemmatimonadetes, Nitrospirae, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes, Thermi, and Verrucomicrobia). OTUs sorted into the candidate phyla of OP11, SBR1093, TM6, TM7, WPS-2, WS3, and WS6 were

combined into an "unclassified phylum" category which comprised between 0-3.7% (average 0.16%) of individual sample libraries.

As evident in Figure 2.1 not only were there differences in the phyla present in the guts of different finfish and sharks, but there was also variability among individuals of the same species. The within-species variability was more marked in some fish, and was particularly extreme for king mackerel and great barracuda. Despite this variability, representatives of the same phyla were found in the guts of all samples of individual fish species, though relative abundance varied. Excluding the category "unclassified phylum," richness (at the phylum level) of the gut microbiomes of different fish species ranged from 7 to 15 phyla (average=11; Supplementary Figure 2.1). Red drum microbiomes contained the greatest richness, whereas mahi-mahi and sandbar shark had the lowest richness. The phyla Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria were found in all 15 fish gut microbiomes. The phyla Spirochaetes and Tenericutes were recovered from 73% and 87% of the fish species.

Proteobacteria ribotypes dominated the gut microbiomes of most species, accounting for 3-98% (mean=61% ± 34%) of the OTUs present. Firmicutes were found in all species, but at lower relative abundance (1.3-45%, mean=17%, ± 22%). Within the Firmicutes, Lactobacillales ribotypes were found in all fish species except mahi-mahi. For most fish species, Lactobacillales ribotypes contributed <1% of the gut microflora OTUs. However, Lactobacillales were more abundant among some species than others: cultured mummichogs (2.2%), crevalle jack (2.1%), and Spanish mackerel (13%).

Spirochaetes contributed <1.1% of the OTUs recovered from all species except for mahi-mahi and barracuda, where Spirochaetes accounted for 64-98% (83% ± 17%) and 0.05-99% (34% ± 57%), respectively, of the OTUs present. Tenericutes accounted for 1.6, 7.9, 2.6 and 1.34% of the OTUs from wild mummichogs, juvenile and adult pinfish, and crevalle jack, respectively, averaged across all samples of a given fish species. Two king mackerel samples contained 18 and 82% Tenericutes OTUs.

Within each fish species, we found that the individual finfish and sharks sampled shared 7 to 60 OTUs, defined at 97% sequence similarity (Table 2.2). For the sake of simplicity we defined the OTUs shared by all of the fish sampled for a given species as the core OTU group for that species, recognizing that this simplification has greater validity for species that had several samples (i.e. mummichogs and pinfish) versus those for which only two fish were sampled (Spanish mackerel) or for which the microbiomes from guts of individual fish were highly divergent (e.g. great barracuda). Many of the OTUs present in one species' core group were also present as members of the core groups of other species; however, no OTU was shared among all species. The three shark species shared a core microbiome containing OTUs assigned to *Cetobacterium* sp., *Photobacterium* sp., and *Vibrio* sp. Most of the core microbiomes contained ribotypes from the phyla Actinobacteria, Fusobacteria, Firmicutes, and Proteobacteria (mainly γ-Proteobacteria).

OTUs binned to the Family Vibrionaceae were present in the core group of all fish guts except Spanish mackerel. With the exception of mummichogs and Spanish mackerel, all species shared ribotypes similar to *Photobacterium* spp. in their core group.

The OTUs assigned to *Propionibacterium* sp., *Vibrio* sp., *Pseudomonas* sp. were present in the core groups of 87%, 67%, and 67% of all target species. OTUs binned as *Escherichia* sp., *Staphylococcus* sp., *Streptococcus* sp., Clostridiaceae, *Clostridium* sp., *Acinetobacter* sp., *Corynebacterium* sp., *Cetobacterium* sp., *Shewanella* sp were also recovered from many of the species (40-53%; Table 2.3). The *Lactobacillales* ribotypes assigned to *Lactobacillus* sp. and *Streptococcus* sp. were part of the core group of mummichogs, pinfish, hogchoker, southern flounder, Spanish mackerel, king mackerel, and crevalle jack. Similar results to our pyrosequencing analysis were also observed in cloning and sequencing amplicons for mummichogs, pinfish, silver perch, black sea bass, spinner shark, and Atlantic sharpnose shark. striped burrfish.

Comparison of Cultured vs. Wild Mummichogs

OTUs retrieved from cultured and wild mummichogs were distributed among 11 and 12 phyla, respectively (Figure 2.2). Proteobacteria ribotypes dominated the samples (48% ± 11% and 72% ±21%, mean +/- S.D. for cultured versus wild fish, respectively). Ribotypes from the phyla Actinobacteria, Fusobacteria, Firmicutes, and Bacteroidetes were also present. Planctomycetes ribotypes were found in a greater relative abundance in cultured fish (2.5%), and Tenericutes (1.0%) were more abundant in wild mummichogs.

Within the phylum Proteobacteria, 67% (± 27%) of OTUs from cultured fish and 74% (± 23%) of the OTUs from wild fish were assigned to the γ-Proteobacteria. OTUs classified as δ-Proteobacteria were only retrieved from wild fish (Figure 2.4). Within the

γ-Proteobacteria, Vibrionales ribotypes were found in both cultured and wild fish at 47% (± 38%) and 68% (± 14%) relative abundance, respectively. Pseudomonadales ribotypes were also common, but at lower relative abundances (35% ± 38%, cultured and 18% ± 16%, wild; Figure 2.5). Vibrionaceae ribotypes accounted for 19% (± 24%) of the total gut microflora of cultured mummichogs and 39% (± 25%) of the gut microflora of wild mummichogs. Of the Vibrionaceae OTUs, 99% (± 24%) and 84% (± 24%) of the gut microflora of wild and cultured fish, respectively, were binned to genus *Vibrio.*

The cultured and wild mummichog core gut microbiomes shared 12 OTUs sorted among the phyla Actinobacteria, Firmicutes, and Proteobacteria (Table 2.4). The core gut microbiome of cultured mummichogs consisted of 27 OTUS and included 15 OTUs not shared with the core of the wild mummichogs. The core group from wild mummichogs consisted of 41 OTUs, of which 29 were not found in the core group of cultured mummichogs.

Comparison of Juvenile and Adult L. rhomboides

OTUs retrieved from juvenile and adult *L. rhomboides* were binned into 11 and 10 phyla respectively (Figure 2.6). Proteobacteria ribotypes dominated both groups, accounting for 87% ± 15% (juvenile) and 79% ± 32% (adult) of the OTUs retrieved. Ribotypes representing Actinobacteria, Firmicutes, and Tenericutes were present in both groups but at lower relative abundances (Figure 2.7). Spirochaete ribotypes contributed 1% of the OTUs found in adult fish, but were not present in juvenile fish.

The Proteobacteria ribotypes retrieved from juvenile *L. rhomboides* gut microbiomes were predominantly γ-Proteobacteria; whereas, adults had additional contributions from β-Proteobacteria (Figure 2.8). Eighty-three percent (± 14%) of the Proteobacteria OTUs retrieved from juvenile pinfish were assigned to the family Vibrionaceae, with those OTUs divided amongst the genera *Enterovibrio* (35% ± 20%) and *Vibrio* (23% ± 35%). Vibrionaceae ribotypes only accounted for 17% (± 24%) of the sequences retrieved from adult pinfish (Figure 2.9) and these could be further sorted into *Enterovibrio* (4.0% ± 6.9%) and *Vibrio* (8.4% ±13%).

Juvenile and adult pinfish shared a core gut microflora consisting of 9 ribotypes. The juvenile pinfish core group contained an additional 34 ribotypes, but the adult core group only contained 5 more ribotypes (Table 2.5). The main difference between the juvenile and adult pinfish core groups is the presence of *Enterovibrio* sp., *Vibrio* sp., and Rhodobacterales ribotypes in the juvenile core group. The adult pinfish core group also includes OTUs assigned to *Halomonas* sp. and *Sphingomonas* sp., neither of which was found in the juvenile core group.

Statistical Analysis

Rarefaction curves for the Chao1, Observed Species, Phylogenetic Diversity (PD) Whole Tree, and Shannon alpha diversity metrics are shown in Figure 2.10. Table 2.3 lists the results of the four alpha diversity metrics for all samples. Table 2.4 averages the alpha diversity metrics across species. Wild mummichogs had the greatest richness (Chao 1=227 ± 60.5) and the most diverse gut microflora assemblage (Observed

Species=166 ± 43.0). Mahi-mahi and sandbar shark had the least diverse assemblage (Observed Species=13.6 ± 3.35, 13.5 ± 10.2 respectively). The gut microbiomes of all three shark species had less richness (Chao1) and diversity (Observed Species) than most finfish species (i.e. mummichog, pinfish, black sea bass, Spanish mackerel, and crevalle jack). The gut microbiome of the sandbar shark had the least diversity (Observed Species=13.5 ± 10.2) and spinner shark had the most diversity (63.7 ± 57.0) of the three shark species.

We compared the gut microflora communities from the fish we sampled using jackknifed analysis weighted UniFrac calculations (Figure 2.11). The analysis indicated that all finfish and shark samples, except barracuda 2 (BR2), cluster together with >75% jackknifed support. Microbiomes from different fish of the same species did not always cluster with each other, reflecting fish-to-fish variability in the composition of their gut microbiomes. There is >75% support that all mahi-mahi specimens cluster together on the same node. This is also true for both sandbar shark specimens.

The core groups of each fish species were also compared using NMDS to visualize groupings (Figure 2.12). MRPP indicates that clusters defined at 20%, 30%, 40%, and 50% similarity are significantly different (p=0.001). The barracuda core group was markedly different from those of the other fish. The remaining fish samples formed two clusters at >20% similarity. One cluster included mahi-mahi, red drum, silver perch, and the shark species. The second cluster included both mackerel; however, there is little similarity between the core groups of the two mackerel species and they do not group together at 30% similarity. Likewise, the flatfish species southern flounder and

hogchoker core microbiomes do not group at >20% similarity. Core groups among the herbivorous and omnivorous species of adult pinfish, mummichog, and hogchoker are > 40% similar.

We also compared the gut microbiomes of cultured and wild mummichogs and juvenile and adult pinfish using a jackknifed analysis based on weighted UniFrac calculations. There is >75% jackknifed support for the cluster that contains wild mummichog 1, 2, and 4 (Figure 2.13) and cultured mummichogs 3 and 4 cluster together with >75% jackknifed support. Adult pinfish 1, 2, and 3 and juvenile pinfish 1, 2, and 3 cluster together with >75% jackknifed support (Figure 2.14). However, the core microbiomes of other juvenile pinfish form an additional cluster (with >75% support) that excludes the adult pinfish samples.

DISCUSSION

Our analysis suggests that both finfish and shark gut microbiomes harbor more diversity than previously suggested by culture-dependent methods and analysis of 16S rRNA clone libraries (Sogin et al. 2006). We recovered ribotypes distributed among 7 to 15 different phyla. OTUs from the phyla of Acidobacteria, Caldithrix, Chlorobi, Chloroflexi, Gemmatimonadetes, Nitrospirae, Thermi, and Verrucomicrobia were found to be minor, rare components (<1%) of the gut microbiomes of several fish. For all fish species, richness ranged among two to six dominant (>1%) phyla. The dominant phyla of Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria, and Tenericutes were consistent with those reported in the Sullam et al.

(2012) meta-analysis of pooled results from previously published fish gut bacteria clone libraries. OTUs from all of these phyla except Tenericutes were also recovered from zebrafish, *Danio rerio*, in a study using 454-pyrosequencing to analyze gut microflora (Roeselers et al. 2011). Similar phyla (i.e. Firmicutes, Bacteroidetes, Proteobacteria) were found in human and other mammalian gut communities (Ley et al. 2008a; Ley et al. 2008b). Firmicutes and Bacteroidetes ribotypes dominate the mammalian gut microbiomes (Ley et al. 2008a), but these ribotypes were less common and did not dominate the gut microbiomes of the fish we analyzed.

Proteobacteria ribotypes dominated (>50%) the gut microbiomes of 67% of the fish species we sampled. However, Firmicutes, Fusobacteria, Spirochaetes, and Tenericutes, not Proteobacteria, were the dominant ribotypes found in the guts of barracuda, mahi-mahi, king mackerel, Spanish mackerel, and southern flounder. Our results also suggest that fish-to-fish variability in gut microbiome composition is significant in some species (i.e. barracuda). This variability has been documented in other studies of gut microflora (Ley et al. 2008b) and suggests that the composition of the gut microflora community responds to external factors such as habitat and diet.

Proteobacteria ribotypes dominated the gut microflora of 32 of the 50 individual fish we sampled, suggesting that they are a core component of most fish species' gut microflora. Within a given species, individual fish contained the same phyla (Proteobacteria, Firmicutes, and Actinobacteria), but at varying contributions to the total gut microflora community. As suggested in previous studies of gut microflora (Eckburg et al. 2005; Tap et al. 2009; Roeselers et al. 2011), these phyla likely represent a "core"

bacteria community. If the core gut microflora is defined as the ribotypes found in all samples of a given species, we did not find a core microbial assemblage that encompassed all of the fish species we sampled. This is consistent with an analysis of fecal microflora reported by Ley et al. (2008), which found that no OTUs were shared by all mammalian species sampled (humans and 59 terrestrial mammals). Our results suggest that the gut microflora of each species assembles in response to the fish's specific physiological demands and dietary needs.

Many of the OTUs present in one species' core group were also found in the core groups of other species. OTUs binned to the Family Vibrionaceae were in the core groups of all fish guts except Spanish mackerel. With the exception of mummichogs and Spanish mackerel, all species also have OTUs similar to *Photobacterium* spp. in their core group. Additionally, the ribotypes *Propionibacterium* sp., *Vibrio* sp., *Pseudomonas* sp., *Escherichia* sp., *Staphylococcus* sp., *Streptococcus* sp., Clostridiaceae, *Clostridium* sp., *Acinetobacter* sp., *Corynebacterium* sp., *Cetobacterium* sp., *Shewanella* sp. were consistently found in the guts of several species. The prevalence of these bacteria within gut microbial assemblages of different fish species suggests that they are important contributors to gut function.

OTUs from genera within the Order Lactobacillales were found in 93% of fish species. This Order includes the lactic acid bacteria species that have been previously documented within the fish gut microflora community (Rawls et al. 2004; Izvekova et al. 2007). The same lactic acid ribotypes were found in both wild and cultured mummichogs, but were twice as abundant in the gut microbiomes of cultured fish.

However, the increased abundance of lactic acid bacteria in guts of cultured mummichog was not statistically significant by t-test. Although the cultured mummichogs were from a fish rearing facility, the fish were not treated with antibiotics. Further the greater relative abundance of Lactobacillales in cultured fish may be an artifact of commercial feed with lactic acid bacteria. Although none of the diets fed to the cultured fish report additions of lactic acid bacteria, we recovered *Streptococcus* sp. OTUs from a freeze-dried plankton diet used in this laboratory (see Chapter 3, Feeding Study). The *Lactobacillales* ribotypes of *Lactobacillus* sp. and *Streptococcus* sp. were members of the core gut microflora of 47% of fish species sampled suggesting that lactic acid bacteria may be an important member of these gut microflora assemblages.

The mahi-mahi gut microflora community was dominated by Spirochaetes ribotypes (83%), most of which were assigned to *Brachyspira* (91%). Spirochaetes were the dominant (99%) ribotype recovered from the gut microbiome of barracuda 2, but they were less abundant in the other two barracuda samples. The mahi-mahi samples were not collected from the same site or at the same time; thus, the dominance of Spirochaetes ribotypes in all three samples indicates that Spirochaetes and *Brachyspira* OTUs are core members of the mahi-mahi gut microflora. The genus *Brachyspira* is known as an "intestinal Spirochaete" and has been classified as a gut pathogen in pigs (Hampson and Ahmed 2009). These bacteria have also been reported in the intestinal tract of various mammals (including humans) and birds, and the genus includes species that are commensals and pathogens (Bellgard et al. 2009). Our sequence data does not allow us

to determine which *Brachyspira* species were present; however, the mahi-mahi we sampled did not display any signs of impaired health.

The previous applications of culture-independent techniques for examining gut microflora have revealed that *Mycoplasma* sp. are abundant in the gut microflora of a variety of hosts (Giebel et al. 1990a; Holben et al. 2002b; Gulmann 2004a; Tanaka et al. 2004a; Hongoh et al. 2006; Bano et al. 2007; Ward et al. 2009a; Meziti et al. 2010a). Tenericutes sequences were recovered from several fish in this study (mummichogs, pinfish, king mackerel, mahi-mahi, and spinner shark); however, they were only members of the core group of king mackerel and mahi-mahi. The Tenericutes ribotypes from mahi-mahi, mummichogs, and pinfish were classified as *Mycoplasma* sp., but ribotypes recovered from king mackerel microflora were binned as *Ureaplasma* sp. Additionally, the contribution of *Mycoplasma* sp. ribotypes to the pinfish gut microbiome was variable, ranging from 0 to 30%, suggesting that their presence within the gut is influenced by environmental factors (i.e. diet, Chapter 3).

The core gut microflora of the shark species we tested (sharpnose, spinner, and sandbar) contained *Cetobacterium* sp., *Photobacterium* sp. and *Vibrio* sp., with *Photobacterium* ribotypes dominating the core group of all three shark species. This is consistent with the findings of Grimes et al. (1985), who used culture-dependent methods and reported that *P. damselae* is a normal member of the gut microflora of sharks. However, our work expands on the Grimes et al. (1985) study with deeper coverage and the application of a culture-independent technique to the survey. Our data indicate that Actinobacteria, Firmicutes (*Clostridium* sp), Fusobacteria (*Cetobacterium* sp.), and other

Proteobacteria (*Campylobacter* sp. and *Vibrio* sp.) are also important members of the shark gut microbiome. Our findings also indicate that shark gut microbiomes have less richness and diversity than most finfish guts we sampled.

Ley et al. (2008) concluded that gut microflora of herbivorous animals have the greatest diversity, and that this diversity would decrease among omnivores and decrease further among carnivores. We found lower values of all four of the diversity metrics (Table 2.7) in gut microbiomes of the fish defined as top piscivores (mahi-mahi, barracuda, and all shark species) (Froese and Pauly 2002; Froese and Pauly 2010). Southern flounder, king mackerel, and Spanish mackerel are also reported to be piscivores (Froese and Pauly 2002; Froese and Pauly 2010) ; however, the gut microbiomes of these fish were more diverse (Observed Species=108 ± 42.7, 121 ± 30.4, 94.2 ± 73.8 respectively) and richer (Chao1=83.5 ± 46.5, 76.7 ± 23.1, 86.2 ± 63.9 respectively) than the other piscivores we sampled. The Kruskal-Wallis one way analysis of variance indicated that there was not a statistically significant difference between the richness and diversity of fish defined as piscivores. However, there was a statistically significant difference in the calculated richness between invertivores/piscivores and piscivores ($p=0.05$) and omnivores and piscivores ($p=0.02$) suggesting that richness may be linked to a more varied diet.

The gut microbiomes of wild mummichogs, the omnivorous fish we sampled had some of the highest diversity indices in accordance with Ley et al. (2008)'s conclusions from studies of mammal feces that suggested that omnivore gut microflora will be more diverse than carnivore gut communities. There was a statistically significant difference

between the diversity of omnivores and piscivores (p=0.006) further suggesting a link between increased gut microflora diversity and a more varied diet. The diversity indices for the herbivorous fish we sampled (adult pinfish) were greater in richness and diversity than those of the piscivores. However, adult pinfish do not appear to have the richest community. The alpha diversity indices of gut microbiomes from the invertivore-piscivores we sampled (red drum) were greater than piscivores and were among the richest (Chao 1=243 ±261). There was no statistically significant difference between the richness or diversity of omnivores, herbivores, and invertivores/piscivores, and these red drum results may be skewed by the sample "red drum 1" whose gut microbiome had much higher diversity indices than the other two red drum fish. The diversity metrics also suggest that silver perch has less diversity (Observed Species=40.7 ± 25.8) much like the piscivore species. This correlates with the results of our silver perch 16S rRNA clone library which was composed of only *Clostridium* spp. and *Photobacterium* spp. ribotypes. Thus, conclusions based on the analysis of both our clone and pyrosequencing suggest a paucity of bacterial diversity within the silver perch gut microflora community. In conclusion, the relationship between gut microbiome diversity and feeding strategy proposed by Ley et al. (2008) is supported by our results.

Our cultured and wild mummichogs shared 12 OTUs (7-58% of total sequences) among the phyla Actinobacteria, Firmicutes, and Proteobacteria. However, the core gut microbiome of cultured mummichogs and wild mummichogs include additional OTUs not shared with the other group. These results are consistent with the findings of Roeselers et al. (2011), who found that although there were differences in the

composition of gut microflora communities between cultured and wild *Danio rerio*, they still shared a core group of microflora.

Juvenile and adult pinfish also shared a core group of gut microflora, consisting of 9 ribotypes (1.1-14% of total sequences). The core group of OTUs from juvenile pinfish contained an additional 34 ribotypes (65-91% of total sequences), and the core group from adult pinfish only contained five additional ribotypes (14-93% of total sequences). These findings are consistent with Luczkovich and Stellwag (1993) who found qualitative shifts in the gut microflora community correlating with the transition from juvenile to adulthood.

The use of 454-pyrosequencing allowed us to delve deeper into this community in an attempt to truly gauge the microbial diversity. The same genera found within the clone library ribotypes of mummichogs, pinfish, silver perch, black sea bass, sharpnose shark, and spinner shark were also present amongst the respective species' pyrosequencing libraries. The longer sequences generated by Sanger sequencing cloned amplicons allowed us to identify ribotypes at the species level. However, pyrosequencing provides a deeper analysis of the composition of the fish gut microflora community. This pyrosequencing study provides an assessment of the resident "core" gut microbiota and of the variable, or transient members of the gut microflora for the fish we sampled. The presence of many different, non-core ribotypes within the same fish species suggests that these bacteria may be opportunistic and the result of varying environmental factors (such as diet, Chapter 3). The fish gut microbiome contributes to digestion and can affect nutrition, growth, reproduction, overall population dynamics, and

vulnerability of the host fish to disease (MacFarlane et al. 1986). Understanding which bacteria groups are core and variable components of the fish gut microflora is an important precursor to further research into the functional role of gut microflora in regards to fish physiology and health.

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Table 2.1: Species Used in Comparison of Gut Microflora

Species	Order	Family	Feeding Strategy[1]	Habitat	Digestive Physiology
Hogchoker (HC) (*Trinectes maculatus*)	Pleuronectiformes	Achiridae	C Omnivore Invertivore	Demersal; Freshwater-Marine	Short Intestine Pyloric Caeca
Silver Perch (SP) (*Bairdiella chrysoura*)	Perciformes	Sciaenidae	C Invertivore	Demersal; Brackish-Marine	Differentiated
Pinfish (PF) (*Lagodon rhomboides*)	Perciformes	Sparidae	C/H** Herbivore Invertivore	Demersal; Brackish-Marine	Differentiated Elongated Intestine
Southern Flounder (FL) (*Paralichthys lethostigma)*	Pleuronectiformes	Paralichthyidae	C Piscivore	Demersal; Brackish-Marine	Short Intestine Pyloric Caeca
Mummichog (MC) (*Fundulus heteroclitus)*	Cyprinodontiformes	Fundulidae	O Omnivore	Benthopelagic; Freshwater-Marine	Simple Tube
Black Sea Bass (BSB) (*Centropristis striata*)	Perciformes	Serranidae	C Invertivore Piscivore	Reef-associated; Marine	Differentiated
Striped Burrfish (SB)*** (*Chilomycterus schoepfi*)	Tetraodontiformes	Diodontidae	C Invertivore	Reef-associated; Marine	Differentiated
Red Drum (RD) (*Sciaeops ocellatus*)	Perciformes	Sciaenidae	C Invertivore Piscivore	Demersal; Brackish-Marine	Folded Intestine Pyloric Caeca
Crevalle Jack (JC) (*Caranx hippos*)	Perciformes	Carangidae	C Invertivore Piscivore	Reef-associated; Brackish-Marine	Pyloric Caeca
Spanish Mackerel (SPM) (*Scomberomorus maculatus*)	Perciformes	Scombridae	C Piscivore	Pelagic-neritic; Marine	Folded Intestine; Pyloric Caeca
King Mackerel (KM) (*Scomberomorus cavalla*)	Perciformes	Scombridae	C Piscivore	Reef-associated Marine	Folded Intestine Pyloric Caeca

Mahi-mahi (MH) (*Coryphaena hippurus*)	Perciformes	Coryphaenidae	C Piscivore	Pelagic-neritic; Brackish-Marine	Short Intestine Pyloric Caeca
Great Barracuda (BR) (*Sphyraena barracuda*)	Perciformes	Sphyraenidae	C Piscivore	Reef-associated Brackish-Marine	Short Intestine
Spinner Shark (SPN) (*Carcharhinus brevipinna*)	Carcharhiniformes	Carcharhinidae	C Piscivore	Reef-associated; Marine	Short Intestine Spiraled Valve
Atlantic Sharpnose Shark (SHP) (*Rhizoprionodon terraenovae*)	Carcharhiniformes	Carcharhinidae	C Piscivore	Demersal; Brackish-Marine	Short Intestine Spiraled Valve
Sandbar Shark (SDB) (*Carcharhinus plumbeus*)	Carcharhiniformes	Carcharhinidae	C Piscivore	Benthopelagic; Brackish-Marine	Short Intestine Spiraled Valve

C-carnivore, H-herbivore, O-omnivore; [1](Froese et al. 1992; Froese & Pauly 2010); ***L. rhomboides* undergo ontogenetic diet shift from carnivorous to herbivorous; *** only 16S rRNA clone library available.

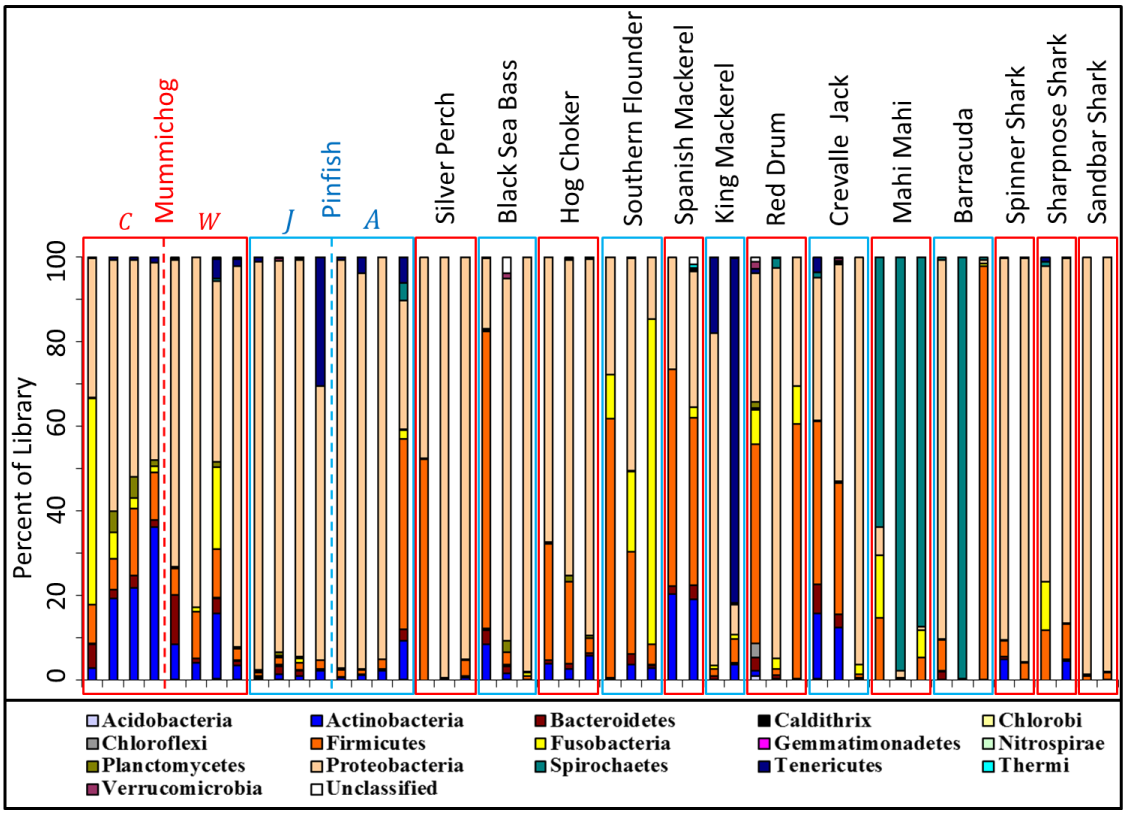

Figure 2.1: Distribution of bacterial phyla (as % of ribotypes retrieved) in individual samples of 12 finfish and 3 shark species determined with 454-pyrosequencing.
C (cultured population), W (wild population), J (juvenile population), and A (adult population).

Table 2.2: Core gut microflora of target species. Core gut microflora included OTUs found among all samples of a species. The top five core phylotypes are listed in order of greatest abundance. Numbers in parentheses indicate that there were multiple OTUs of this ribotype.

Species	# of Shared OTUS (Core) % of total sequences	Top 5 Core Ribotypes (in abundance)
Cultured Mummichogs	27 (50-68%)	*Cetobacterium* sp., *Propionibacterium* sp., *Vibrio* sp., *Acidovorax* sp., *Pseudomonas* sp.
Wild Mummichogs	41 (28-76%)	*Vibrio* sp., *Photobacterium* sp., *Pseudomonas*, *Halomonas* sp., *Propionibacterium* sp.
Mummichogs (All)	12 (7-58%)	*Vibrio* sp., *Propionibacterium* sp., *Pseudomonas* sp., Moraxellaceae, *Acidovorax* sp.
Juvenile Pinfish	43 (65-91%)	*Vibrio* sp., *Enterovibrio* sp., Vibrionaceae, *Staphylococcus* sp., *Propionibacterium* sp.
Adult Pinfish	14 (14-93%)	*Shewanella* sp., *Halomonas* sp., *Photobacterium* sp., *Propionibacterium* sp., *Corynebacterium* sp.
Pinfish (All)	10 (1.1-14%)	*Photobacterium* sp., *Propionibacterium* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Corynebacterium* sp.
Silver Perch	20 (69-99%)	*Photobacterium* sp. (2), Clostridiaceae, Vibrionaceae (2)
Black Sea Bass	12 (9-81%)	*Photobacterium* sp., *Propionibacterium* sp., *Ruegeria* sp., *Corynebacterium* sp., *Escherichia* sp.
Hogchoker	36 (61-92%)	*Shewanella* sp., *Halomonas* sp., *Propionibacterium* sp., *Pseudomonas* sp. (2)
Southern Flounder	21 (12-41%)	*Photobacterium* sp., Clostridiaceae, *Clostridium* sp., Clostridiaceae (2)
Spanish Mackerel	26 (57-62%)	*Alicyclobacillus* sp., *Propionibacterium* sp., *Pseudomonas* sp. (2), *Corynebacterium* sp.
King Mackerel	60 (94-96%)	*Photobacterium* sp., *Ureaplasma* sp., *Acinetobacter* sp., *Cetobacterium* sp., *Alicyclobacillus* sp.
Red Drum	15 (16-74%)	*Photobacterium* sp., *Cetobacterium* sp., Clostridiaceae (2), *Vibrio* sp.
Crevalle Jack	20 (20-91%)	*Photobacterium* sp., *Alicyclobacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Propionibacterium* sp.
Mahi-mahi	13 (98-99%)	*Brachyspira* sp., Spirochaetes, Ruminococcaceae, *Cetobacterium* sp., *Photobacterium* sp.

Barracuda	7 (0.10-74%)	*Photobacterium* sp., *Acinetobacter* sp. (2), *Escherichia* sp., Enterobacteriaceae
Sharpnose Shark	19 (69-74%)	*Photobacterium* sp. (2), *Vibrio* sp., *Campylobacter* sp., *Propionibacterium* sp.
Spinner Shark	14 (82-90%)	*Photobacterium* sp., *Propiongenium* sp., Clostridiaceae, *Clostridium* sp., *Vibrio* sp.
Sandbar Shark	8 (97-98%)	*Photobacterium* sp. (2), *Vibrio* sp., *Cetobacterium* sp., *Vibrio* sp.

Table 2.3: **Comparison of the core group of gut microflora between sampled fish species.**

Species	# of Core OTUs	Corynebacterium	Propionibacterium	Alicyclobacillus	Clostridiaceae	Clostridium	Staphylococcus	Streptococcus	Cetobacterium	Acinetobacter	Escherichia	Photobacterium	Pseudomonas	Shewanella	Vibrio
Mummichog (C)	27	X	X	X			X	X	X				X	X	X
Mummichog (W)	41	X	X				X	X		X	X	X	X		X
Pinfish (J)	43	X	X			X	X	X			X	X	X	X	X
Pinfish (A)	14	X	X			X	X	X				X	X	X	
Silver Perch	20		X	X	X	X						X		X	X
Black Sea Bass	12	X	X								X	X	X		
Hogchoker	36	X	X	X			X			X	X	X	X	X	
S. Flounder	21		X	X	X	X	X			X	X	X	X		X
Sp. Mackerel	26	X	X	X	X		X			X	X		X		
King Mackerel	60	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Red Drum	15			X	X				X		X	X	X		X
Crevalle Jack	20		X	X			X	X		X	X	X	X	X	X
Mahi-mahi	13				X				X	X		X			X

Barracuda	7									X	X	X			
Sharpnose Shark	19	X	X			X			X			X	X		X
Spinner Shark	14				X	X			X			X			X
Sandbar Shark	8								X			X			X
Occurrence		9	12	8	7	7	9	6	7	8	10	15	12	7	12

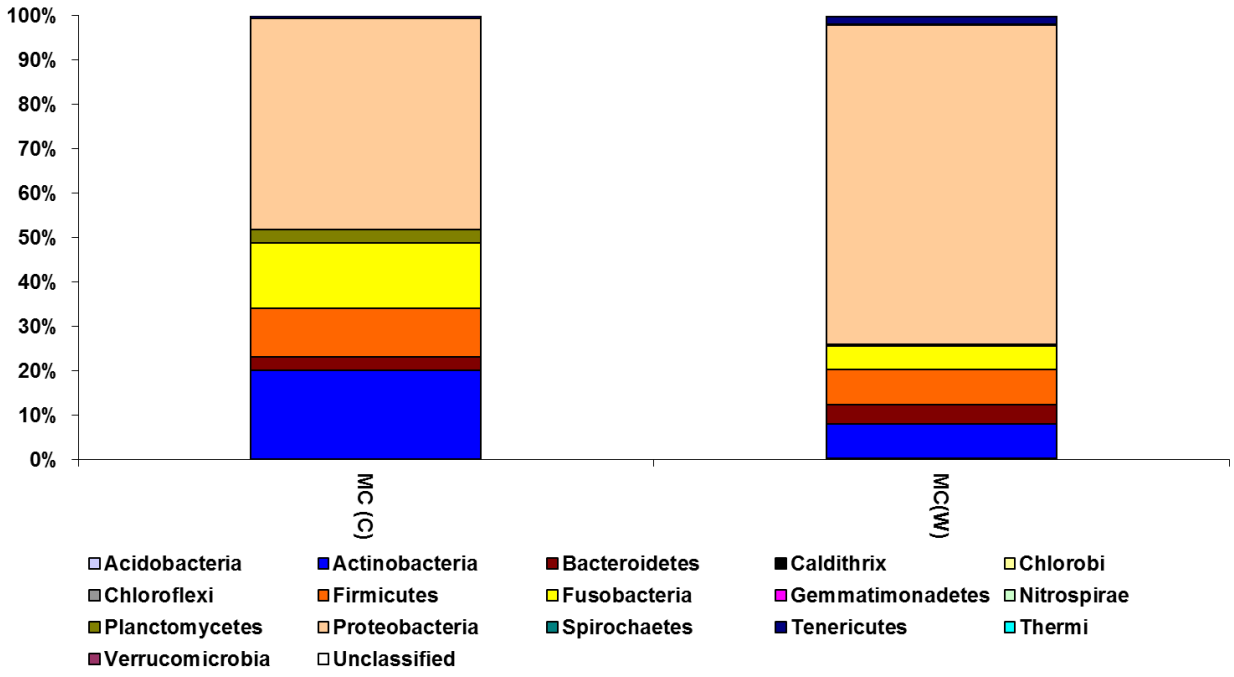

Figure 2.2: Composition of the gut microbiome (phylum level, % of ribotypes retrieved) for cultured (MC (C)) and wild (MC (W)) mummichog.

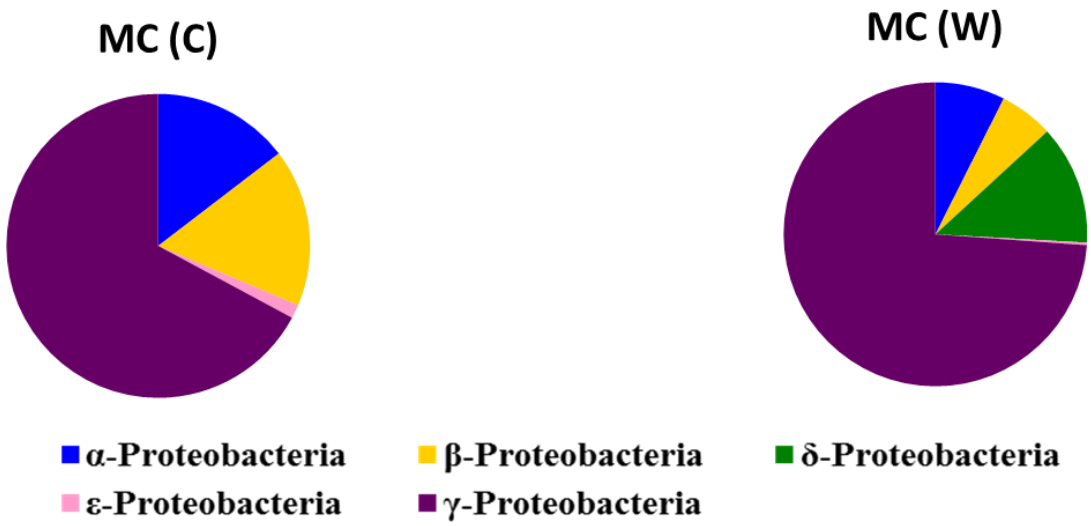

Figure 2.3: Proteobacteria composition (%) in cultured and wild mummichogs

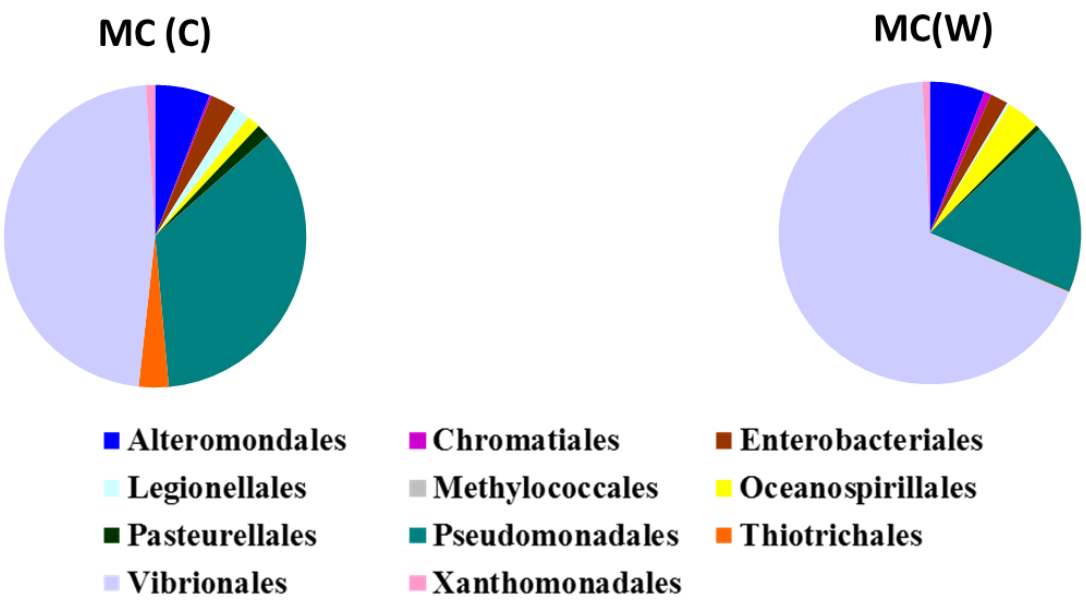

Figure 2.4: γ-Proteobacteria composition (%) in cultured and wild mummichogs

Table 2.4: Core OTUs within the mummichog gut microbiome.
Core gut microflora included OTUs found among all samples of a species. Numbers in parentheses indicate that there were multiple OTUs of this ribotype.

Species	# of Shared OTUS (Core)	Core OTUs
Cultured Mummichogs	27	*Corynebacterium* sp. (5), *Propionibacterium* sp., *Alicyclobacillus* sp., *Staphylococcus* sp., *Lactococcus* sp., *Streptococcus* sp. (2), *Anaerococcus* sp., *Cetobacterium* sp., Pirellulales, *Rhodopirellula* sp., *Planctomyces* sp. (2), *Aquabacterium* sp., Comamonadaceae, *Acidovorax* sp., *Shewanella* sp., Moraxellaceae, *Pseudomonas* sp. (3), *Vibrio* sp., *Stenotrophomonas* sp.
Wild Mummichogs	41	*Brevibacterium* sp., *Corynebacterium* sp. (7), *Micrococcus* sp., *Mycobacterium* sp., *Propionibacterium* sp., Flavobacteriaceae *Staphylococcus* sp., *Lactobacillus* sp., *Streptococcus* sp., *Finegoldia* sp., Caulobacteraceae, *Methylobacterium* sp., Sphingomonadaceae *Sphingopyxis* sp., Burkholderiales, *Acidovorax* sp., *Comamonas* sp., *Delftia* sp., Rhodocyclales, *Pseudoalteromonas* sp. (2), *Escherichia* sp., *Halomonas* sp., Moraxellaceae, *Acinetobacter* sp. (2), *Pseudomonas* sp. (4), *Enterovibrio* sp., *Photobacterium* sp., *Vibrio* sp. (3)
Mummichogs (All)	12	*Corynebacterium* sp. (4), *Propionibacterium* sp. (1), *Staphylococcus* sp., *Streptococcus* sp., *Acidovorax* sp., Moraxellaceae, *Pseudomonas* sp. (2), *Vibrio* sp.

Table 2.5: Core gut microflora within pinfish
Core gut microflora included OTUs found among all samples of a species. Numbers in parentheses indicate that there were multiple OTUs of this ribotype.

Species	# of Shared OTUS (Core)	Top 5 Core Phylotypes (in abundance)
Juvenile Pinfish	43	*Vibrio* sp., *Enterovibrio* sp., Vibrionaceae, *Staphylococcus* sp., *Propionibacterium* sp.
Adult Pinfish	14	*Shewanella* sp., *Halomonas* sp., *Photobacterium* sp., *Propionibacterium* sp., *Corynebacterium* sp.
Pinfish (All)	10	*Photobacterium* sp., *Propionibacterium* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Corynebacterium* sp.

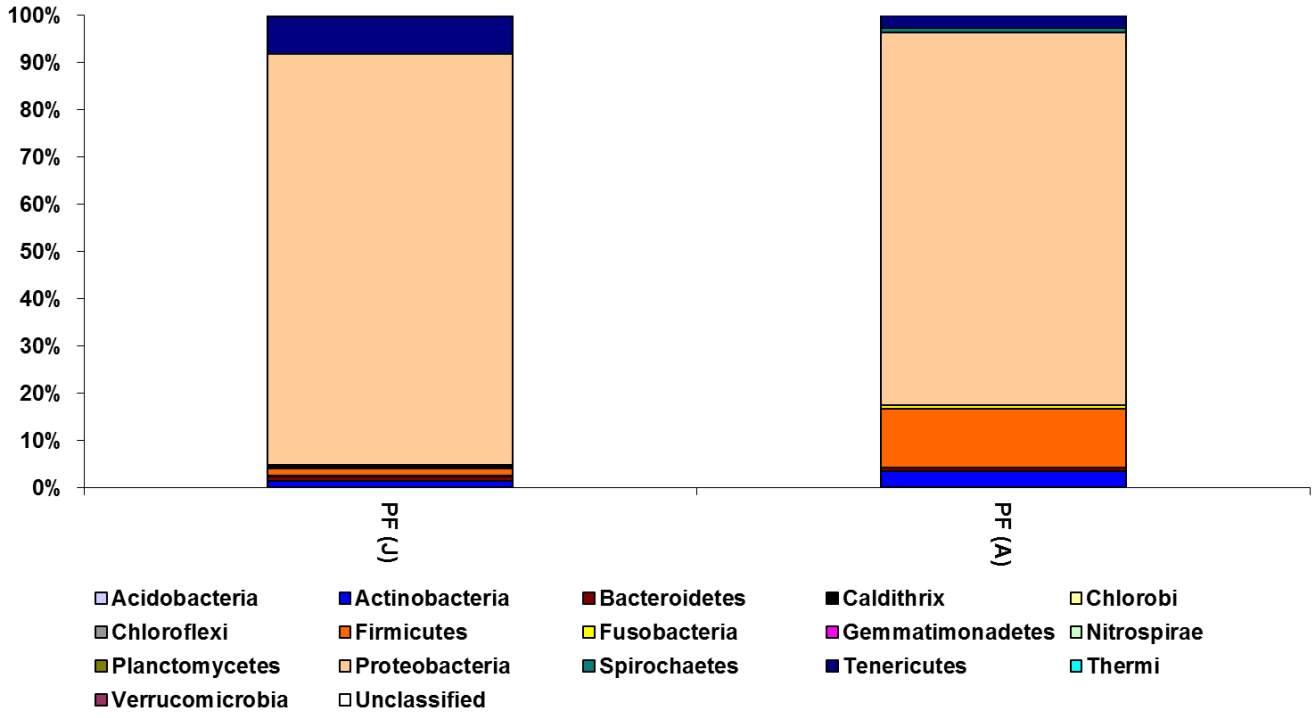

Figure 2.5: Composition of the gut microbiome (phylum level, % of ribotypes retrieved) for juvenile (PF-J) and adult pinfish (PF-A)

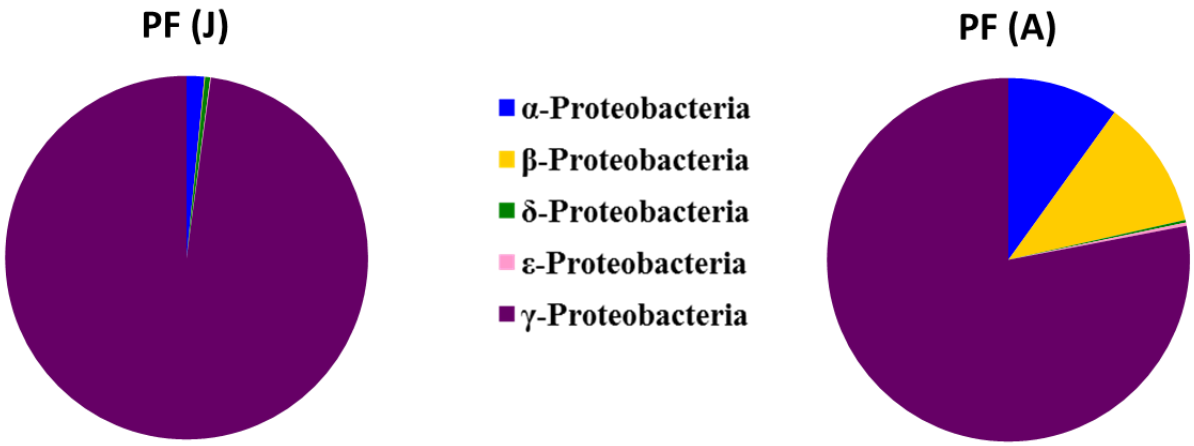

Figure 2.6: Proteobacteria composition (%) in juvenile and adult pinfish

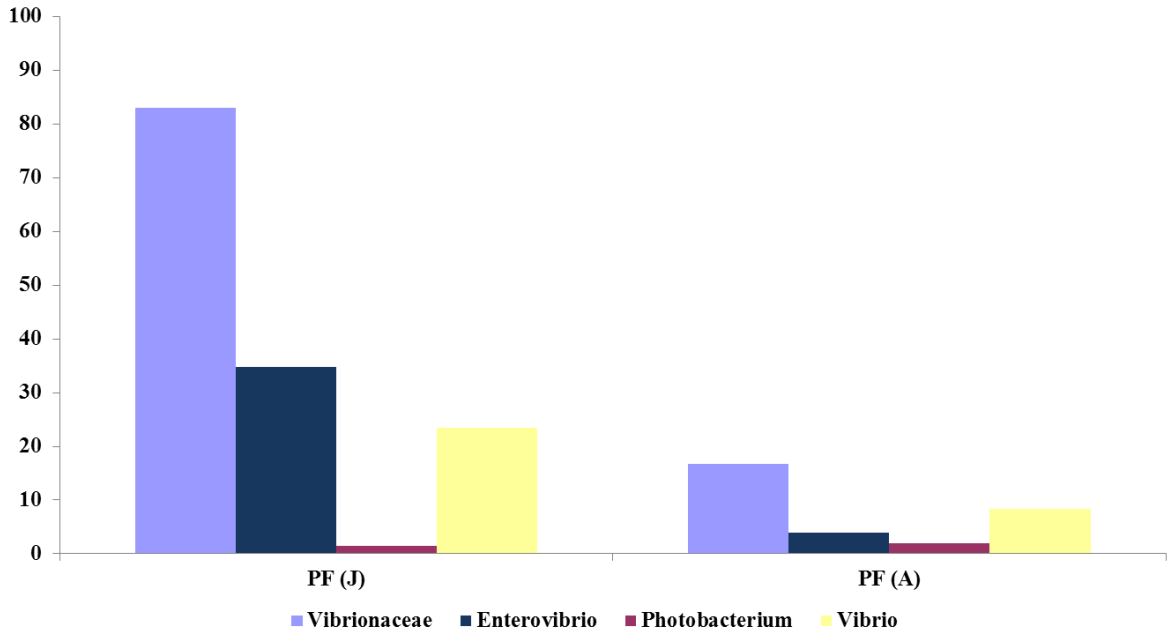

Figure 2.7: Composition of Vibrionaceae ribotypes retrieved from juvenile and adult pinfish gut microbiomes (genus level, % of all ribotypes retrieved)

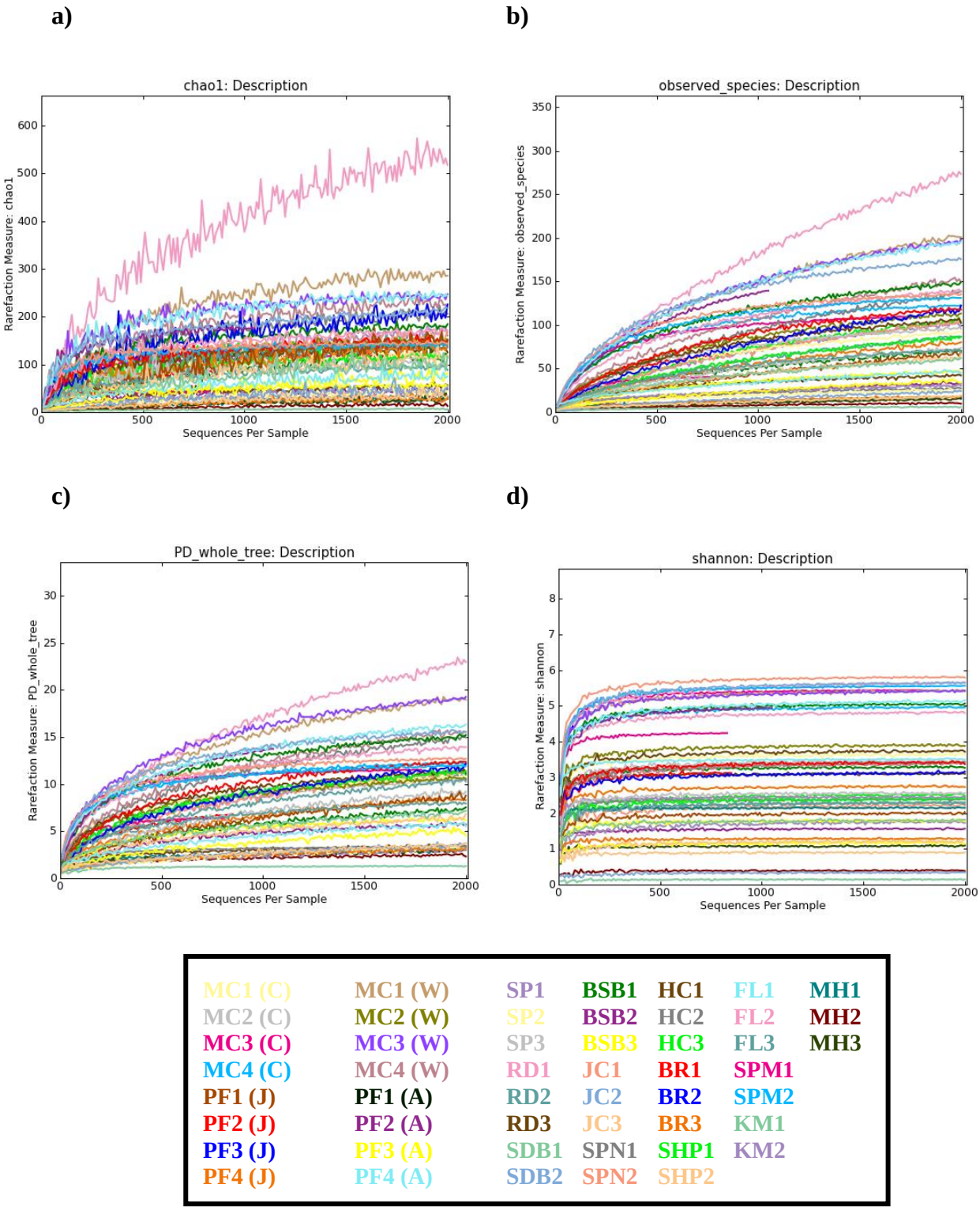

Figure 2.8: Rarefaction curves for alpha-diversity metrics of a) Chao1, b) Observed Species, c) PD_Whole Tree, and d) Shannon index

Table 2.6: Alpha Diversity Metrics of Chao1, Observed Species, PD_Whole Tree, and Shannon index. Diversity values were determined for individual samples at 2000 sequences/sample. For those samples with < 2000 sequences, values were recorded at the maximum sequence length.

Sample	Sequences/ Sample	Chao1	Observed Species	PD_Whole Tree	Shannon
MC1-C	2000	113	90.7	11.2	3.54
MC2-C	2000	125	97.7	10.1	3.46
MC3-C	1710	108	102	11.3	5.32
MC4-C	2000	136	123	11.5	4.96
MC5-W	2000	286	201	19.0	5.53
MC6-W	2000	142	110	10.2	4.07
MC7-W	2000	243	197	18.5	5.33
MC8-W	2000	236	155	15.4	3.24
PF1-J	2000	142	66.7	8.56	1.63
PF2-J	2000	143	117	11.7	3.26
PF3-J	2000	193	115	11.3	3.06
PF4-J	2000	137	80.1	8.90	2.72
PF1-A	2000	109	65.5	6.84	3.03
PF2-A	2000	45.6	33.3	5.70	1.58
PF3-A	2000	54.1	44.7	5.89	1.76
PF4-A	2000	230	184	14.9	5.02
SP1	2000	44.4	30.4	2.80	2.51
SP2	2000	37.7	21.6	2.62	1.76
SP3	2000	95.1	70.1	8.53	2.57
BSB1	2000	179	148	14.4	5.04
BSB2	1030	168	127	13.2	4.80
BSB3	2000	79.9	30.5	4.59	3.06
HC1	2000	126	105	11.1	3.71
HC2	2000	193	131	14.3	3.36
HC3	2000	120	85.4	11.4	2.41
FL1	2000	75.0	44.8	4.56	3.33
FL2	2000	156	135	14.0	4.85
FL3	2000	93.4	70.5	7.46	2.17
SPM1	810	42.0	41.0	6.42	4.23
SPM2	2000	146	131	11.8	5.57
KM1	2000	100	60.3	6.40	1.74
KM2	2000	143	93.0	10.5	1.68
RD1	2000	541	278	23.3	5.68
RD2	2000	134	82.9	10.2	2.29
RD3	2000	52.9	41.4	3.24	3.12
JC1	2000	145	135	12.4	5.73
JC2	2000	204	176	13.8	5.61
JC3	2000	97.1	60.2	5.81	1.20
MH1	2000	28.7	16.1	2.99	2.07
MH2	2000	14.3	9.80	2.42	0.37
MH3	2000	27.5	14.9	2.64	1.07
BR1	840	72.7	59.0	6.22	2.98
BR2	2000	13.1	7.60	1.77	0.05
BR3	2000	25.6	18.6	2.93	1.30
SHP1	2000	136	98.6	10.9	2.67
SHP2	2000	30.2	17.6	3.43	0.91
SPN1	2000	27.3	23.4	3.11	2.32

SPN2	2000	149	104	11.5	2.22
SDB1	2000	7.80	6.30	1.42	0.13
SDB2	2000	42.5	20.7	3.03	0.28

Table 2.7: Alpha Diversity Metrics for each species.
Listed values are averages among samples with standard deviations in parentheses.

Species	Chao1	Observed Species	PD_Whole Tree	Shannon
MC-C	120 (12.4)	103 (13.8)	11.0 (0.62)	4.32 (0.96)
MC-W	227 (60.5)	166 (43.0)	15.7 (4.07)	4.54 (1.08)
PF-J	154 (26.3)	94.6 (25.0)	10.1 (1.61)	2.67 (0.72)
PF-A	110 (84.9)	81.9 (69.5)	8.33 (4.40)	2.85 (1.59)
SP	59.1 (31.4)	40.7 (25.8)	4.65 (3.37)	2.28 (0.45)
BSB	142 (54.4)	102 (62.7)	10.7 (5.36)	4.30 (1.08)
HC	146 (40.3)	107 (22.8)	12.3 (1.81)	3.16 (0.67)
FL	108 (42.7)	83.5 (46.5)	8.67 (4.82)	3.45 (1.34)
SPM	94.2 (73.8)	86.2 (63.9)	9.13 (3.83)	4.90 (0.95)
KM	121 (30.4)	76.7 (23.1)	8.44 (2.89)	1.71 (0.04)
RD	243 (261)	134 (126)	12.3 (10.2)	3.70 (1.77)
JC	149 (53.6)	124 (58.9)	10.7 (4.29)	4.18 (2.58)
MH	23.5 (8.02)	13.6 (3.35)	2.68 (0.28)	1.17 (0.86)
BR	37.2 (31.4)	28.4 (27.1)	3.64 (2.31)	1.44 (1.47)
SHP	83.2 (75.0)	58.1 (57.3)	7.20 (5.30)	1.79 (1.25)
SPN	88.4 (86.4)	63.7 (57.0)	7.32 (5.95)	2.27(0.07)
SDB	25.2 (24.5)	13.5 (10.2)	2.22 (1.14)	0.21 (0.11)

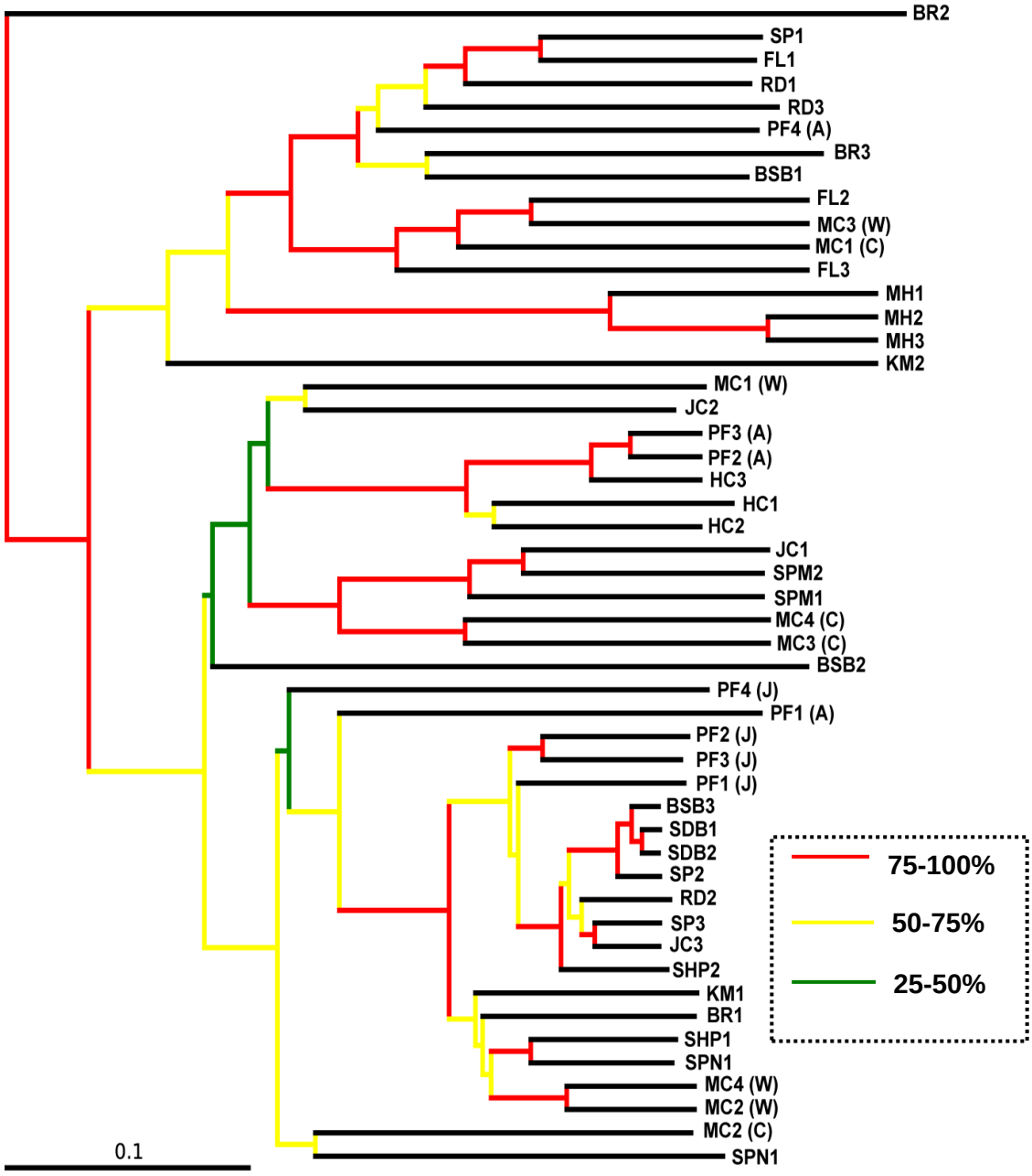

Figure 2.9: Differences in the composition of the microbial assemblages between diets. Cluster analysis with jackknife support based on weighted UniFrac distances and UPGMA clustering.

Red-colored nodes had 75-100% support, yellow-colored nodes had 50-75% support, and green-colored nodes had 25-50% support. Weighted UniFrac distances were calculated from OTUs based on 97% similarity level. MC (C) (cultured mummichog), MC (W) (wild mummichog), PF (J) (juvenile pinfish), PF (A)(adult pinfish), SP (silver perch), BSB (black sea bass), HC (hogchoker), FL (southern flounder), SPM (Spanish mackerel),

KM (king mackerel), RD (red drum), JC (crevalle jack), MH (mahi-mahi), BR (barracuda), SHP (Atlantic sharpnose shark), SPN (spinner shark), and SDB (sandbar shark).

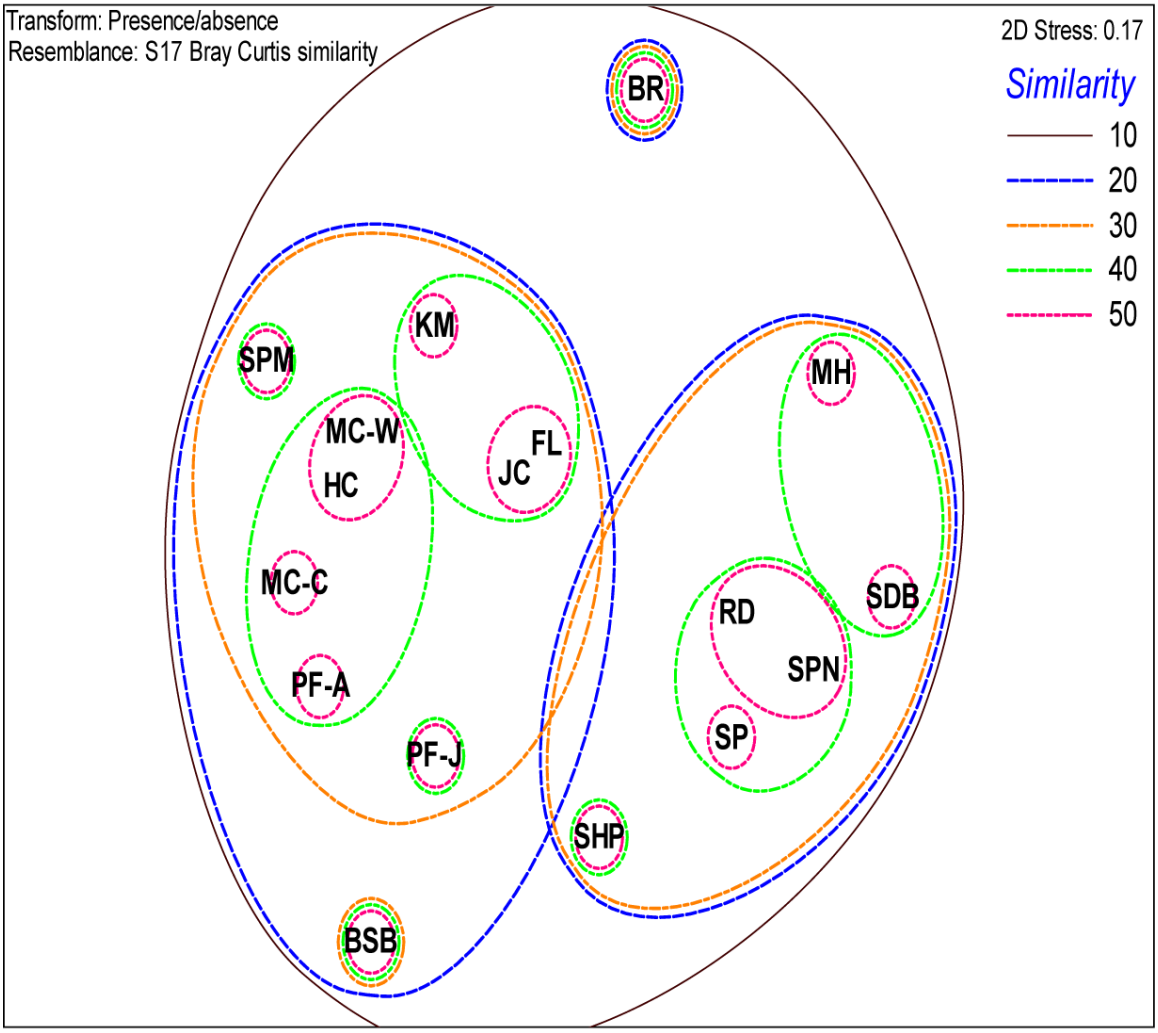

Figure 2.10: Non-Metric Multidimensional Scaling comparison of Core groups of the Gut Microbiomes from 12 finfish and 3 shark species. Data was transformed as Presence/Absence with Bray Curtis similarity resemblance.

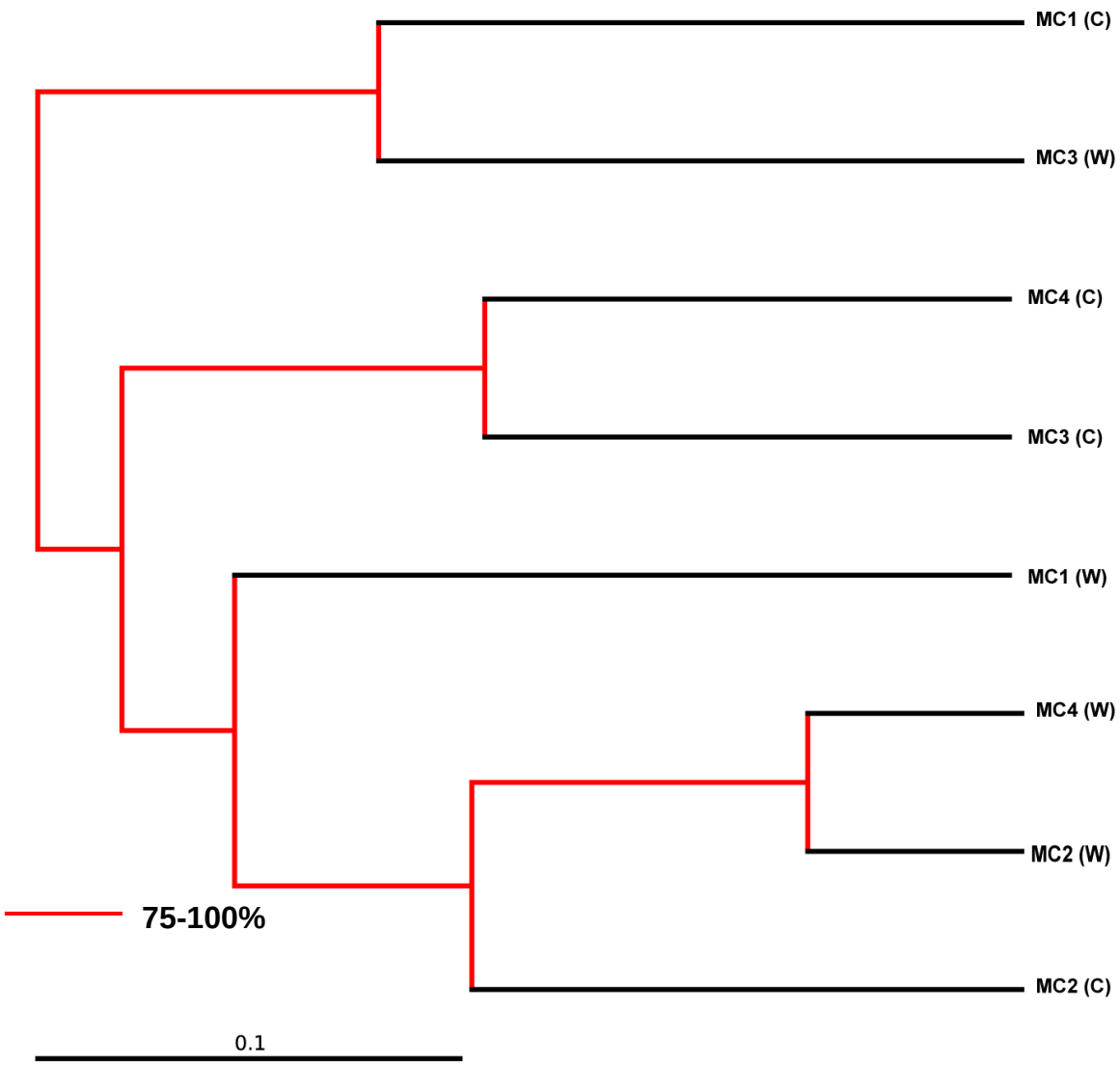

Figure 2.11: Differences in the composition of the microbial assemblages between diets. Cluster analysis with jackknife support based on weighted UniFrac distances and UPGMA clustering.

Red-colored nodes had 75-100% support. Weighted UniFrac distances were calculated from OTUs based on 97% similarity level. (C) refers to cultured fish and (W) refers to wild fish.

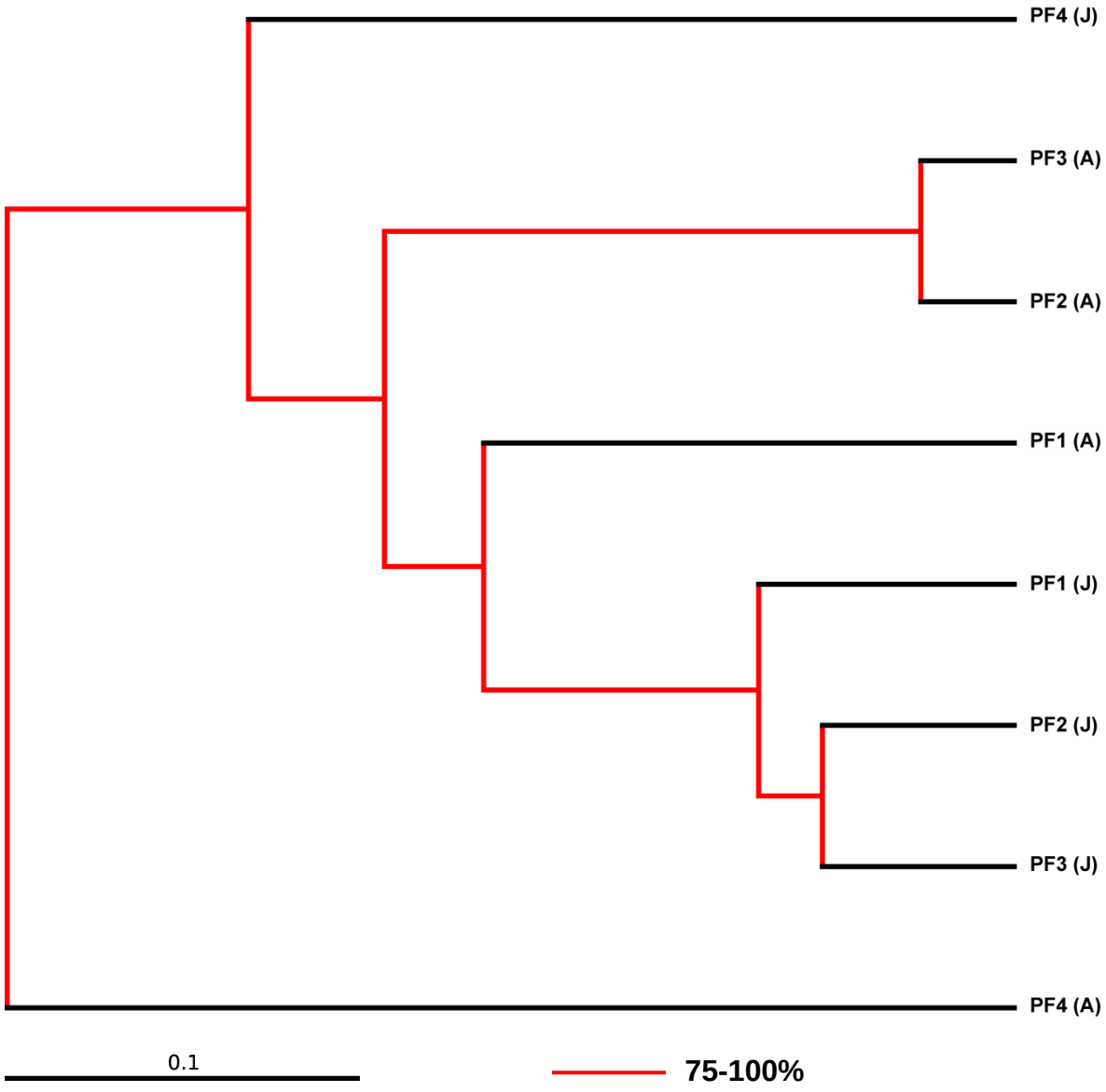

Figure 2.12: Differences in the composition of the microbial assemblages between diets. Cluster analysis with jackknife support based on weighted UniFrac distances and UPGMA clustering.
Red-colored nodes had 75-100% support. Weighted UniFrac distances were calculated from OTUs based on 97% similarity level. (J) refers to juvenile fish and (A) refers to adult fish.

CHAPTER 3

ALTERING THE BALANCE: THE EFFECTS OF MODIFIED DIET ON THE GUT MICROFLORA OF *FUNDULUS HETEROCLITUS* AND *LAGODON RHOMBOIDES*[1]

[1] Givens, C.E. and J.T. Hollibaugh. To be submitted to The International Society of Microbial Ecology Journal.

ABSTRACT

Food resources may be a major factor influencing the composition of fish gut microflora. Food sources might influence the composition of the gut microflora in two ways: first by altering the nutritional characteristics of the material in the gut lumen; and second by inoculating the gut with diet-associated microbes, which may or may not become established in the gut leading to an ecological succession. We completed two feeding experiments with *Fundulus heteroclitus* and *Lagodon rhomboides* to gauge the contribution of diet-associated microbes to the gut microflora community. We used massively parallel sequencing (pyrosequencing) to survey the bacterial 16S rRNA ribotypes present in five different diets, and then followed the changes in the composition of gut microflora among fish fed different diets. The results from feeding studies with both species indicate that diet and diet-associated bacteria impact the composition of the microflora community. However, both fish retained a core Proteobacteria gut community, regardless of diet. These results provide insight into how the gut microflora community responds to dietary change and are important to understanding the underlying mechanisms of species succession in gut microflora. They also emphasize the point that the apparent composition of the gut microbiome as assessed by ribotyping can be strongly influenced by microorganisms associated with recently consumed food, even if these cells are not alive.

INTRODUCTION

The composition of fish gut microflora is influenced by a variety of factors including diet (Ringø and Strøm 1994; Uchii et al. 2006), environmental conditions

(Yoshimizu and Kimura 1976; MacFarlane et al. 1986), and developmental stage (Verner-Jeffreys et al. 2003; Romero and Navarrete 2006). In fact, the gut microflora community present at larval and juvenile stages is strongly influenced by bacteria associated with diet and the surrounding water (Hansen et al. 1992; Hansen and Olafsen 1999; Ringø and Birkbeck 1999; Skjermo and Vadstein 1999).

Sera and Ishida (1972b) suggested that fish with undeveloped intestines have gut flora resembling that associated with food, but fish with more structured digestive tracts maintain a gut microflora distinct from food-associated bacteria. A recent study of Burmese python gut microflora found that pythons and their diet (rats) had different microbial community composition; however the researchers could not discount a small exchange of microbes to the python gut from ingested rats (Costello et al. 2010). Yet in a different study that compared 16S rRNA gene sequences retrieved from terrestrial mammalian gut (fecal) samples, diet was an important factor in grouping patterns (Ley et al. 2008b). Ribotypes from non-human omnivorous primates grouped together and were distinct from those from herbivorous primates (Ley et al. 2008). This same study found that bacterial diversity was lowest in carnivores, greater in omnivores, and greatest in herbivores.

Although feeding may alter gut microflora by the introduction of food-associated bacteria, starvation or fasting may also impact gut physiology and hence its microbial community. Starvation of the Red Sea surgeonfish *Acanthurus nigrofscus* resulted in a reduced gut tract length, which reverted back to normal once these fish resumed feeding (Montgomery and Pollak 1988). A one-day (24-hour) fast in mice resulted in shifts in the composition of their gut microflora (Crawford et al. 2000). Likewise, the gut microbial

community of fasting Burmese pythons has decreased diversity with an increased abundance of Bacteroides, Rikenella, Synergistes, and Akkermansia (Costello et al. 2010).

Some fish have increased gut length to increase nutrient absorption and assimilation from an energy-poor, often herbivorous, diet (Rimmer 1986; Sibly and Calow 1986; Penry and Jumars 1987; Horn 1989). Many herbivorous fish begin their life as carnivores (Horn 1989). Juveniles consume a primarily animal-based diet that allows them to meet the high energy demands associated with this early life stage (Pough 1973; White 1985; Zimmerman and Tracy 1989; Benavides et al. 1994). This ontogenetic diet shift is linked to an increase in intestinal length when compared to overall body length (Stoner and Livingston 1984; Benavides et al. 1994; Muñoz and Ojeda 2000; Gallagher et al. 2001; Moran et al. 2005).

The ontogenetic shift is mirrored by an increase in microbial abundance within the hindgut of herbivorous adults (Rimmer 1986), which is suggested as the main site of microbial fermentation (Mountfort et al. 2002). The ontogenetic shift in the diet of pinfish, *Lagodon rhomboides*, correlates with a qualitative and quantitative transition in the composition of the gut microflora and results in increased occurrence of bacteria capable of hydrolyzing carboxymethylcellulose within the gut of herbivorous adults (Luczkovich and Stellwag 1993). In the herbivorous fish *Kyphosus sydneyanus*, Moran et al. (2005) observed that the total OTU diversity increased along the gut tract of the largest (deemed the oldest) size class. Additionally, the smallest *K. sydneyanus* had the lowest gut microbial diversity, suggesting that increased diversity of the gut microbiome

may be linked to increasing size class or age (Moran et al. 2005). For many herbivorous fish, increased size results in an ontogenetic diet shift (Luczkovich and Stellwag 1993)

Based on these results, it is likely that fish species with different feeding strategies (i.e. herbivores, omnivores, carnivores) will also host different gut microflora. This was established (Chapter 2 of this dissertation) by comparing the composition of the gut microflora of 15 finfish and 3 shark species, captured from the wild. Although we documented differences in the composition of the gut microbiome among these species, we also noted variability, and sometimes dramatic differences, in the composition of the gut microflora from different individuals of the same species. This led us to hypothesize that the composition of the gut microflora as we assessed it (ribotyping) might be influenced by ribotypes associated with recently consumed food items that varied from fish to fish. We completed two feeding experiments to test this hypothesis. Both studies aimed to investigate the effect of diet-associated bacteria on the assessed composition of the gut microflora community. The first study used cultured mummichogs *Fundulus heteroclitus,* which are stomachless and have a simple tube intestine (Burnett et al. 2007). The second study used wild juvenile and adult pinfish, *Lagodon rhomboides,* which were chosen because they undergo an ontogenetic diet shift from carnivore to herbivore with the transition from juvenile to adult life stages (Luczkovich and Stellwag 1993; Gallagher et al. 2001). We used massively parallel sequencing (pyrosequencing) to survey bacterial 16S rRNA ribotypes present in the guts of test fish that were all conditioned to the same diet then followed changes in the relative abundance of the gut microflora community among subsets fed different diets. We found that food-associated ribotypes persisted in fish guts, even when fish were fed sterilized diets, suggesting that microbial DNA

associated with food is not rapidly digested. The influence of food-associated ribotypes was greatest for mummichogs and least for adult pinfish; however, all three treatment groups maintained a "core microflora," regardless of diet.

METHODS

These studies were completed in compliance with Animal Use Protocol (AUP) #2008-10017 approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC).

Sample Collection and Feeding Study 1 – Fundulus heteroclitus

F. heteroclitus (n=125) were taken from a population at the Aquatic Biotechnology and Environmental Lab, University of Georgia, that has been reared in captivity for 11 generations. Fish were held in recirculating seawater culture tanks and were conditioned to a diet of brine shrimp (San Francisco Bay Brand), freeze-dried plankton (San Francisco Bay Brand), and Otohime EP1 (Aquatic Ecosystems) for one month before introducing experimental diets. Five fish were selected randomly and sacrificed to establish the initial composition of the gut microflora. The remaining fish were divided randomly into 12 tanks (10 fish/tank). Fish were fed twice daily and all uneaten food was removed after 15 minutes. Fish were fed three sterilized diets of either: 1) San Francisco Bay Brand Freeze-Dried Plankton (SPK; 64% protein); 2) Ocean Nutrition Formula One Flakes (SFL; 49% protein); or 3) Julian Sprung's SeaVeggies Mixed Seaweed Flakes (SSV; 25% protein); or an unsterilized diet of San Francisco Bay Brand Freeze-Dried Plankton (PK). Diets were sterilized by gamma

irradiation at 10 kilogray (kGy) with a Cobalt-60 source at the University of Georgia Center for Applied Isotope Studies (Athens, GA), and upon post-irradiation testing exhibited no growth in LB medium incubated at 50 rpm at 25°C for 72 hours. Unsterilized diets exhibited vigorous bacterial growth under these conditions. Three tanks were assigned for each test diet. Tanks were equipped with Power Filters with two filter cartridges and recirculated water at a rate of 125 gallons per hour. Tank salinity and water temperature were kept constant at 16-17 psu and 26-27 °C, and levels of nitrite, nitrate, ammonia, and pH levels were monitored regularly to ensure optimal conditions for fish.

The study lasted a total of 42 days and was sampled on days 0, 14, 28, and 42. Nine fish from each diet (three fish per tank) were sacrificed at each time point. All specimens were euthanized with tricaine methanesulfonate (MS-222; Sigma). Each fish was cleaned with 95% ethanol prior to dissection. The mid-to-hind-gut region of the intestine was removed, sliced open with a flame-sterilized scalpel, and placed into a PowerBead tube (MoBio Laboratories; Solana Beach, CA). DNA extraction from the dissected gut was completed using the MoBio PowerSoil DNA Extraction Kit.

Sample Collection and Feeding Study 2 – Lagodon rhomboides

L. rhomboides (n=172) were collected by trawl from the Gulf of Mexico (29° 52' N 84° 29' W) by personnel from the Florida State University Coastal Marine Laboratory (St. Teresa, FL), transported back to the University of Georgia campus, and placed in quarantine for 30 days. Fish were classified as juveniles if they were under 100 mm total body length, with adults measuring over 101 mm length. Juvenile and adult fish were placed in separate quarantine tanks to avoid crossover of microflora between the two

groups. Fish were fed twice daily and all uneaten food was removed after 15 minutes. During quarantine all fish were fed a mixed diet of unsterilized Julian Sprung's SeaVeggies Green Seaweed and San Francisco Bay Brand Freeze Dried Krill. Juvenile and adult fish were quarantined in a 100-gallon tank and 800-gallon tank, respectively. Following quarantine, five juvenile and five adult fish were selected randomly and sacrificed (t=0 days) to establish the initial composition of the gut microflora. The remaining juvenile and adult fish were randomly divided into six 10-gallon juvenile and six 100-gallon adult tanks (n=14 juveniles or 13 adults per tank). All tanks were equipped with double filter Power Filters rated for tank size. Diets were not sterilized and were a) freeze-dried krill (K, 62% protein), b) green seaweed (GSW, 33% protein), and c) equal parts freeze-dried krill and green seaweed (GSWK). Two juvenile and two adult tanks were fed the same diet for the duration of the experiment. Tank salinity and water temperature were kept constant at 25 psu, 25 °C, and nitrite, nitrate, ammonia, and pH levels were monitored.

The study lasted a total of 20 days with fish sampled at 0, 2, 5, 9, 14, and 20 days. Four juveniles and four adults from each diet (two fish per tank) were sacrificed at each time point. All fish were euthanized and dissected as described above. Body length and gut length were measured for each fish. All DNA extractions were completed using the MoBio PowerSoil DNA Extraction Kit.

Diet DNA Extractions

The five unsterilized diets (mixed seaweed flakes, green seaweed, Formula 1, plankton, and krill) used in both feeding studies were placed into individual PowerBead

tubes. These DNA extractions were completed using the MoBio PowerSoil DNA Extraction Kit.

16S rRNA Sequencing and Analysis

We determined the distribution of 16S rRNA ribotypes by massively parallel sequencing (pyrosequencing) using a Roche 454/FLX instrument running Titanium chemistry. All samples were amplified, purified, and quantified separately. DNA was amplified using universal 16S rRNA primers 27F and 338R-I and II (Roeselers et al. 2011) that were modified with Titanium adaptors (Lib-L) and sample-specific barcodes. Samples corresponding to the same treatment (experimental diet and sampling day) were amplified using the same barcode. PCR amplifications for each sample were performed in triplicate using Phusion High Fidelity Polymerase (Thermo Scientific) and 1 μM forward (27F) and reverse (pooled 338R I & II) primers with the following conditions: denaturation at 95 °C for 10 minutes; 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 10 minutes.

Following amplification PCR products from each fish were pooled and purified using Agencourt Ampure XP (Beckman Coulter) with a modified 1:1 volume of PCR product to Ampure XP beads. Purified amplicons were quantified (Quant-iT PicoGreen; Invitrogen) and amplicons representing a given diet type and sampling period (4 fish) were pooled in equal amounts before submission to the Georgia Genomics Facility (University of Georgia; Athens, GA) for sequencing. A total of 1,282,893 sequences were obtained. These were filtered, denoised, checked for chimeras, and then assigned to

operational taxonomic units (OTUs) based on ≥97% sequence similarity using the Greengenes classifier through the Qiime software pipeline (Caporaso et al. 2010; Caporaso et al. 2011). All sequences not assigned to OTUs to at least the kingdom level were removed from the dataset before further analyses.

Rarefaction curves were prepared for all samples using the alpha_rarefaction.py script in Qiime and the Chao1, Shannon, Phylogenetic Diversity (PD) Whole Tree, and Observed Species richness and diversity metrics. We used the jackknifed_beta_diversity.py workflow script in Qiime (Caporaso et al. 2010; Caporaso et al. 2011) to compare the gut microbiomes of fish fed different diets. This analysis assesses the robustness of our sequencing effort (Caporaso et al. 2010; Caporaso et al. 2011) and determines how likely it is that treatments are clustered randomly (Lozupone et al. 2011).The analysis used weighted UniFrac (based on normalized abundance data) distances from our complete OTU table, at an even sampling depth for all samples. A consensus tree was constructed from 999 jackknifed iterations using UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering. T-tests were performed in R (R Core Team 2009) using the vegan statistical package (Oksanen et al. 2009).

RESULTS

Phylogenetic composition of diet-associated bacteria

We obtained a total of 136, 567 sequences that sorted into 651 OTUs (Figure 3.1) from the five different diets. Similar ribotypes were associated with mixed seaweed flakes and green seaweed diets, which were dominated by sequences from the Order

Rhodophyta (85% and 63% respectively), representing chloroplast sequences. OTUs binned to the Phylum Cyanobacteria were likely chloroplasts and will be noted as such for the rest of these results. Additionally, both the mixed seaweed flake and green seaweed libraries had small contributions (3.9 and 2.7% of OTUs) from Flavobacteria within the genus *Olleya*. The sequences retrieved from the plankton and krill diets were also similar to each other and were dominated by Proteobacteria ribotypes, mostly belonging to the genera of *Halomonas* (39%, 32%) and *Shewanella* (37%, 33%). The sequences retrieved from the Formula 1 diet were assigned to Firmicutes and chloroplast ribotypes (57%, 32%). The majority (57%) of Firmicutes ribotypes were assigned to *Lactobacillus* sp., *Lactococcus* sp., and *Weissella* sp. OTUs. Chloroplast ribotypes were predominately from the Phylum Streptophyta.

Alpha diversity calculations suggest the Formula 1 library contained the greatest bacterial diversity (Table 3.1). Rarefaction curves for diet samples plateaued around 2000 sequences are shown in Figure 3.2. Jackknife analysis indicated that bacterial ribotypes associated with the diets form three clusters (with >75% jackknifed support) of 1) Mixed Seaweed Flakes and Green Seaweed; 2) Formula 1, and 3) Plankton and Krill (Figure 3.3).

Feeding Study 1: F. heteroclitus

We retrieved a total of 201,658 sequences from the *F. heteroclitus* study that were binned into 806 OTUs. The initial sample collected from fish immediately prior to changing their diet contained 71% of Proteobacteria and 28% of Fusobacteria ribotypes.

Ninety-nine percent of the Fusobacteria ribotypes belonged to a *Propionigenium* sp. OTU. The majority (83%) of Proteobacteria ribotypes were *Vibrio* spp. OTUs.

Core OTUs were defined as those found in all mummichogs (initial and diet manipulation samples) and are listed in Table 3.2. In contrast to the prevalence of *Propionigenium* sp. OTUs in the initial sample, this OTU only contributed <0.5% to the other feeding study samples. Several OTUs were retrieved from all feeding study samples, but were not retrieved from the initial sample. These OTUs include *Staphyloccus* sp., *Pseudoalteromonas* sp., *Stentrophomonas* sp., and several from the Order Lactobacillales (*Lactobacillus*, *Streptococcus*, *Lactococcus*, and *Weisella*). Fusobacteria ribotypes decreased in abundance and Actinobacteria, Cyanobacteria (chloroplasts), Firmicutes, and Proteobacteria ribotypes increased in all feeding study samples relative to the initial sample (Figure 3.4).

The gut microflora of mummichogs in the feeding study were dominated by Proteobacteria ribotypes (71-96%, average 87%) except for the fish from Days 14 and 28 fed on a diet of mixed seaweed flakes. Of those Proteobacteria ribotypes, *Vibrio* spp. OTUs contributed 10-70% (average 40%) of the total reads per sample. Samples from the Day 14 and 28 mixed seaweed flake-fed and Day 42 sterilized plankton-fed treatments contained OTUs similar to chloroplasts 22-54%, average 42%). Day 14 and 28 seaweed-fed fish had chloroplasts ribotypes which were predominantly within the Orders Rhodophyta (44 and 54% of sample library) and Phylum Streptophyta (4.9 and 0.23% of sample library). The Day 42 sterilized plankton-fed fish had chloroplast ribotypes that were derived primarily from the genera *Arthospira* sp. (20% of total).

Alpha diversity metrics of Chao1, Observed Species, Phylogenetic Diversity Whole Tree, and Shannon (Figure 3.5) suggest that the initial sample had the lowest diversity (Table 3.2). Diversity of the gut microflora increased as the feeding study progressed in all fish except those fed on seaweed flakes. Jackknife analysis indicates that the gut samples from the Day 14 and Day 28 seaweed-fed fish cluster together and separate (at >75% support) from the remaining initial rest of the treatments and from the sample (Figure 3.6). Within the main cluster (includes 11 samples), the initial sample segregates separately from the feeding study samples.

The two main chloroplast ribotypes of Rhodophyta and Streptophya that were associated with the mixed seaweed flake diet contributed 44% and 4.9% and 54 % and 0.23% of the Day 14 and Day 28 seaweed flake-fed samples, respectively. By Day 42, the seaweed flake-fed fish were almost devoid of Rhodophyta and Streptophyta ribotypes with respective contributions of 0.02 and 0.01% of these OTUs. Jackknife analysis indicated that Days 14 and 28 seaweed flake-fed fish cluster with the mixed seaweed flake diet at >75% support (Figure 3.7) and separately from the initial and Day 42 seaweed-fed fish.

Ribotypes associated with the Formula 1 diet (*Lactobacillus* sp., *Lactococcus* sp., and Streptophyta), are only minimally present (<0.53%) in samples from Formula 1-fed fish. Jackknife analysis showed that the all Formula 1-fed fish cluster with the initial sample at >75% support and separate from the Formula 1 diet (Figure 3.8).

Shewanella sp. and *Halomonas* sp. OTUs increase by 25 and 17%, respectively, in the Day 42 plankton-fed fish. Neither *Shewanella* sp. nor *Halomonas* sp. OTUs were found in the initial *F. heteroclitus* fish, but both were retrieved from the plankton diet.

The initial and other feeding study samples (except Day 42 sterilized plankton-fed fish) clustered with 75% support (Figure 3.9). The plankton diet and the Day 42 plankton-fed fish form an additional cluster at >75% support. The Day 28 sterilized and unsterilized plankton-fed fish further cluster together with >75% support.

Feeding Study 2: Juvenile L. rhomboides

We retrieved a total of 446,876 sequences from juvenile *L. rhomboides* that were sorted into 801 OTUs. At the beginning of the diet manipulation, the gut microbiome of juvenile *L. rhomboides* was dominated by Proteobacteria (95%) (Figure 3.10). These fish also contained minimal contributions from additional phyla including Firmicutes (1.5%) and Bacteroidetes (1.0%) ribotypes. The core gut microbiome of juvenile pinfish (Table 3.2), which included OTUs shared between the initial fish and these fish following the diet shift, consisted predominately of *Vibrio* sp. and *Photobacterium* sp. ribotypes.

Fish fed the green seaweed diet had increased relative abundance of Actinobacteria (average 8.3%) and Firmicutes (1.9%) within their gut microbiome. Gut microbiomes of Day 2 green seaweed-fed fish also contained more chloroplast ribotypes (8.0%). Seaweed-fed fish from later sampling points did not have an increased relative abundance of chloroplast ribotypes when compared to the initial sample. Some green seaweed/krill-fed fish had increased relative abundances of Actinobacteria, Cyanobacteria, and Firmicutes ribotypes; however, this pattern was not consistent across all seaweed/krill-fed fish. Additionally, *Bacillus* sp., *Facklamia* sp., *Tenacibaculum* sp. *Pseudoalteromonas* spp., Oceanospirillales, and Sphingobacteriales ribotypes were

absent in initial samples, but were present as minor components of the gut microflora in fish from all feeding trials.

The chloroplast OTUs associated with the green seaweed diet were retrieved from all green seaweed-fed fish except the Day 2 fish. Although retrieved from the other feeding study samples, these OTUs were present at low relative abundances (0.01-0.44%, average 0.18%). The gut microflora of green seaweed-fed fish cluster together with >75% jackknife support and separate from the initial sample and the seaweed diet (Figure 3.11a).

Several of the OTUs (*Aquabacterium* sp. *Pseudomonas* sp., and *Vibrio* spp.) associated with the krill diet were retrieved from the initial sample and in the guts of krill-fed fish. One of the *Vibrio* sp. OTUs associated with the krill diet was part of the core gut microbiome of juvenile *L. rhomboides*. This OTU increased in relative abundance from 1.8% in the initial sample to 1.4-92% (average 55%) among krill-fed samples. There was a statistically significant difference ($p=0.05$) between the relative abundance of this *Vibrio* spp. OTU in the initial and later krill-fed samples. The *Halomonas* sp. ribotype associated with the krill diet was not retrieved from any of the krill-fed fish. The *Shewanella* sp. OTU associated with the krill diet was found in several other samples of krill-fed fish at minimal contribution (<0.12%). Cluster analysis indicated that the krill fed-fish samples were more similar to each other (with >70% jackknifed support) than to either the initial sample or to the krill diet (Figure 3.11b).

Like the gut microbiomes of the green seaweed-fed fish, gut microbiomes from fish fed the seaweed/krill diet shared few (<1.4%) OTUs with the seaweed diet. Cluster

analysis suggested that the gut microbiomes of seaweed/krill-fed fish were more similar to the krill diet (with >75% jackknifed support) than to the initial sample or the seaweed diet (Figure 3.11c). The gut microbiome of seaweed/krill-fed fish form an additional cluster (with >75% support) separate from the krill diet.

Feeding Study 2: Adult L. rhomboides

We retrieved 263,861 sequences from adult *L. rhomboides* gut microbiomes that were binned into 719 OTUs. The initial sample of the gut microflora of adult *L. rhomboides* was dominated by Proteobacteria (61%). In contrast to juveniles, the adult *L. rhomboides* microbiome had larger contributions of Firmicutes (27%), chloroplast (7.6%), and Actinobacteria (3.4%) ribotypes. The core group of the adult *L. rhomboides* gut microbiome (Table 3.2) defined as above, contained predominantly *Vibrio* sp. and *Photobacterium* sp. ribotypes. These were the same *Vibrio* sp. and *Photobacterium* sp. OTUs that were dominant in the juvenile core group. The adult core group includes *Enterobacter* sp., *Shewanella* sp., and *Pseudomonas* sp. ribotypes, which were not present in the core group of the juvenile gut microbiome.

As in the juvenile fish gut microbiomes, Proteobacteria ribotypes were present in and dominated most gut microbiomes of fish fed different diets (26-99%, average 81%) (Figure 3.12). *Photobacterium* spp. OTUs contributed 3.1% of the OTUs in initial adult samples. *Photobacterium* spp. relative abundance increased in gut microflora of fish fed the seaweed/krill diet, up to 97% in adult fish. The relative abundance of these OTUs also increased by 6.2% in fish fed the krill diet. In seaweed-fed fish, the relative abundance of these OTUs decreased by 1.1% relative to the initial fish. The decrease of

Photobacterium spp. in the seaweed-fed fish and the increase in the seaweed/krill-fed fish was statistically significant (p=0.05). As in juvenile samples, *Bacillus* sp., *Facklamia* sp., *Tenacibaculum* sp. *Pseudoalteromonas* spp., Oceanospirillales, and Sphingobacteriales ribotypes were absent in the initial fish, but were present as minor components of the gut microbiomes of fish fed all three diets.

The main OTUs associated with the seaweed diet were present in all seaweed-fed samples except the Day 2 fish. Although present among the other feeding study samples, these OTUs were at low relative abundances (<0.04%). Jackknifed analysis indicates that all seaweed-fed fish were more similar on an OTU level (with >75% support) to the initial fish than to the seaweed diet (Figure 3.13a). All seaweed-fed fish were more similar to each other (with >75% support) than to the initial sample.

Several of the OTUs (*Pseudomonas* sp. and *Vibrio* sp.) associated with the krill diet were also found in the adult core microbiome. The *Vibrio* sp. OTU associated with the krill-diet increased in the Day 14 and Day 20 krill-fed fish at 22 and 8.7% relative to the initial fish. However, this same diet-associated OTU decreased in relative abundance in the guts of Day 2, 5, and 9 fish. This is in contrast to what we observed with this OTU in the juvenile gut microbiome. There was a 35% increase in the relative abundance of the *Halomonas* sp. ribotype associated with the krill diet in the Day 20 fish. This *Halomonas* sp. ribotypes was not present among any of the other krill-fed fish. The *Shewanella* sp. OTU associated with the krill diet was also found in several of the krill-fed samples at minimal percent compositions (<1.3%). Day 2, 5, 14, and 20 krill-fed fish were more similar to the krill diet than to the initial fish (with >75% support). However, none of the krill-fed fish clustered directly with the krill diet (Figure 3.13b). As in the

juvenile seaweed/krill-fed samples, the gut microbiomes of adult seaweed/krill-fed fish had minimal, if any seaweed-diet associated OTUs. Day 2, 9, and 20 seaweed/krill-fed fish cluster with the krill diet at >75% support (Figure 3.13c). With the exception of Day 14 seaweed/krill-fed fish, all seaweed/krill-fed fish and the initial fish were more similar to the krill diet than to the seaweed diet.

Rarefaction curves for the alpha diversity metrics are shown in Figure 3.14. Table 3.4 lists alpha diversity metrics for *L. rhomboides* samples. Results indicate that the gut microbiomes of juvenile and adult initial fish have greater richness than the microbiomes of subsequent feeding study fish. The Observed Species metric (Figure 3.15) indicated that fish fed the seaweed diet had the greatest richness. With the exception of Day 9 krill-fed adult fish, the microflora assemblages from other krill-fed fish had lower richness. For most fish, adults had greater richness and diversity than the juvenile cohort.

DISCUSSION

The results from these studies indicate that diet and diet-associated bacteria affect the composition of fish gut microflora. However, in both experiments, fish appeared to retain a core Proteobacteria gut community. This suggests that gut variations resulting from diet-associated ribotypes have a greater influence on the transient (or non-core) microflora community. We were able to identify *Shewanella* sp. and *Halomonas* sp. OTUs from the plankton and krill diets that were retrieved from the gut microbiomes of plankton- and krill-fed fish. These OTUs were absent in initial samples and were only found within fish fed either of these particular diets. Additionally, fish fed the mixed

seaweed flake and green seaweed diets had higher relative abundances of chloroplast OTUs within their gut microflora. These same chloroplast OTUs were identified in diet samples and increased in relative abundance in the fish gut microbiomes after the fish were fed either the mixed seaweed flake or green seaweed diets. Fish fed sterilized diets (of Formula 1, mixed seaweed flake, and plankton) also had diet-associated OTUs within their gut microflora. Our post gamma-irradiation analyses indicated that there was no bacterial growth associated with the sterilized diets, suggesting that these OTUs did not persist by growing in fish guts. However, gamma-irradiation does not destroy DNA, and our ability to retrieve these OTUs from gut microbiomes suggests that the DNA in diet-associated microbes (or chloroplasts) is not degraded rapidly in fish guts, thus persisting to influence the apparent composition of the gut microbiome.

Our results also indicate that changing diets impact the relative abundance of some ribotypes within the fish gut microbiome. The abundance of Actinobacteria (*Propionibacterium* sp. and *Corynebacterium* sp), Firmicutes (*Clostridium* sp. and Lactobacillales), and Proteobacteria (*Aquabacterium* sp, γ-Proteobacteria and *Vibrio* spp.) increased in *F. heteroclitus* fed Formula 1 and plankton diets. The *Corynebacterium* sp, *Clostridium* sp., some of the Lactobacillales, and most of the *Vibrio* spp. ribotypes were not found within the Formula 1 or plankton diets, and thus, changes in relative abundance of these ribotypes in the gut microflora may be a response to changes in the gut, for example in the suite of potential growth substrates available to bacteria, as a result of the fish being fed different diets. Additionally, we observed diet-dependent shifts in the relative abundance of Bacteroidetes and *Photobacterium* spp. ribotypes in samples from *L. rhomboides* guts following feeding trials. The relative

abundance of Lactobacillales ribotypes that were not found in diet-associated bacteria increased in all samples from the *F. heteroclitus* and *L. rhomboides* feeding study. These shifts in the gut microflora community may be a consequence of competition among the microbial assemblage.

We intentionally used two fish that differed in digestive physiology for these experiments. *F. heteroclitus* has a simple tube gut and no stomach. *L. rhomboides* has a more developed, differentiated gut tract that presumably results in greater processing of the food, longer retention time and a more differentiated gut microflora. If we apply Sera et al. (1972)'s conceptual model to the comparison, the composition of the *F. heteroclitus* gut microflora should have been affected more by diet-associated bacteria than the gut microflora of *L. rhomboides*. We found that the gut microflora from *L. rhomboides* fed on the green seaweed diet only had minimal contributions (average 0.37%) from diet-associated ribotypes. In contrast, one chloroplast ribotype from the mixed seaweed flake diet accounted for 44 and 54% of the ribotypes retrieved from *F. heteroclitus* guts fed this diet. Bacteria associated with the mixed seaweed flake diet were more abundant in the seaweed flake-fed *F. heteroclitus* than those bacteria associated with the green seaweed diet in the seaweed-fed *L. rhomboides.* Similarly, the contribution of ribotypes from the plankton diet to *F. heteroclitus* gut microflora was much greater than the contribution of ribotypes from the krill diet to *L. rhomboides* gut microflora. We conclude that the bacterial community within the simple gut of *F. heteroclitus* was more directly influenced by diet-associated bacteria than the community of the more differentiated gut of *L. rhomboides*. Further, because this difference was observed with diets that had been sterilized by gamma-irradiation as well as with

unsterilized diets, it must be due to more complete digestion of microbial biomass by *L. rhomboides* than *F. heteroclitus*, rather than as a result of differences in the ability of diet-associated bacteria to reproduce in the guts of these two fish.

Diversity of the gut microflora community was lowest among *L. rhomboides* fed an invertebrate diet (krill), greater among *L. rhomboides* fed an omnivorous diet (seaweed and krill), and greatest among those fed a vegetarian (seaweed) diet. This agrees with the conclusions of Ley et al. (2008), who found that diversity was lowest in the fecal microflora of carnivores in a study of the fecal microflora of humans and terrestrial mammals. This generality was not as clearly delineated within the *F. heteroclitus* gut microflora, possibly as a result of the greater contribution of diet-derived ribotypes to the gut microflora assemblage, which may have masked differences in the core microbiome. Alpha diversity metrics indicated that microflora diversity was greatest in fish fed the Formula 1 diet. However, the diversity of the ribotypes associated with the Formula 1 diet was also greater than for the other diets we tested. Since the gut microflora communities of *F. heteroclitus* were more directly impacted by diet-associated bacteria than those of *L. rhomboides*, the increased diversity of gut microflora in Formula 1-fed samples may simply be attributed to the greater diversity of bacteria in the Formula 1 diet.

On average, richness and alpha diversity metrics were greater for gut microflora communities of the adult *L. rhomboides* than in that of the juvenile population. A study of the herbivorous *K. sydneyanus* showed that increased gut microflora diversity was linked to increased fish size and to an ontogenetic shift in diet (Moran et al. 2005). Thus,

it seems to be a general rule that as fish undergo an ontogenetic shift in diet from carnivorous juveniles to herbivorous adults, their gut microflora increases in diversity.

In summary, we found that diet influences the microbial assemblage present in fish guts directly via the contribution of diet-associated ribotypes to the community and also through proliferation of bacterial ribotypes that become more prevalent as a result of digestive and nutrient requirements. We were able to identify a core microbiome that consistently contributed to the bulk of the respective fish's gut microbiome. Diet-associated ribotypes were recovered from the gut, but they were not always present and did not increase in relative abundance in samples from later time-points, suggesting that they were not able to persist within this community. Even when diets contained potentially viable bacteria, these bacteria were unable to proliferate within the gut. Our findings provide insight into the environmental factors regulating the composition of the gut microflora community and contribute to understanding the underlying mechanisms that influence digestion and nutrient adsorption in fish.

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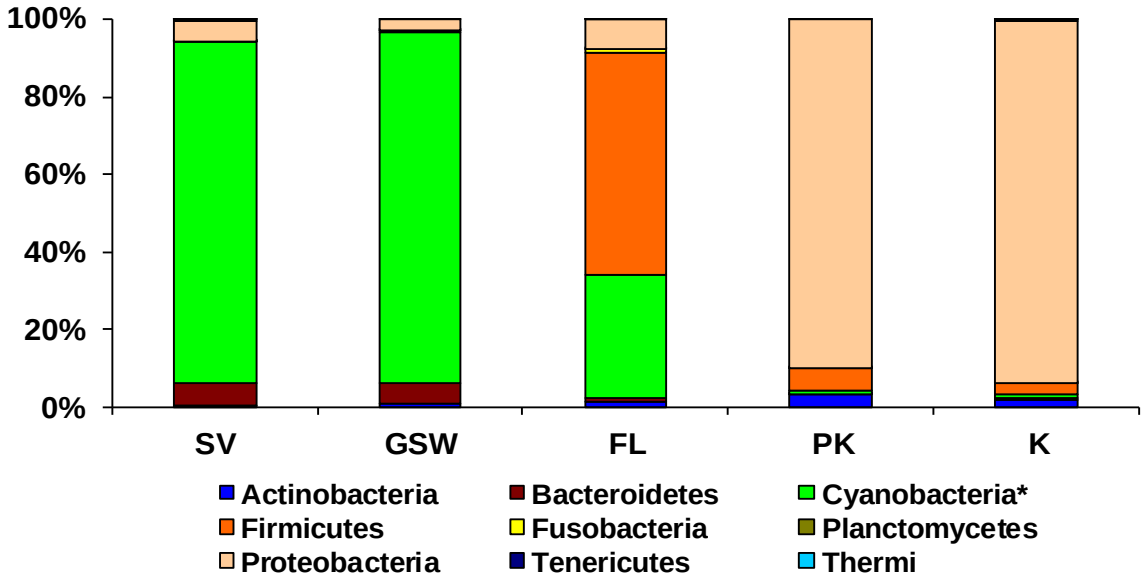


Figure 3.1: Composition of the Bacterial assemblage (at the phylum level, % of ribotypes) retrieved from samples of the diets used in this study. Diets are: Julian Sprung's SeaVeggies Mixed Seaweed Flakes (SV); Julian Sprung's SeaVeggies Green Seaweed (GSW); Ocean Nutrition Formula One Flakes (FL); San Francisco Bay Brand Freeze-Dried Plankton (PK); and San Francisco Bay Brand Freeze-Dried Krill (K).

*Ribotypes binned as Cyanobacteria were likely chloroplasts.

Table 3.1: Richness and Alpha Diversity Metrics for Diet samples used in both Feeding Studies.

Alpha diversity measures were calculated for 6000 sequences/sample.

Sample	Sequences/ Sample	Chao1	Observed Species	PD Whole Tree	Shannon
Formula 1	6000	318	260	17.6	4.72
Krill	6000	117	107	10.5	3.17
Mixed Seaweed Flakes	6000	205	136	13.0	1.31
Plankton	6000	108	88.3	9.26	2.60
Green Seaweed	6000	168	93.9	9.38	1.68

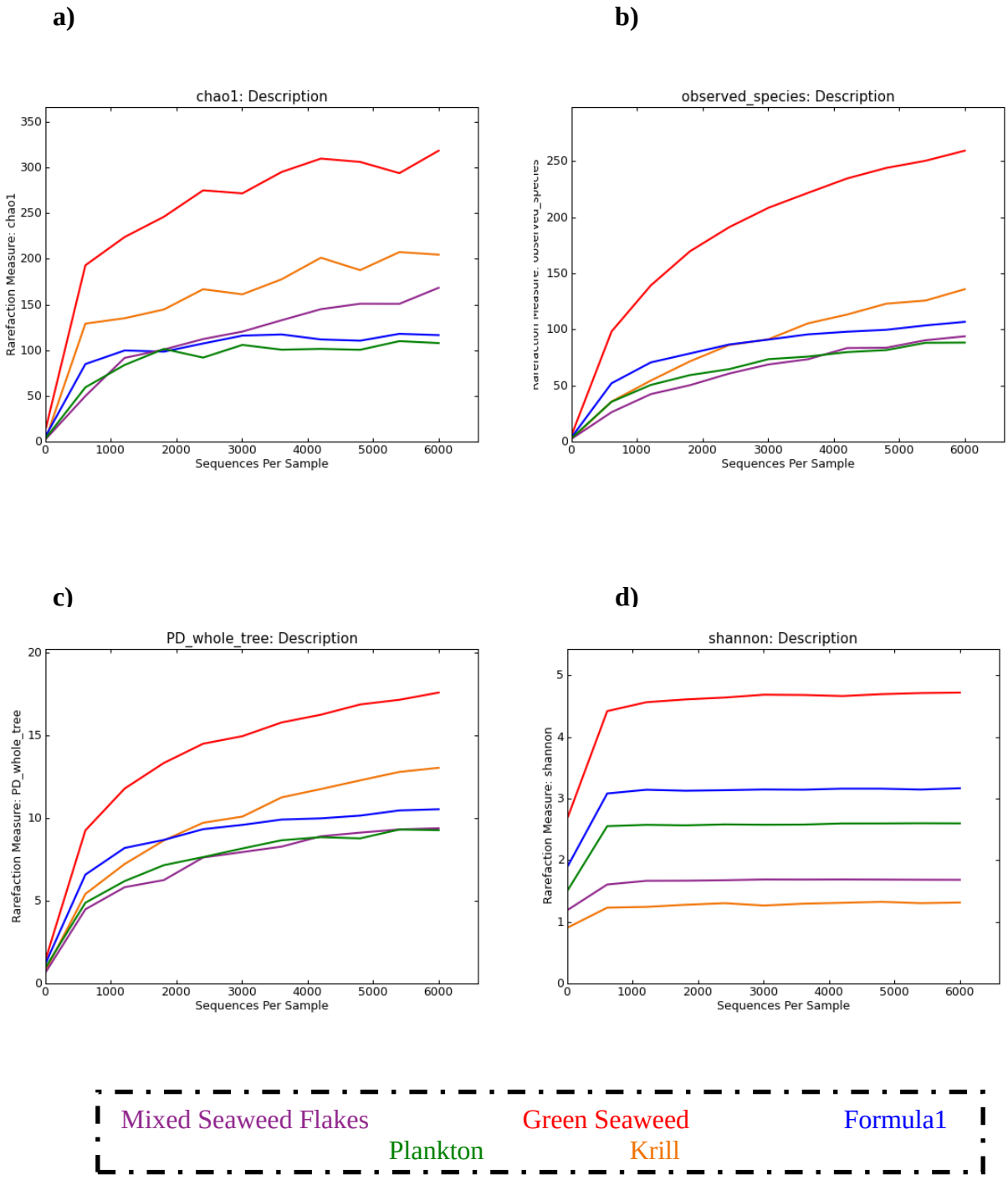


Figure 3.2: Rarefaction curves of richness and alpha-diversity metrics for diets used in this study. a) Chao1, b) Observed Species, c) PD Whole Tree, and d) Shannon index.

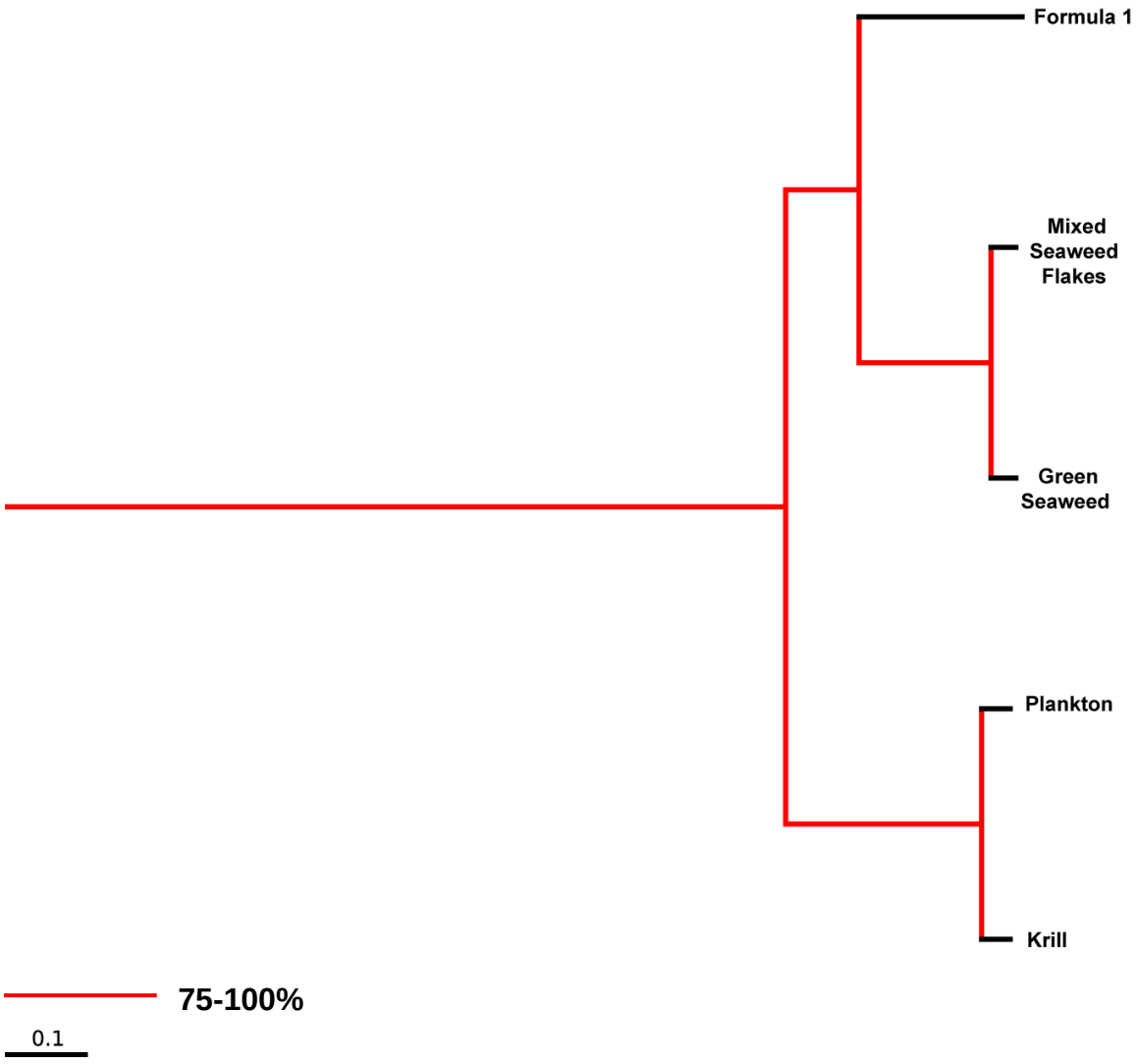


Figure 3.3: Differences in the composition of the microbial assemblages between diets. Cluster analysis with jackknife support based on weighted UniFrac distances and UPGMA clustering. Red-colored nodes had 75-100% support. Weighted UniFrac distances were calculated from the distribution OTUs defined at a 97% similarity level.

Table 3.2: Core gut microflora of *F. heteroclitus* and *L. rhomboides*.

Core gut microflora are defined as OTUs found in all samples of an experimental group, regardless of diet. The top five core ribotypes are listed in order of greatest relative abundance in the microbiome (not corrected for genome dosage).

Species	# of Core OTUs	Core Phylotypes (in order of abundance)
F. heteroclitus	19	*Vibrio* sp., Vibrionaceae, *Aquabacterium* sp., *Shewanella* sp., *Vibrio* sp., γ-Proteobacteria, *Photobacterium* sp., *Vibrio* sp., *Propionibacterium* sp., *Pseudomonas* sp., Vibrionaceae, Rhodobacteraceae, Vibrionaceae, *Corynebacterium* sp., *Bradyrhizobium* sp., *Acinetobacter* sp., *Pseudomonas* sp.
Juvenile *L. rhomboides*	6	*Vibrio* sp., *Photobacterium* sp., Vibrionaceae, *Vibrio* sp., *Propionibacterium* sp., *Staphylococcus* sp.
Adult *L. rhomboides*	7	*Vibrio* sp., *Photobacterium* sp., *Vibrio* sp., *Propionibacterium* sp., *Enterobacter* sp., *Shewanella* sp., *Pseudomonas* sp.

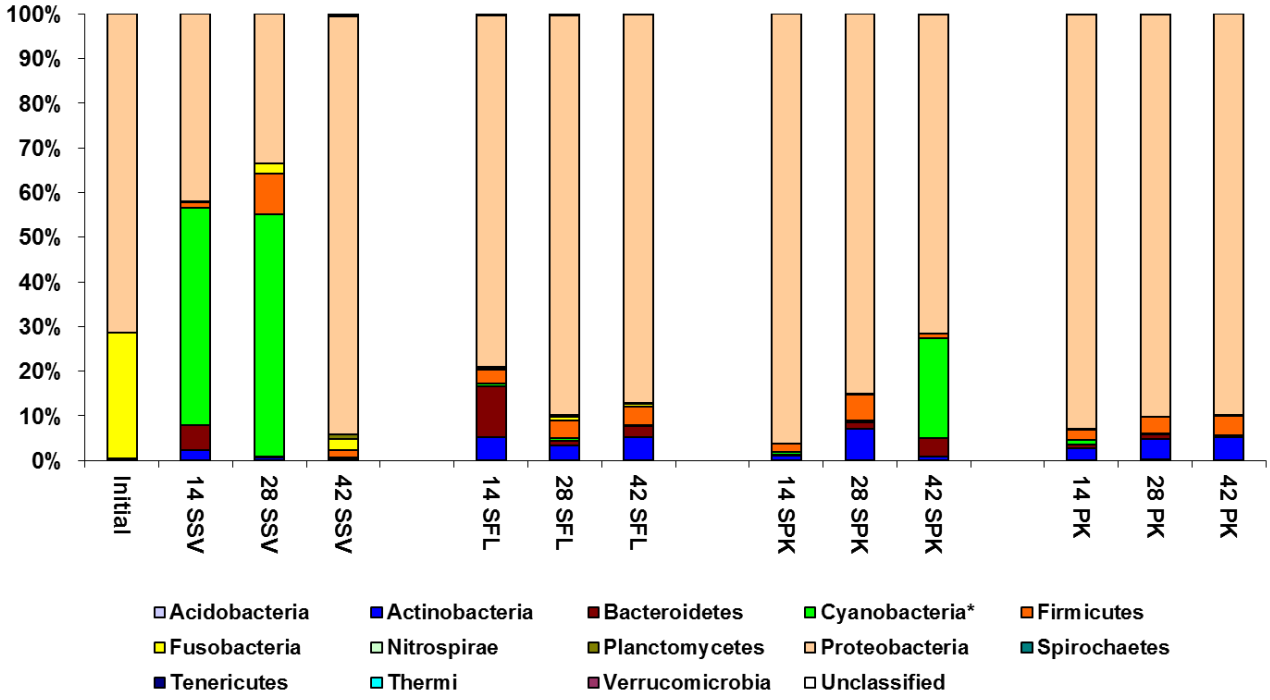


Figure 3.4: Composition of the Bacterial assemblage (at the phylum level, % of ribotypes) retrieved from samples of the *F. heteroclitus* Feeding Study samples
Percent composition of phyla within the guts of *F. heteroclitus* from Day 0, 14, 28, and 42. SSV samples were fed sterilized seaweed flake. SFL fish were fed a diet of sterilized Formula One (F1) flakes. SPK fish were fed sterilized plankton and PK fish were fed unsterilized plankton. *Ribotypes binned as Cyanobacteria were likely chloroplasts.

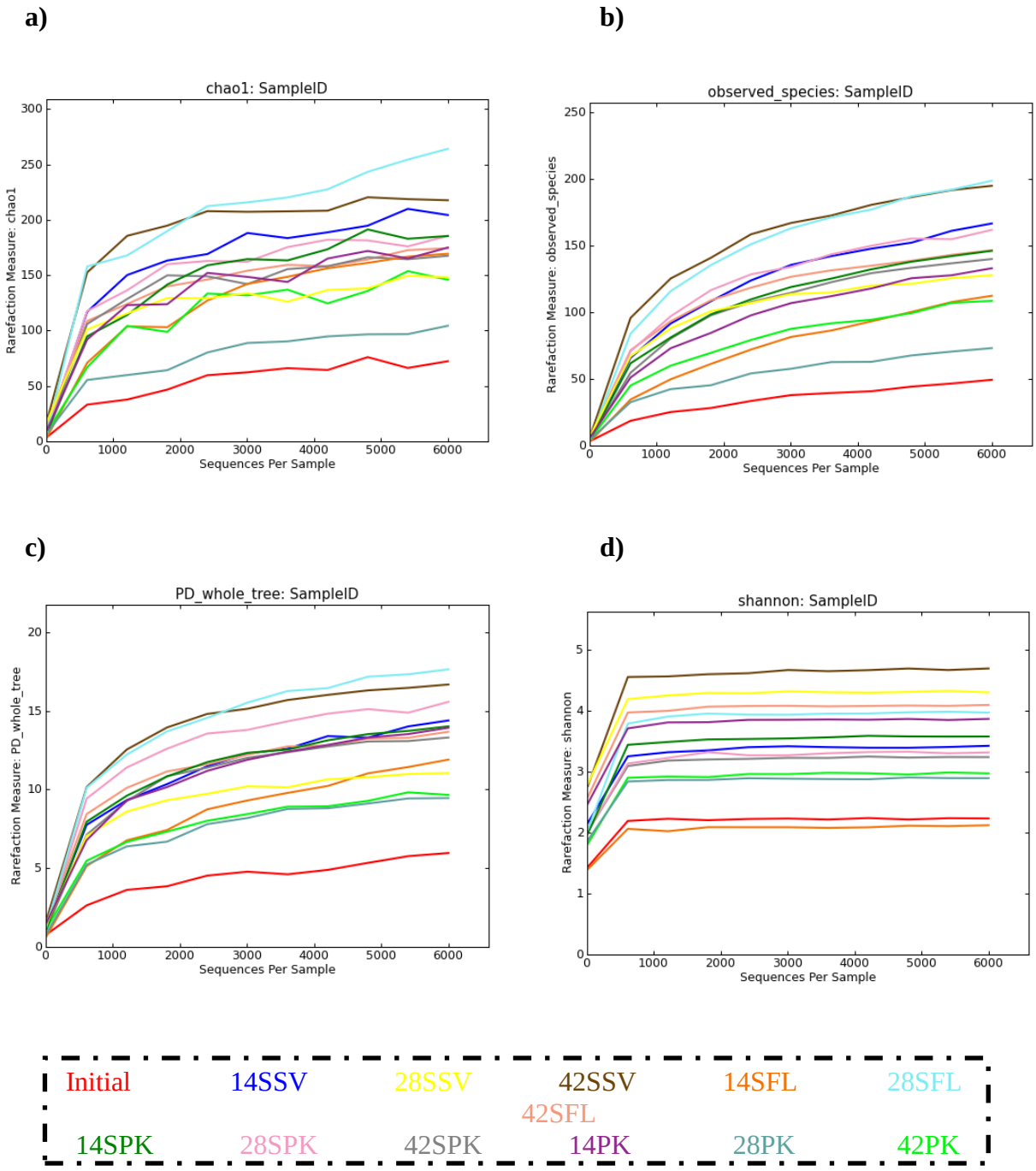


Figure 3.5: Rarefaction curves of richness and alpha-diversity metrics for diets used in this study. a) Chao1, b) Observed Species, c) PD Whole Tree, and d) Shannon index.

Table 3.3: Richness and Alpha Diversity Metrics for the *F. heteroclitus* Feeding Study.

Indices were calculated for each sample using 6000 sequences/sample. SSV samples were fed sterilized seaweed flake. F1 fish were fed a diet of sterilized Formula 1 (SFL) flakes. SPK fish were fed sterilized plankton and PK fish were fed unsterilized plankton.

Sample	Sequences/ Sample	chao1	Observed Species	PD Whole Tree	Shannon
Initial	6000	72.4	49.4	5.96	2.23
14 SSV	6000	204	167	14.4	3.42
28 SSV	6000	146	109	9.65	2.97
42 SSV	6000	104	73.2	9.45	2.89
14 SFL	6000	218	195	16.7	4.69
28 SFL	6000	186	162	15.6	3.32
42 SFL	6000	264	199	17.6	3.97
14 SPK	6000	169	113	11.9	2.12
28 SPK	6000	174	147	13.7	4.10
42 SPK	6000	175	133	13.9	3.87
14 PK	6000	168	140	13.3	3.24
28 PK	6000	185	146	14.0	3.58
42 PK	6000	148	128	11.0	4.30

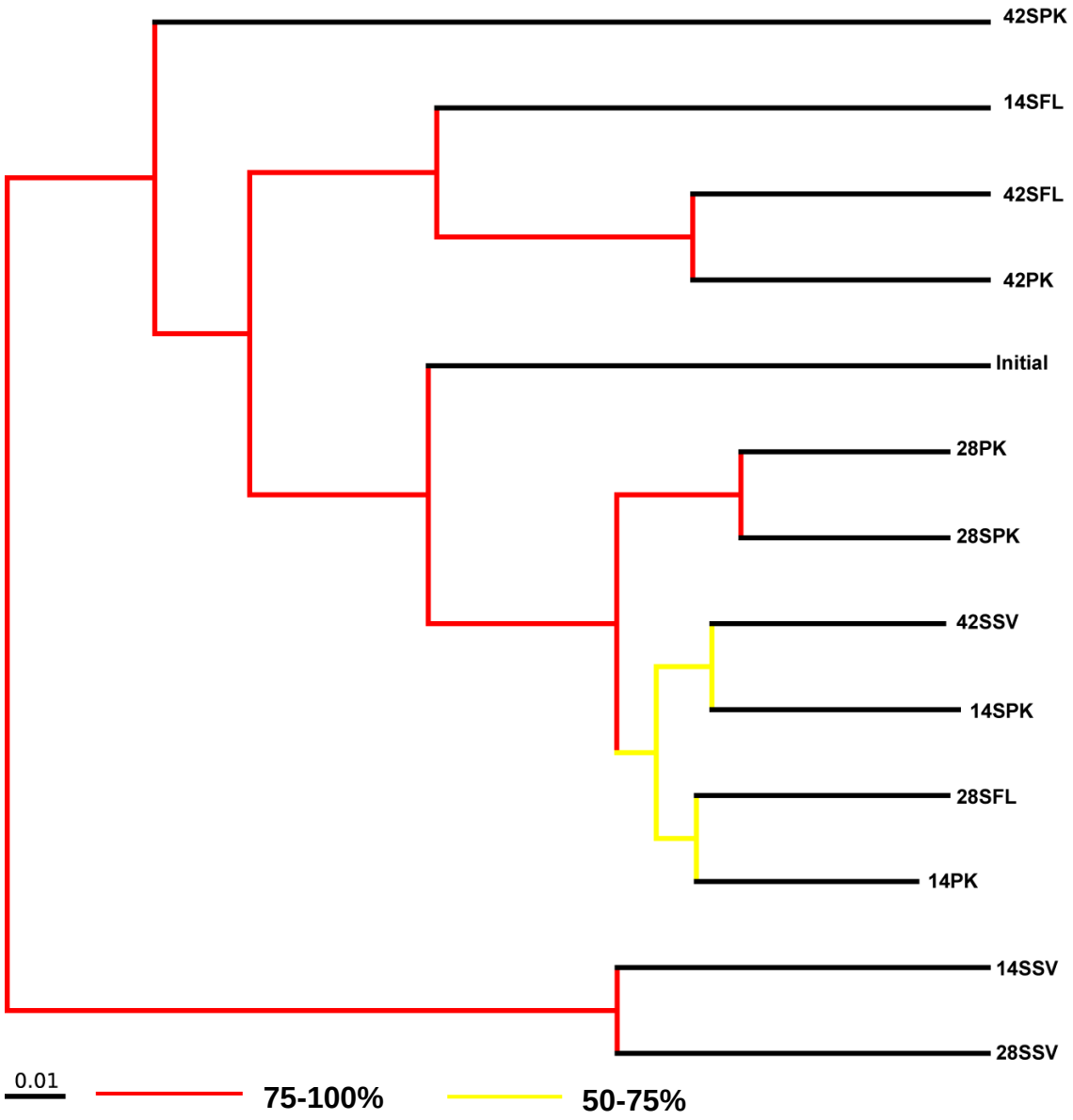


Figure 3.6: Differences in the composition of the microbial assemblages between *F. heteroclitus* Feeding Study samples. Cluster analysis with jackknife support based on weighted UniFrac distances and UPGMA clustering. Red-colored nodes had 75-100% support. Yellow-colored nodes had 50-75% support. Weighted UniFrac distances were calculated from the distribution OTUs defined at a 97% similarity level. 14, 28, and 42 indicate sampling day. SPK fish were fed sterilized plankton, PK fish were fed unsterilized plankton, SFL fish were fed sterilized Formula 1, and SSV fish were fed sterilized mixed seaweed flake.

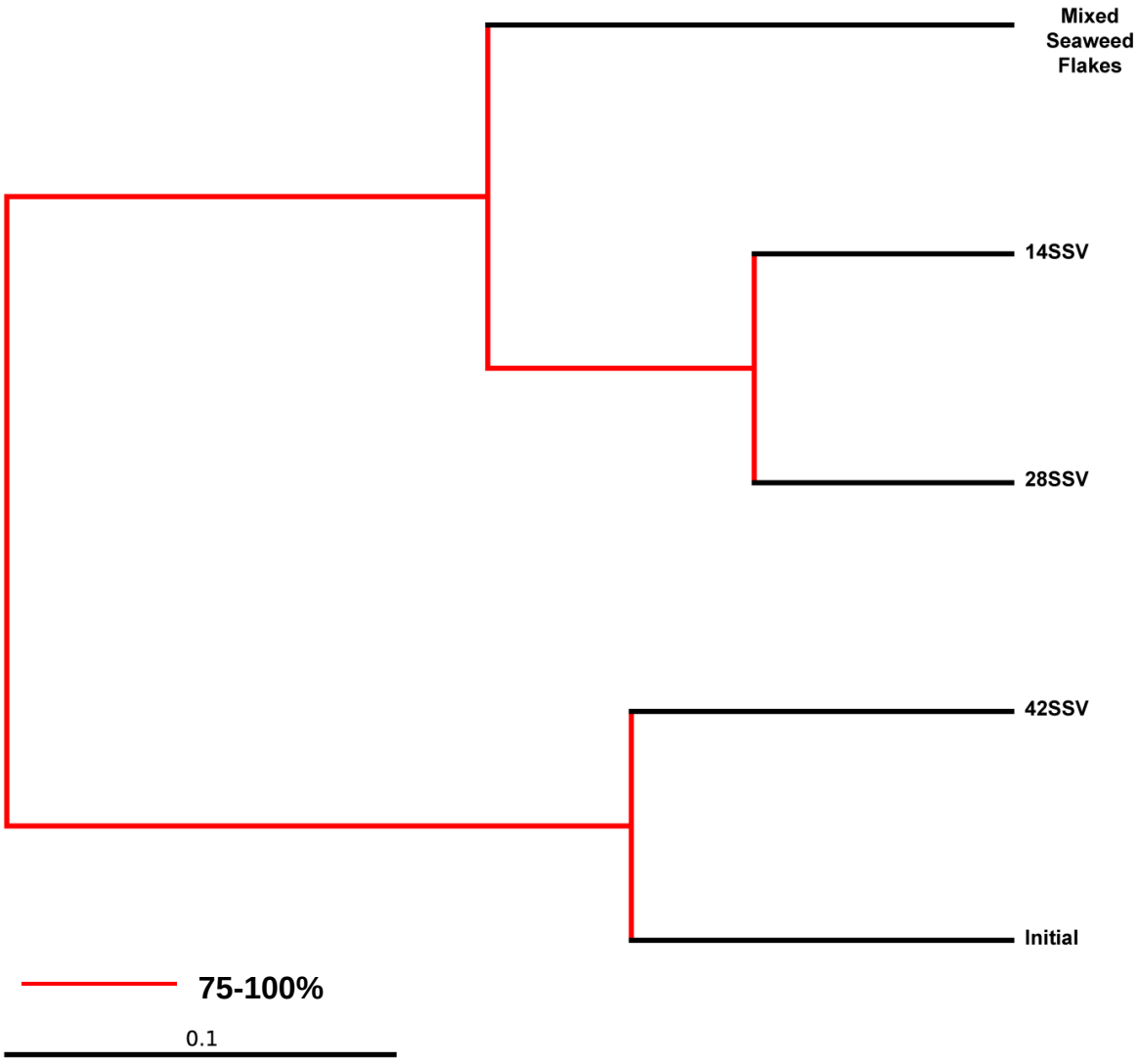


Figure 3.7: Bootstrapped tree of jackknifing analysis based on weighted UniFrac and UPGMA clustering for all *F. heteroclitus* initial, mixed seaweed flake-fed fish, and mixed seaweed flake diet. Red-colored nodes had 75-100% support. Weighted UniFrac distances were calculated from the distribution OTUs defined at a 97% similarity level. 14, 28, and 42 indicate sampling day. SSV fish were fed sterilized mixed seaweed flake.

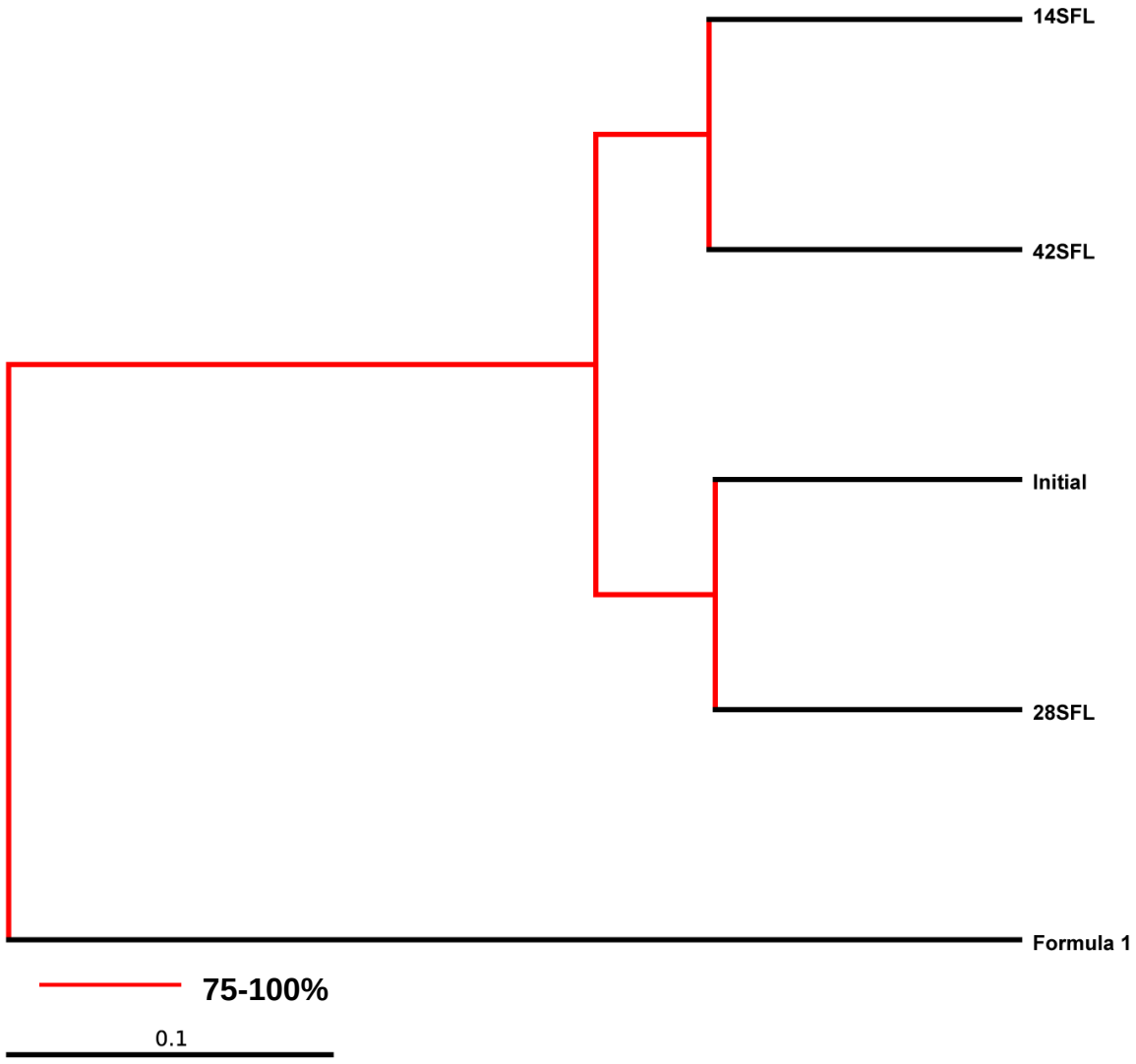


Figure 3.8: Bootstrapped tree of jackknifing analysis based on weighted UniFrac and UPGMA clustering for all *F. heteroclitus* initial, Formula 1-fed fish, and Formula 1 diet.
Red-colored nodes had 75-100% support. Weighted UniFrac distances were calculated from the distribution OTUs defined at a 97% similarity level. 14, 28, and 42 indicate sampling day. SFL fish were fed sterilized Formula 1.

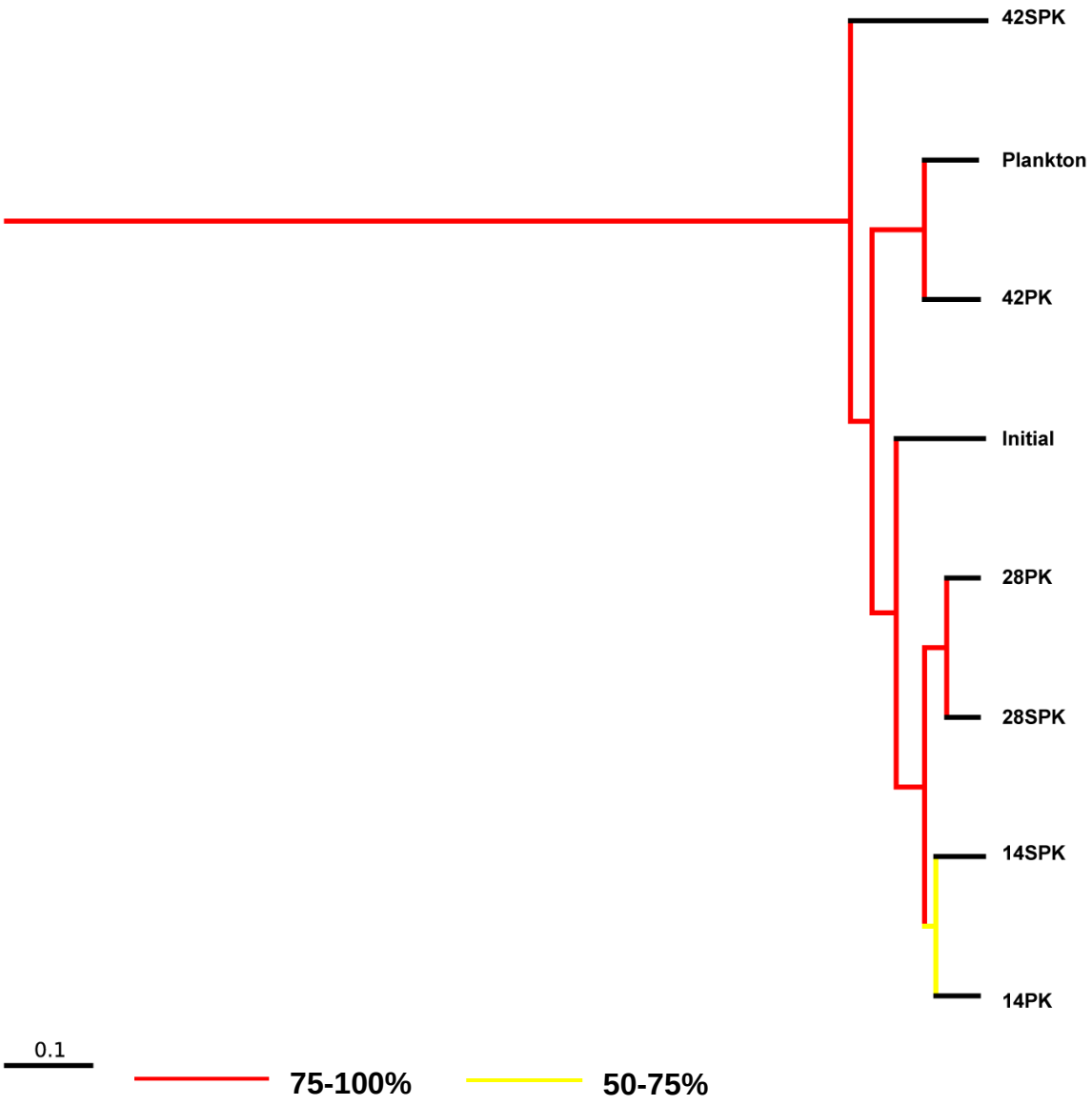


Figure 3.9: Bootstrapped tree of jackknifing analysis based on weighted UniFrac and UPGMA clustering for all *F. heteroclitus* initial, sterilized and unsterilized plankton-fed fish, and plankton diet.
Red-colored nodes had 75-100% support. Yellow-colored nodes had 50-75% support. Weighted UniFrac distances were calculated from the distribution OTUs defined at a 97% similarity level. 14, 28, and 42 indicate sampling day. SPK fish were fed sterilized plankton and PK fish were fed unsterilized plankton.

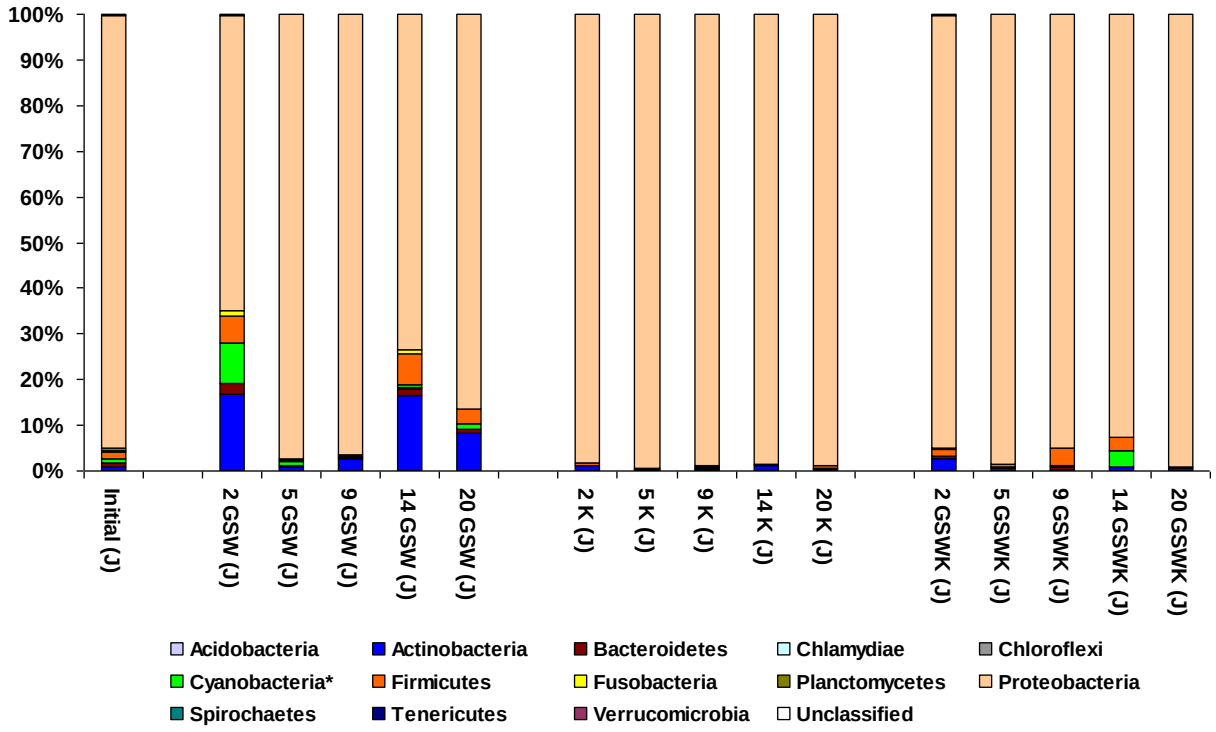
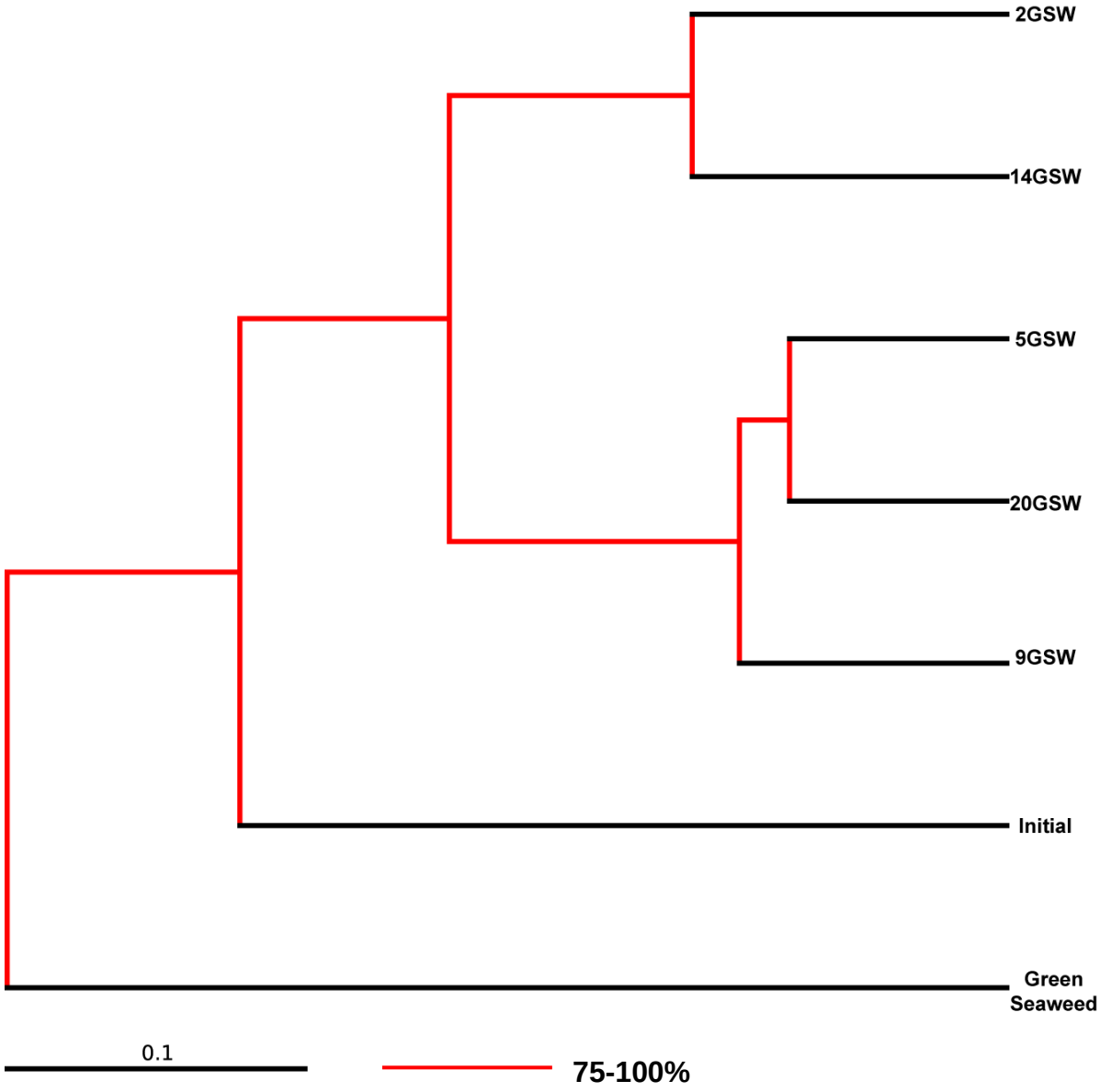


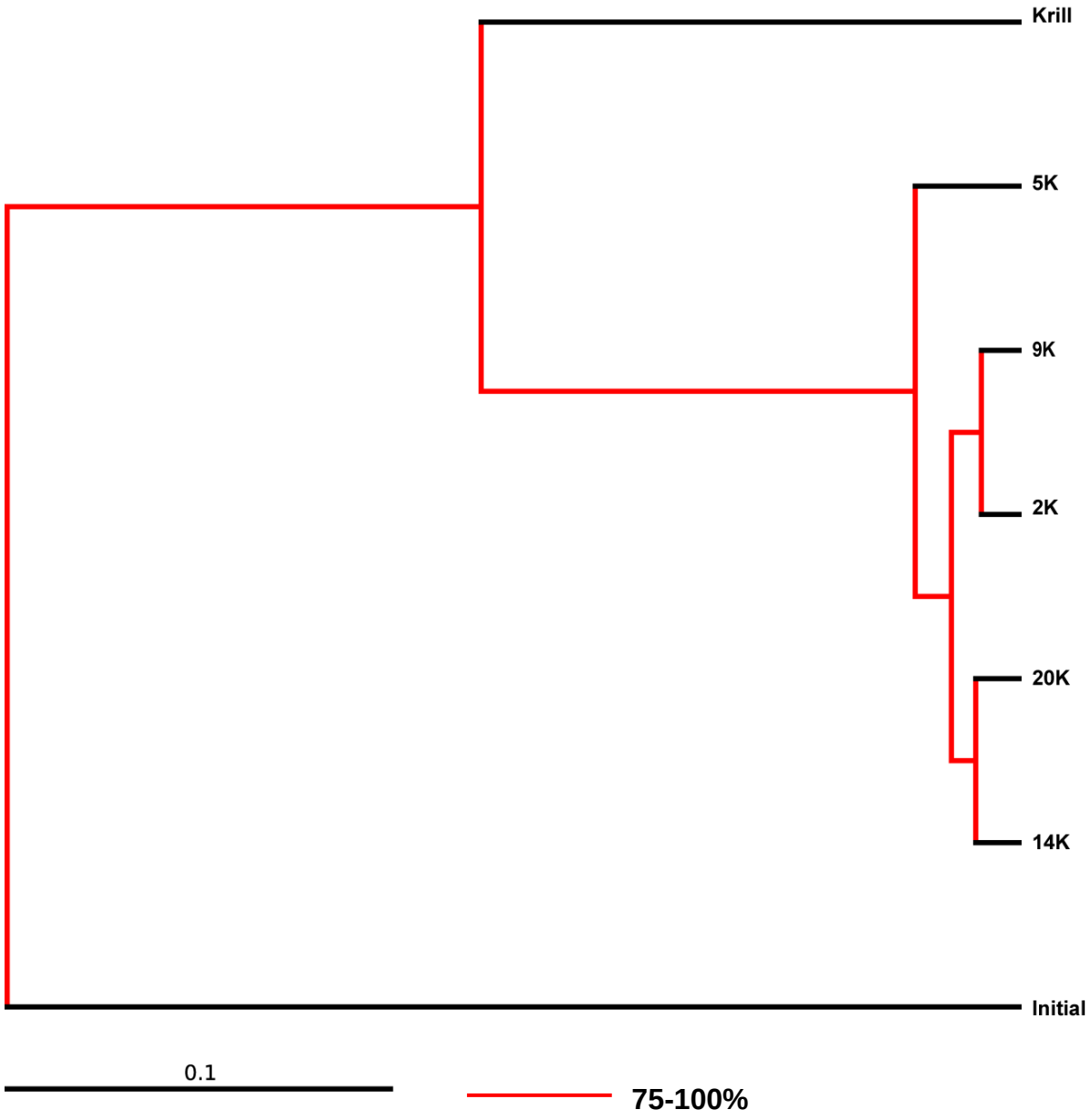
Figure 3.10: Composition of the Bacterial assemblage (at the phylum level, % of ribotypes) retrieved from samples of the juvenile *L. rhomboides* Feeding Study samples

Percent composition of phyla within the guts of juvenile *L. rhomboides* from Day 0, 2, 5, 9, 14, and 20. GSW samples were fed green seaweed. K fish were fed krill. GSWK fish were fed equal parts green seaweed and krill. *Ribotypes binned as Cyanobacteria were likely chloroplasts.

a)



b)



c)

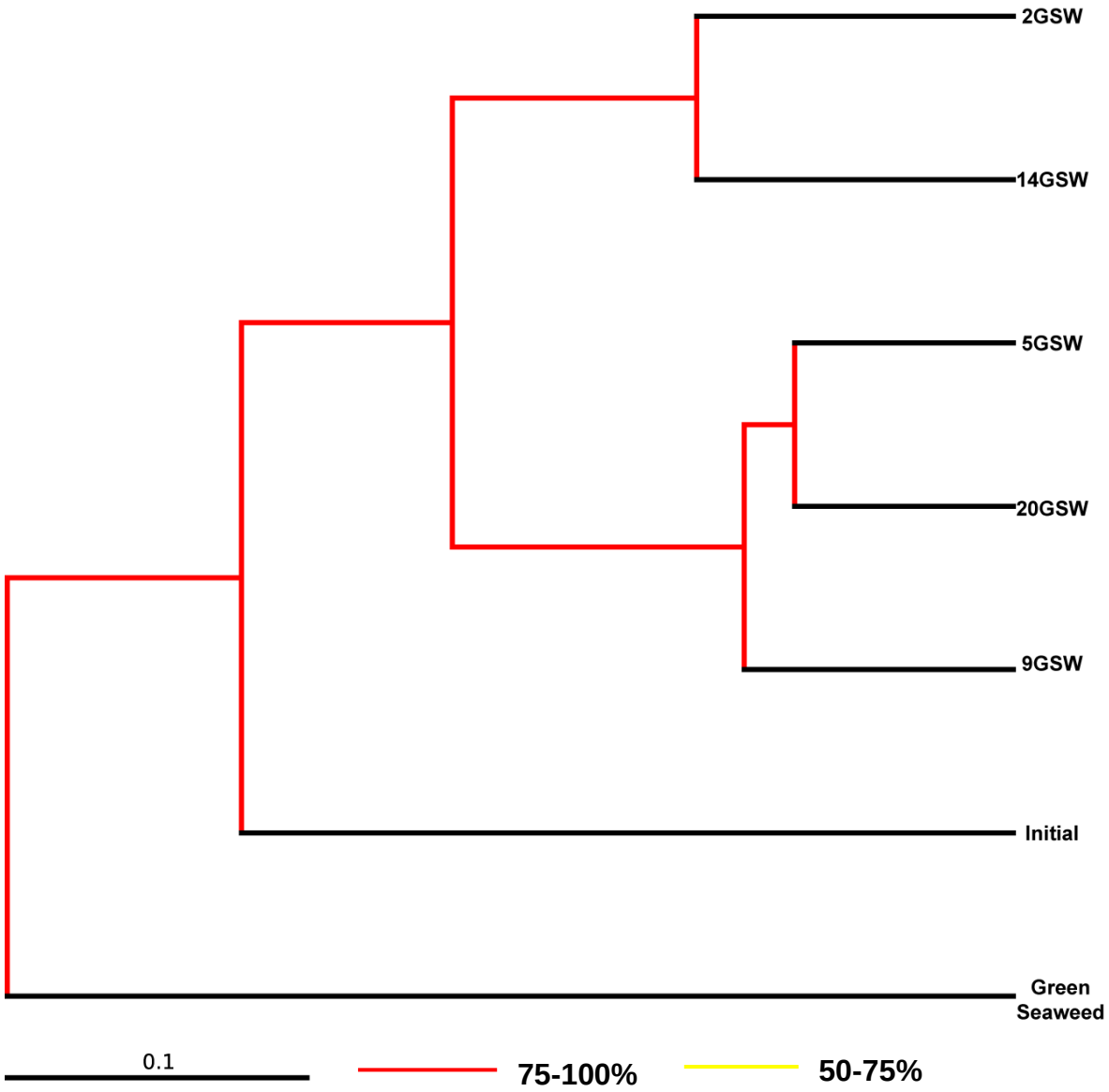


Figure 3.11: Bootstrapped tree of jackknifing analysis based on weighted UniFrac and UPGMA clustering for a) juvenile *L. rhomboides* initial fish, GSW-fed fish, and green seaweed diet, b) juvenile *L. rhomboides* initial fish, K-fed fish, and krill diet, and c) juvenile *L. rhomboides* initial fish, GSWK-fed fish, and green seaweed and krill diet.

Red-colored nodes had 75-100% support. Yellow-colored nodes had 50-75% support. Weighted UniFrac distances were calculated from the distribution OTUs defined at a 97% similarity level. K fish were fed krill, GSW fish were fed green seaweed diet, and GSWK fish were fed green seaweed and krill diet.

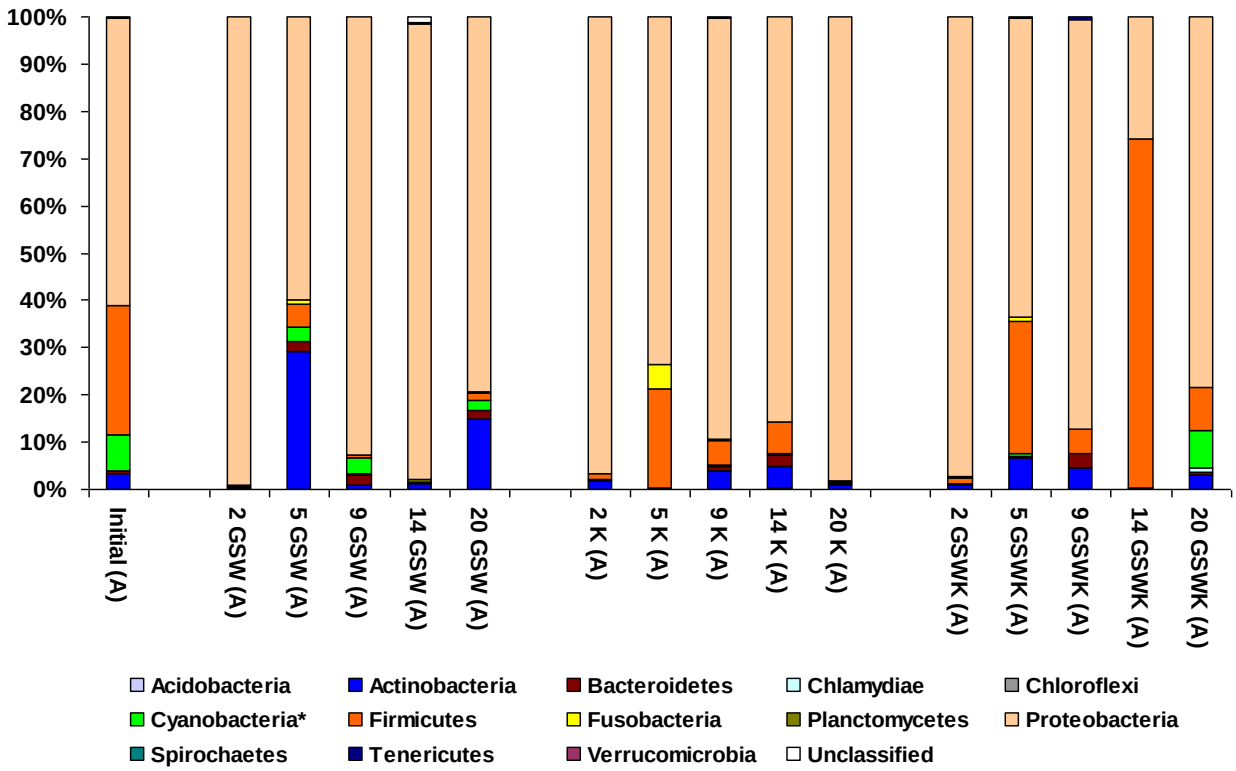
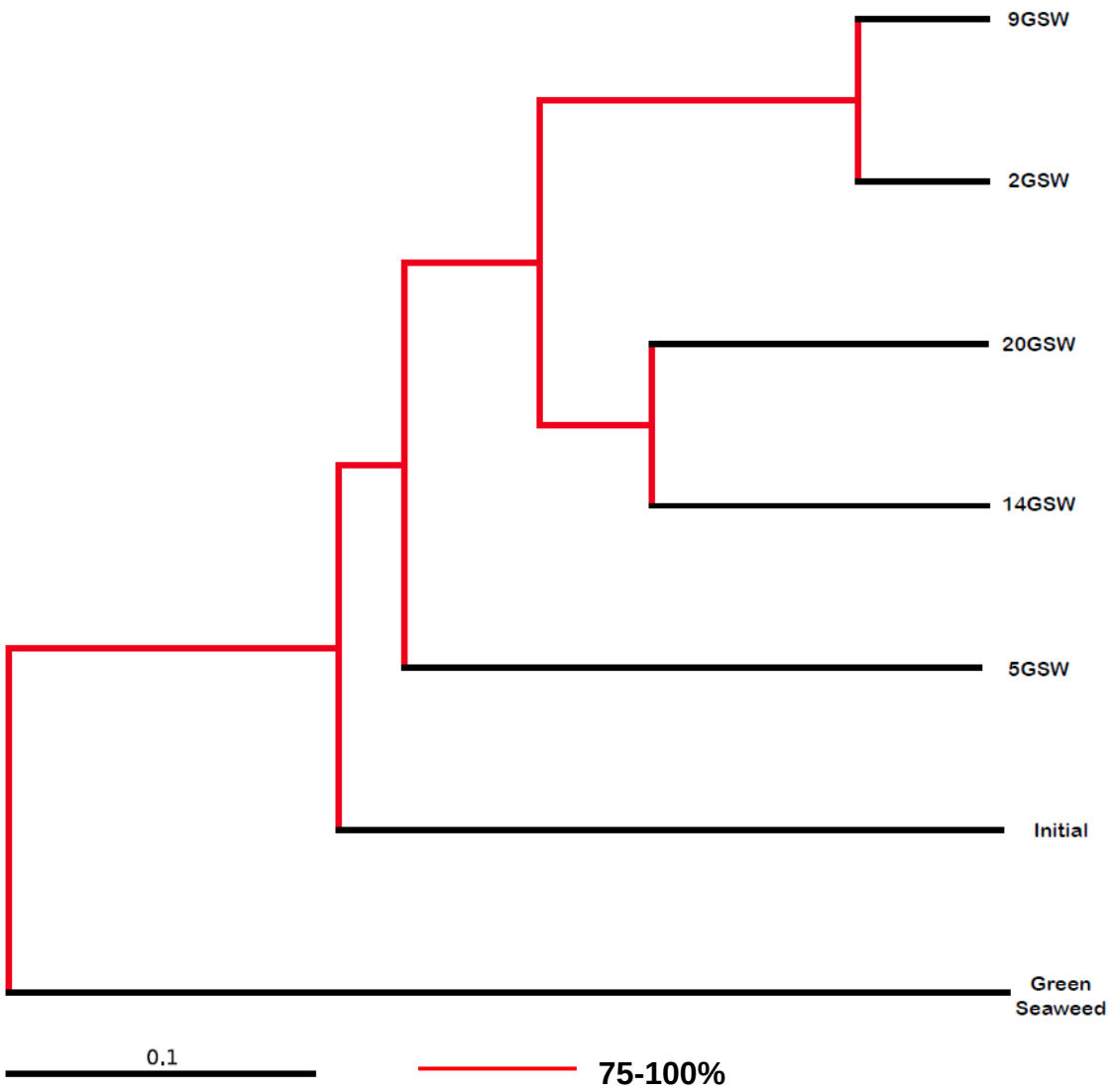
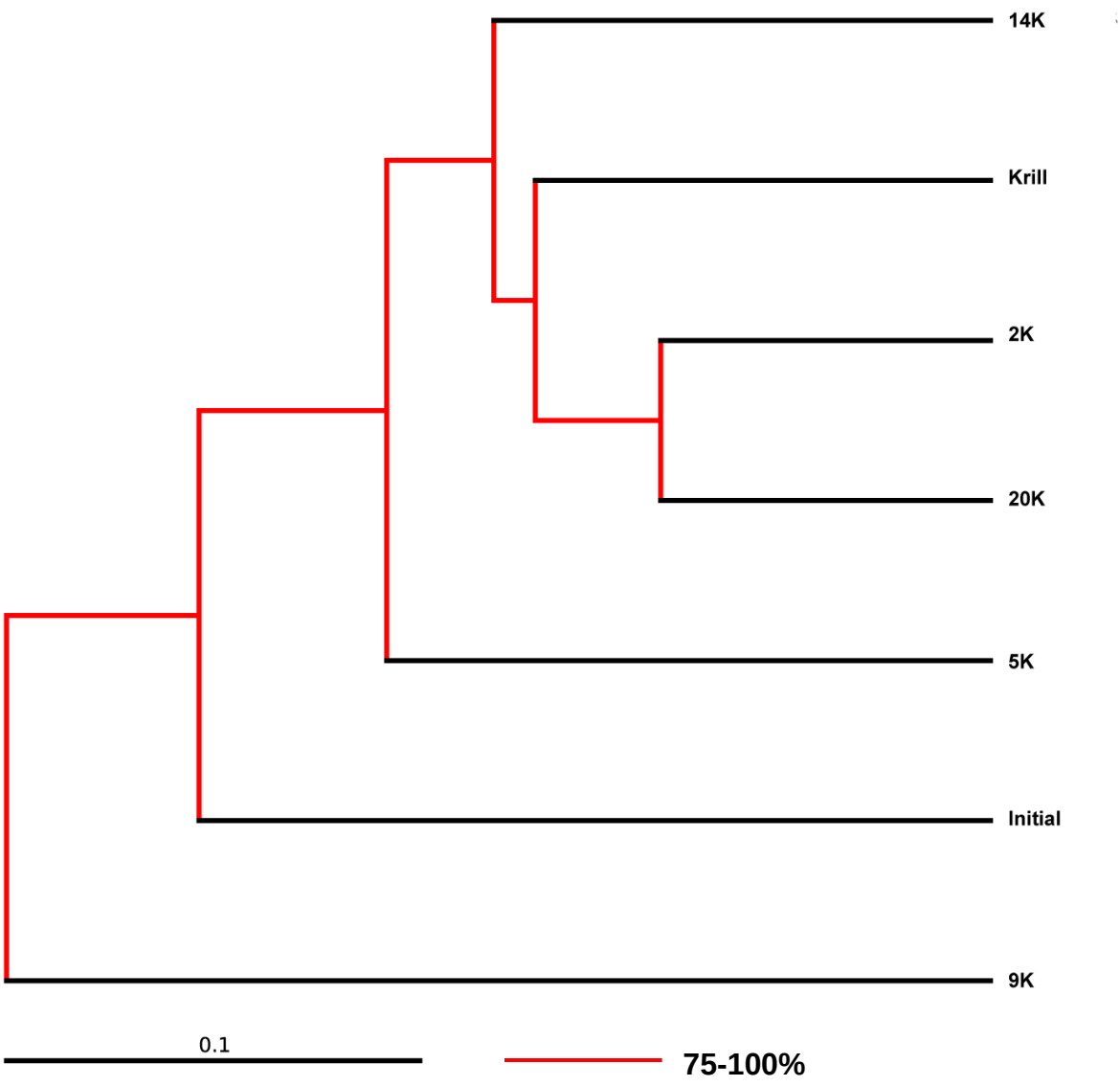


Figure 3.12: Composition of the Bacterial assemblage (at the phylum level, % of ribotypes) retrieved from samples of the adult *L. rhomboides* Feeding Study samples
Percent composition of phyla within the guts of adult *L. rhomboides* from Day 0, 2, 5, 9, 14, and 20. GSW samples were fed green seaweed. K fish were fed krill. GSWK fish were fed equal parts green seaweed and krill. *Ribotypes binned as Cyanobacteria were likely chloroplasts.

a)



b)



c)

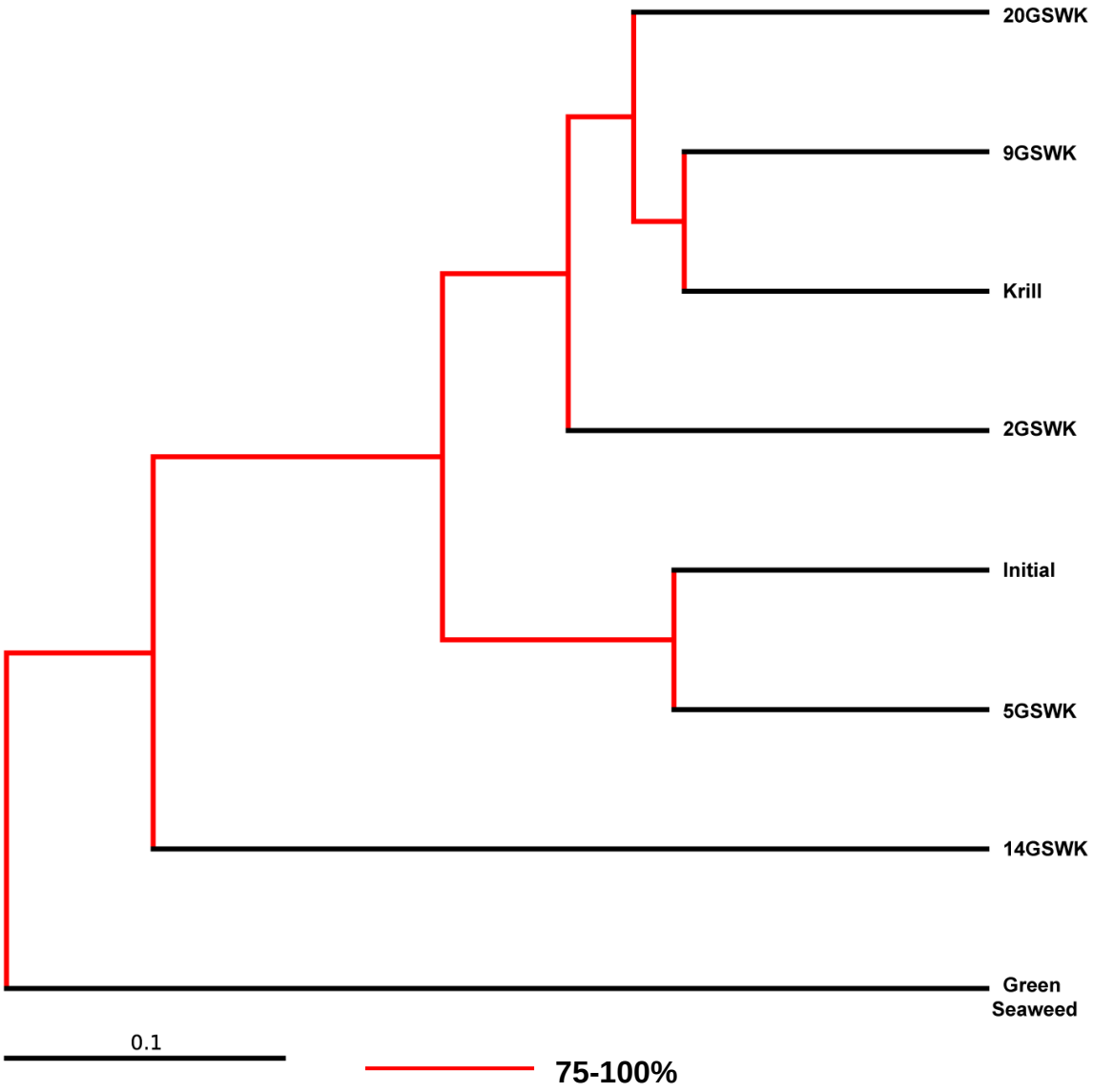


Figure 3.13: Bootstrapped tree of jackknifing analysis based on weighted UniFrac and UPGMA clustering for a) adult *L. rhomboides* initial fish, GSW-fed fish, and green seaweed diet, b) adult *L. rhomboides* initial fish, K-fed fish, and krill diet, and c) adult *L. rhomboides* initial fish, GSWK-fed fish, and green seaweed and krill diet. Red-colored nodes had 75-100% support. Weighted UniFrac distances were calculated from the distribution OTUs defined at a 97% similarity level. K fish were fed krill, GSW fish were fed green seaweed diet, and GSWK fish were fed green seaweed and krill diet.

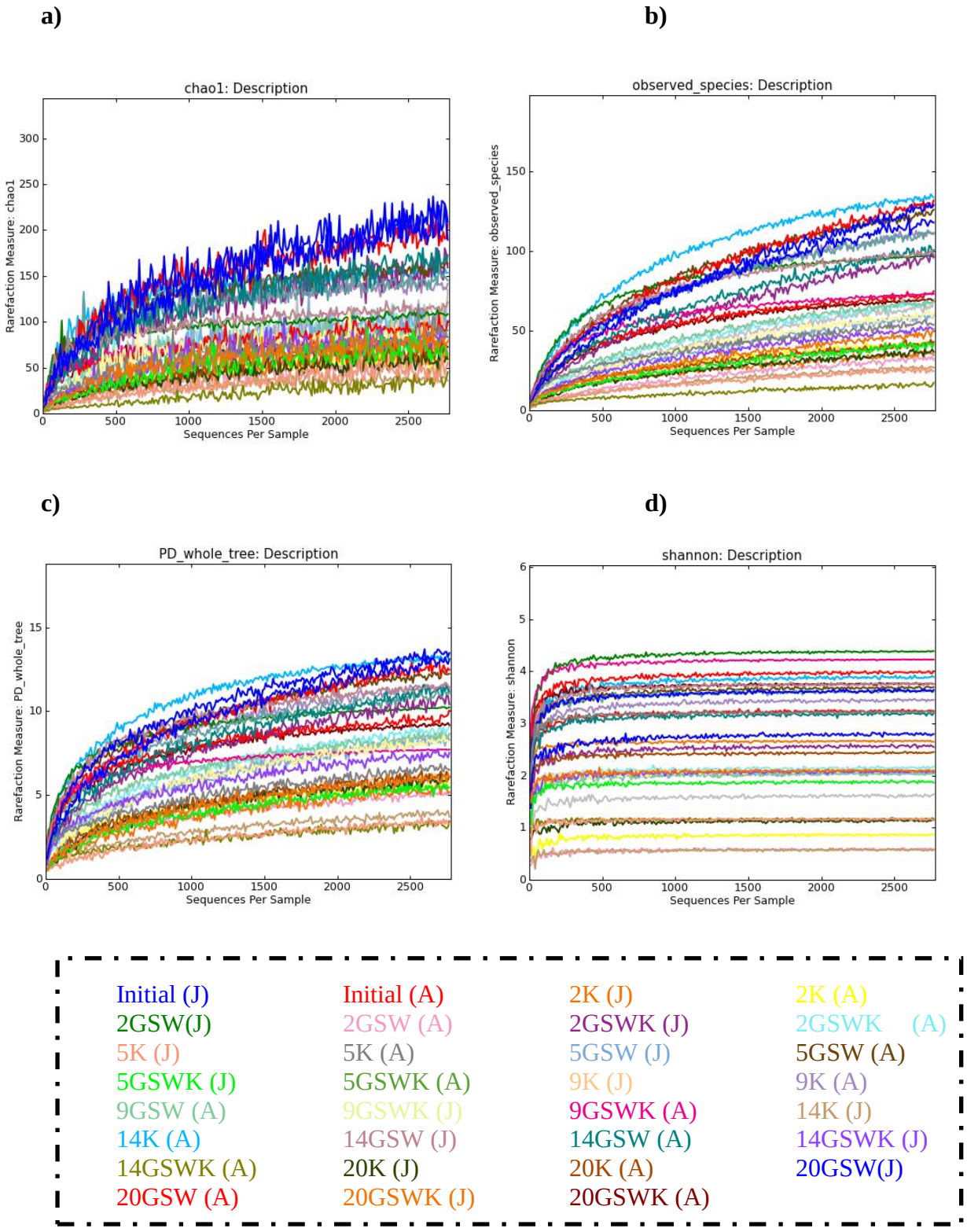


Figure 3.14: Rarefaction curves of alpha-diversity metrics of a) chao1, b) Observed Species, c) PD Whole Tree, and d) Shannon index for *L. rhomboides* Feeding Study samples.

Table 3.4: Alpha Diversity Metrics of Chao1, Observed Species, Phylogenetic Diversity (PD) Whole Tree, Shannon index for the *L. rhomboides* Feeding Study.

Indices were calculated for each sample using 2700 sequences/sample GSW samples were fed seaweed flake. K samples were fed krill. GSWK samples had a diet of equal parts krill and seaweed.

Sample	Sequences/ Sample	Chao1	Observed Species	PD Whole Tree	Shannon
Initial (J)	2700	193	120	13.0	3.65
Initial (A)	2700	183	124	11.7	3.97
2 GSW (J)	2700	102	93.4	9.63	4.36
5 GSW (J)	2700	103	60.6	7.84	2.07
9 GSW (J)	2700	128	67.6	8.32	1.64
14 GSW (J)	2700	116	98.5	10.9	3.78
20 GSW (J)	2700	196	125	13.0	2.84
2 GSW (A)	2700	57.7	33.6	5.10	0.58
5 GSW (A)	2700	158	124	11.7	3.70
9 GSW (A)	2700	93.0	67.6	7.84	2.04
14 GSW (A)	2700	177	102	11.2	3.21
20 GSW (A)	2700	95.1	77.5	9.30	3.29
2 K (J)	2700	76.6	46.6	5.88	2.09
5 K (J)	2700	40.2	23.7	3.49	1.16
9 K (J)	2700	83.0	41.6	6.15	2.12
14 K (J)	2700	36.0	26.8	4.02	0.58
20 K (J)	2700	57.8	39.0	5.55	1.15
2K (A)	2700	51.9	40.3	5.53	0.86
5 K (A)	2700	87.4	52.6	6.31	3.19
9 K (A)	2700	137	110	11.3	3.45
14K (A)	2700	151	131	12.7	3.87
20 K (A)	2700	67.7	42.7	6.01	2.46
2 GSWK (J)	2700	108	67.9	8.72	2.14
5 GSWK (J)	2700	59.5	38.1	5.18	1.86
9 GSWK (J)	2700	92.8	61.0	7.97	2.08
14 GSWK (J)	2700	78.3	51.1	7.34	2.09
20 GSWK (J)	2700	62.6	36.6	4.85	2.61
2 GSWK (A)	2700	148	91.8	10.5	2.58
5 GSWK (A)	2700	133	109	10.8	3.65
9 GSWK (A)	2700	73.3	69.8	7.55	4.21
14 GSWK (A)	2700	33.6	16.4	3.06	1.18
20 GSWK (A)	2700	74.1	69.5	8.93	3.95

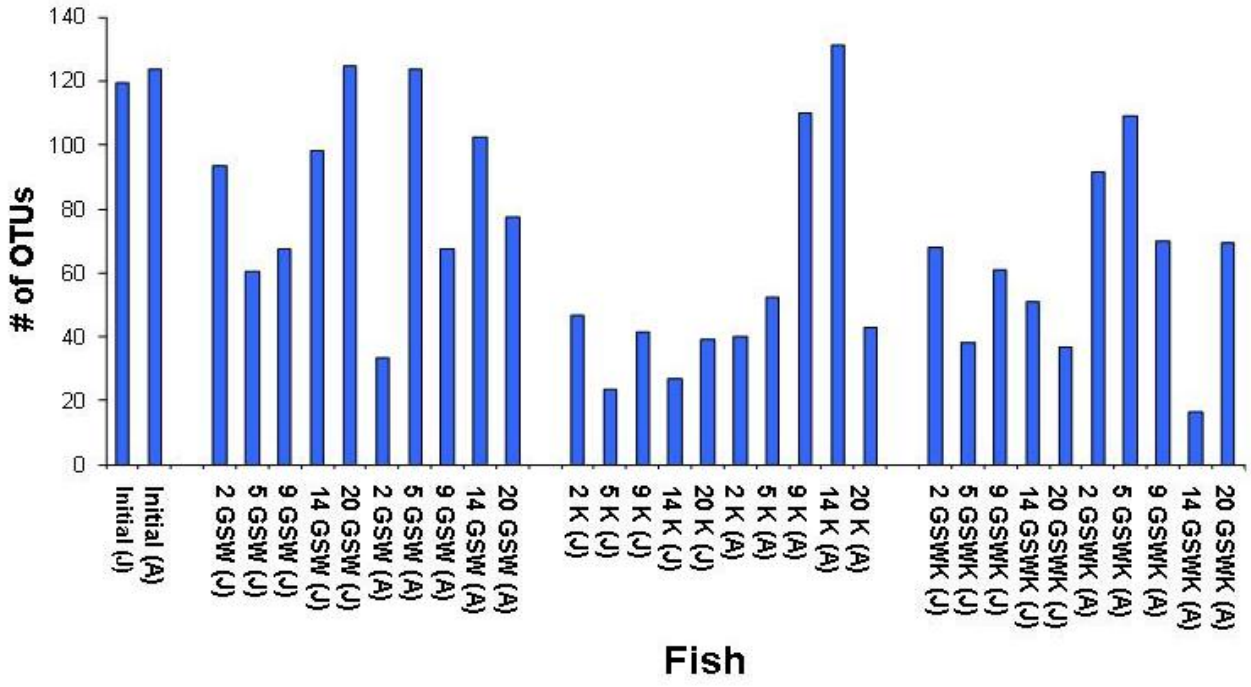


Figure 3.15: Observed Species metric for *L. rhomboides* Feeding Study fish.

CHAPTER4

PUSHING THE LIMIT?

EXAMINING THE EFFECTS OF INCREASED WATER TEMPERATURE ON THE GUT MICROFLORA OF *FUNDULUS HETEROCLITUS* AND *LAGODON RHOMBOIDES*[1]

[1] Givens, C.E. and J.T. Hollibaugh. To be submitted to FEMS Microbiology Ecology.

ABSTRACT

Vibrio species are often the dominant bacteria associated with marine fish and are commonly found in the gut microflora of both farmed and wild fish. Our previous work indicated that the gut microflora of the mummichog (*Fundulus heteroclitus*) and pinfish (*Lagodon rhomboides*) are dominated by γ-Proteobacteria ribotypes (57% and 41% respectively), mainly members of the family Vibrionaceae (34% and 41% of all bacteria), including some that are closely related to potential pathogens. We used the distribution of ribotypes in clone libraries and quantitative PCR (qPCR) assessments of the relative abundance of *Vibrio* spp. and *V. vulnificus* within the gut microflora community to assess whether environmental temperature affected the occurrence of *Vibrio* species within fish guts. Clone libraries from the *L. rhomboides* temperature study were dominated by Vibrionaceae ribotypes; including several that are documented fish pathogens. The presence of potential pathogens in fish at the start of the study, in addition to the observation that several fish became ill and/or died during the study, suggests that these fish were stressed and more susceptible to gut pathogens. Sequences retrieved from *F. heteroclitus* indicate that the *Vibrio* spp. within the gut microflora are dominated by bacteria closely related (98.1-100%) to *V. vulnificus* (212 clones, 47%) with additional contributions from *V. ponticus* (105 clones, 23%). Changes in relative abundance of these two ribotypes indicate that there may be shifts within the *Vibrio* spp. community with time and temperature. *V. ponticus* ribotypes peaked non-synchronously to *V. vulnificus* ribotypes, suggesting an interaction between dominant *Vibrio* species. qPCR analysis of *F. heteroclitus* gut microbiomes suggest an overall trend of increased *Vibrio* spp. and *V. vulnificus* abundance with temperature.

INTRODUCTION

Global climate change will impact all ecosystems and may have serious repercussions for animal, human and environmental health. At the time the *Climate Change 2007* report was published, 11 of the last 12 years (1995-2006) were ranked among the top 12 warmest years since estimates of annual mean of global surface temperature were available (IPCC 2007; Parry et al. 2007). Climatologists predict a 1.8 to 4.0 ° C increase in mean temperatures over the next 100 years (Solomon et al. 2007), with trends of increased temperature varying regionally. This temperature trend extends to the global ocean. Although land regions seem to be warming more quickly than the ocean, the upper 3000 meters of the ocean has warmed by approximately 0.4 °C since 1950 (Barnett et al. 2001; Levitus et al. 2001).

Small increases in water temperature can have serious impacts on aquatic organisms. Elevated water temperatures may cause shifts in the distribution and abundance of various organisms, in addition to causing changes in ice cover, salinity, stratification, oxygen and nutrient levels and circulation (IPCC 2007; Parry et al. 2007). The abundance of *Vibrio* spp. in coastal waters increases seasonally in response to warmer water temperatures, and is greatest when the water temperatures are between 20 and 30 °C (Tantillo et al. 2004). As a result of this temperature preference, most outbreaks of pathogenic *Vibrio* spp. occur during summer in temperate or subtropical regions such as the Gulf Coast of the United States (DePaola et al. 2000). However, recent reports of pathogenic *Vibrio* spp. outbreaks in new locales seem to indicate that their ranges are expanding and incidences of virulence are increasing as a consequence of warming oceans. These reports include *V. vulnificus* outbreaks in Israel (Paz et al. 2007)

and Denmark (Dalsgaard et al. 1996). *V. parahaemolyticus* outbreaks in Spain (Martinez-Urtaza et al. 2008), Chile (González-Escalona et al. 2005), and Alaska (McLaughlin et al. 2005) have occurred at locales previously unaffected by this pathogen. *Vibrio* spp. infections are increasing and appear to follow regional temperature trends with outbreaks correlating with periods of unusually warm weather (Baker-Austin et al. 2010).

Vibrio species in general are often the dominant bacteria associated with marine fish and are common members of the gut microflora in both farmed and wild fish (MacFarlane et al. 1986; Cahill 1990; Sakata 1990; Blanch et al. 1997; Martin-Antonio et al. 2007; Ward et al. 2009a). De Paola et al. (1994) isolated *V. vulnificus* from the intestines of 18 different fish species from the Gulf of Mexico, suggesting that *V. vulnificus* is a common member of the fish gut microbiome. This same study also found that *V. vulnificus* densities in sheepshead (*Archosargus probatocephalus*) intestines were 2-4 orders of magnitude more abundant than in oysters and sediment collected at the same time. The concentration of *V. vulnificus* in fish guts was five orders of magnitude greater than in the surrounding seawater (DePaola et al. 1994).

Our previous work indicated that the gut microflora of the mummichog (*Fundulus heteroclitus*) and pinfish (*Lagodon rhomboides*) are dominated by γ-Proteobacteria ribotypes (57% and 41% respectively), mainly members of the family Vibrionaceae (34% and 41% of all Bacteria), including some that are closely related to potential pathogens. Thus, these two species were ideal for use in experiments to assess how changing environmental conditions, specifically temperature, might affect the occurrence of potentially pathogenic bacteria in fish intestines. Since environmental monitoring has

indicated a correlation between the occurrence of pathogenic Vibrios and warmer temperatures, we hypothesized an increase in the relative abundance of potential pathogens within the gut in response to elevated water temperature. We used the distribution of ribotypes in clone libraries and quantitative PCR (qPCR) to assess the relative abundance of *Vibrio* spp. and *V. vulnificus* within the gut microflora community to test this hypothesis.

METHODS

These studies were completed in compliance of Animal Use Protocol (AUP) #2008-10017 approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC).

Lagodon rhomboides *Collection*

L. rhomboides (n=40) were collected by trawl from the Gulf of Mexico (29° 52' N 84° 29' W) near the Florida State University Coastal Marine Laboratory (St. Teresa, FL), transported to the University of Georgia campus, and held in quarantine in a pre-cleaned 800-gallon tank at 15-18 °C for 30 days. Fish body length ranged between 80 and 130 mm and according to our previous size classifications (see Chapter 2 and 3) were considered to be old juveniles and young adults. Following quarantine, four fish were selected randomly and sacrificed to establish the initial composition of the gut microflora. The remaining 36 fish were divided among 6 100-gallon tanks kept at 18 °, 25 ° or 32 °C (2 tanks per temperature). Tanks were equipped with Power Filters with two filter cartridges and recirculated water at a rate of 125 gallons per hour. The study

lasted a total of 12 days with fish sampled every four days. All fish were fed a diet of San Francisco Bay Brand Freeze-Dried Krill that was sterilized at 10 kGy with a Cobalt-60 source gamma irradiator at the University of Georgia Center for Applied Isotope Studies (Athens, GA). Food not consumed within 15 minutes of feeding was removed from the tank. Tank salinity was kept constant and nitrite, nitrate, ammonia, and pH levels were monitored.

Two fish were taken from each tank at each time point. All specimens were euthanized with tricaine methanesulfonate (MS-222; Sigma). The mid- to hind-gut region of the intestine was removed, sliced open, and placed in a PowerBead tube (MoBio; Solana Beach, CA). DNA extraction was completed using the MoBio PowerSoil DNA Extraction Kit.

All fish were outwardly healthy before beginning the experiment. Within the first three days of the study, four fish from the 32 °C tanks developed skin lesions, suffered skin degradation around their lower jaws, developed red fins, and died. Another 6 fish (two from each temperature treatment) developed red streaking through pectoral and tail fins. One of those six fish (from the 32 °C treatment) also developed skin lesions, which were sampled by swabbing.

Fundulus heteroclitus *Sample Collection*

F. heteroclitus (n=175) were collected from Sapelo Island, Georgia, and placed into quarantine in a pre-cleaned 800-gallon tank at 15-18 °C for 30 days. Following quarantine, five fish were selected randomly and sacrificed to establish the initial composition of the gut microflora. The remaining 170 fish were divided randomly

among 12 ten-gallon tanks maintained at four different temperatures of 20 °, 24 °, 28 °, 32 °C (3 tanks per temperature). Tanks were equipped with Power Filters and recirculated as mentioned above. The study lasted a total of 28 days with fish sampled every 4 days. Fish husbandry, tank maintenance, and fish dissections and DNA extractions were completed as described in the previous study.

16S rRNA Sequencing and Analysis

Extracted DNA from *L. rhomboides* gut samples was amplified using Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare) with the Bacteria-specific 16S rRNA primers 27F/1492R (Lane 1991a) with the following PCR conditions: initial denaturation at 95 °C for 5 minutes; 35 cycles of: denaturation at 95 °C for 45 seconds, annealing at 62 °C for 30 s, and extension at 72 °C for 1 minute; finishing with a final extension at 72 °C for 45 minutes. Extracted DNA from *F. heteroclitus* gut samples was amplified using Bacteria-specific 16S rRNA primer 27F and *Vibrio* spp. 16S rRNA primer 680R (Lane 1991a; Thompson et al. 2004b) and cycling parameters previously described (Thompson et al. 2004b). Amplified DNA was electrophoresed on a 1% agarose gel, bands of the expected product size were excised, and the DNA was extracted and purified using QIAGEN QIAquick gel extraction kits. Gel-purified DNA was cloned with the TOPO TA cloning kits (Invitrogen) using the pCR 4.0-TOPO TA vector and competent *E. coli* cells. Clones were selected randomly and sequenced using the 27F primer by Genewiz (South Plainfield, NJ). Clone libraries for *L. rhomboides* aimed to analyze the total bacteria community (Bacteria 16S rRNA); however, *F. heteroclitus* libraries only considered the *Vibrio* spp. 16S rRNA community. The previous clone

libraries for *L. rhomboides* included Vibronaceae ribotypes that were further identified (at >97% similarity) to both *Photobacterium* spp. and *Vibrio* spp. ribotypes. The *Vibrio* spp. 16S rRNA primers do not target *Photobacterium* spp. and we aimed to gauge the effects of increased water temperature on all Vibrionaceae ribotypes. This was not an issue for *F. heteroclitus* clone libraries since all Vibrionaceae ribotypes in the previous libraries were binned as *Vibrio* spp. ribotypes. All sequences were checked for chimeras using the Bellerophon server (Huber et al. 2004). Sequences were then assigned to ribotypes by BLAST (Johnson et al. 2008) against the non-redundant nucleotide database (NCBI GenBank), aligned with ClustalW (Larkin et al. 2007), and phylogenetic trees were constructed using Geneious Pro 4.8.5 (Biomatters Ltd; Auckland, New Zealand) and MEGA 5.05(Tamura et al. 2011).

Quantitative PCR

Quantitative PCR (qPCR) was completed using a BioRad iCycler (Hercules, CA). Reactions were run in 25 μL volume with 1 X iQ SYBR Green Supermix (BioRad Laboratories; Hercules, CA), forward and reverse primers, nuclease free water, and 2 μL of template DNA. The primers Bact1369F and Prok1492R (Suzuki et al. 2000b) were used for qPCR of 16S rRNA genes, with cycling parameters previously as described (Buchan et al. 2009a). Primers 567F and 680R were used for *Vibrio*-specific qPCR of 16S rRNA genes, with published cycling parameters (Thompson et al. 2004b). Primers F-vvh and R-vvh (Panicker et al. 2004) were used for qPCR of *V. vulnificus vvh* genes., (Panicker et al. 2004). All reactions were run in triplicate with standards ranging from 10^1 to 10^7 copies per μL. Since many bacteria have more than one copy of the 16S

rRNA gene per genome, the data were adjusted by dividing the number of gene copies per reaction (copies/μL) by an average number of 16S rRNA genes per genome (3.94 for bacteria and 9.75 for Vibrios) (Klappenbach et al. 2001a; Lee et al. 2009b). The average 16S rRNA gene copy number was determined by evaluating the operon copy number of ribotypes identified in both 16S rRNA and Vibrio 16S rRNA clone libraries.

Statistical Analysis

Kruskal-Wallis one way analysis of variance, and pairwise Wilcoxon rank sum tests were performed in R (R Core Team 2009) using the vegan statistical package (Oksanen et al. 2009) to test whether differences in relative abundances measured by quantitative PCR were significant among temperature treatments.

RESULTS

Temperature Study I –L. rhomboides

A total of 270 clones were sequenced, of which 29 were discarded because they were of poor quality or chimeric. Of the remaining 241 sequences, 52% were γ-Proteobacteria (Figure 4.1). These γ-Proteobacteria were primarily either *Photobacterium* spp. or *Vibrio* spp., which contributed 27% and 23%, respectively, to the total bacteria sequences retrieved. *Photobacterium* spp. ribotypes were >97% similar to *P. mandapamensis* and *P. damselae* subsp. *piscida*. *Vibrio* spp. ribotypes were >97% similar to *V. ichthyoenteri, V. ponticus*, and *V. harveyi*.

Most (58%) of the *Photobacterium* spp. and *Vibrio* spp. ribotypes were retrieved from fish in the 18 °C treatments. The gut microbiome of fish from the 32 °C treatment

contained primarily *P. mandapamenis* ribotypes (47%). Ribotypes (>97% similarity cutoff) retrieved from the guts of fish in the 25 °C treatments were assigned to *Methylobacterium jeotgali* (29%), uncultured Burkholderiales (29%), and *Saccharococcus* sp. (24%). Ribotypes from the lesions on fish from the 32 °C treatment were classified as *V. harveyi* (64%), Oxalobacteraceae (18%), *Methylobacterium jeotgali* (14%) and uncultured Clostridia (4%).

Vibrio spp. ribotypes accounted for approximately 50% of the Bacteria ribotypes in the samples taken at the beginning of the experiment (Figure 4.2). Although the relative abundance of Vibrio ribotypes varied over the time-course of the experiment, the differences between time points and treatments were not statistically significant.

Temperature Study II – Vibrios in F. heteroclitus

A total of 550 sequences were retrieved from *F. heteroclitus* gut microbiomes. Twelve percent of those sequences were discarded because they were of poor quality. Six percent of the remaining sequences were identified as *Enterovibrio* spp. and were also discarded since they did not classify within the genus *Vibrio*. Of the remaining 455 sequences, 47% were >98% similar to *V. vulnificus* and 23% were >98% similar to *V. ponticus* (Figure 4.3). The remaining clones (30%) were classified as a variety of *Vibrio* spp. including *V. natriegens*, *V. shilonii*, *V. sinaloensis*, *V. hepatarius*, and *V. alginolyticus*.

V. vulnificus ribotypes were found in fish from all four temperature treatments. Twenty-five percent of these ribotypes were from the 20 °C, 27% from the 24 °C, 17% from the 28 °C, and 36% from the 32 °C treatment. The *V. ponticus* ribotypes were

predominately from either the 24 °C (47%) or 28 °C treatments (38%). *V. vulnificus* ribotypes were found in the baseline fish (data not shown) and persisted through the entire time course of the study. However, *V. ponticus* ribotypes were not found in the initial samples and were only minimally abundant in the clone library prior to Day 12.

The relative abundance of both *Vibrio* 16S and *vvh* genes increased during the experiment in all treatments (Figure 4.5). There was a statistically significant difference in the relative abundances of *Vibrio* 16S rRNA with temperature (p=0.018). There was also a statistically significant difference in the relative abundance of *V. vulnificus vvh* genes with temperature (p=0.008). This difference was statistically significant between the 20 °C and 32 °C treatments for both the relative abundance of *Vibrio* 16S rRNA and *V. vulnificus vvh* (p=0.037 and 0.012 respectively).

DISCUSSION

These studies were conducted to determine if and how elevated temperature regimes might affect the relative abundance of *Vibrio* spp. in fish gut microbiomes. Libraries from the *L. rhomboides* temperature study were dominated by Vibrionaceae ribotypes; including several (*P. damselae* subsp. *piscida*, *V. harveyi*, and *V. ichythoenteri*) that are documented fish pathogens (Austin and Austin 1999; Buller 2004). Several of the ribotypes associated with lesions were also similar to the potentially pathogenic *V. harveyi*. The presence of potential pathogens in fish at the start of the study, in addition to the observation that several fish became ill and/or died during the study, suggests that these fish were stressed and more susceptible to gut pathogens.

This increased susceptibility may have masked any effects of increased water temperature on the gut microbiome.

We did not see a correlation between the relative abundance of *Vibrio* spp. 16S rRNA genes and increased temperature in the *L. rhomboides* study. The relative abundance of *Vibrio* 16S rRNA increased with temperature during the first 8 days of the experiment. The subsequent decrease after Day 8 may indicate that other bacteria were becoming more abundant with time and/or increased temperature.

Sequences retrieved from *F. heteroclitus* indicate that the *Vibrio* spp. within the gut microflora were dominated by bacteria closely related (98.1-100%) to *V. vulnificus* (212 clones, 47%) with additional contributions from *V. ponticus* (105 clones, 23%). There was significant variation in the relative abundance of *Vibrio* 16S rRNA between different time points. These changes may reflect shifts within the composition of the *Vibrio* spp. community with time. This is supported by changes we observed in the contribution of *V. ponticus* ribotypes to the Vibrio community in our samples. This ribotype was not found in the initial sample but increased in relative abundance in clone libraries with time. The relative abundance of *V. ponticus* ribotype in clone libraries peaked in opposition to *V. vulnificus* ribotypes, suggesting a shift in dominating *Vibrio* species. The relative abundance of other *Vibrio* spp. ribotypes present in the clone libraries (*V. alginolyticus*, *V. natriegens*, *V. shilonii*) did not vary temporally. The relative abundance of *V. vulnificus vvh* genes varied significantly between the 20 °C and 32 °C treatments. Kelly (1982) found that this bacterium had an optimal growth rate in vitro of 37 °C. They also reported that *V. vulnificus* grew well at 30-35°C, but grew

more slowly at 25 °C. This may explain the increased abundance of *V. vulnificus vvh* in the 32 °C treatment when compared to the 20 °C treatments (especially post-day 20).

Increased abundance of potentially pathogenic bacteria in intestinal microflora may have implications for both fish health and human health. Although most of the *V.vulnificus*-related illnesses and deaths in humans stem from oyster or shellfish consumption, many fish species harbor this potential pathogen in their guts (DePaola et al. 1994; Chapter 4). Previous studies have suggested that it is possible for Gram-negative bacteria such as the *Vibrio* spp. to move from the fish intestine to blood and muscle (Buras et al. 1985; DePaola et al. 1994) and pathogenesis of some *Vibrio*-spp. infections in mammals originates as a gut infection (Ringø et al. 2003). Thus, there may be a danger of increased *V.vulnificus* infections in areas where consumption of lightly-cooked and raw fish are popular (i.e. Eastern Europe, Japan, and IndoPacific regions) (DePaola et al. 1994). Fish feces contain gut microflora, which are then released into the environment (data not shown), thus fish harboring pathogens within their guts may also serve as vectors capable of transferring pathogenic gut bacteria to new hosts within the same environment.

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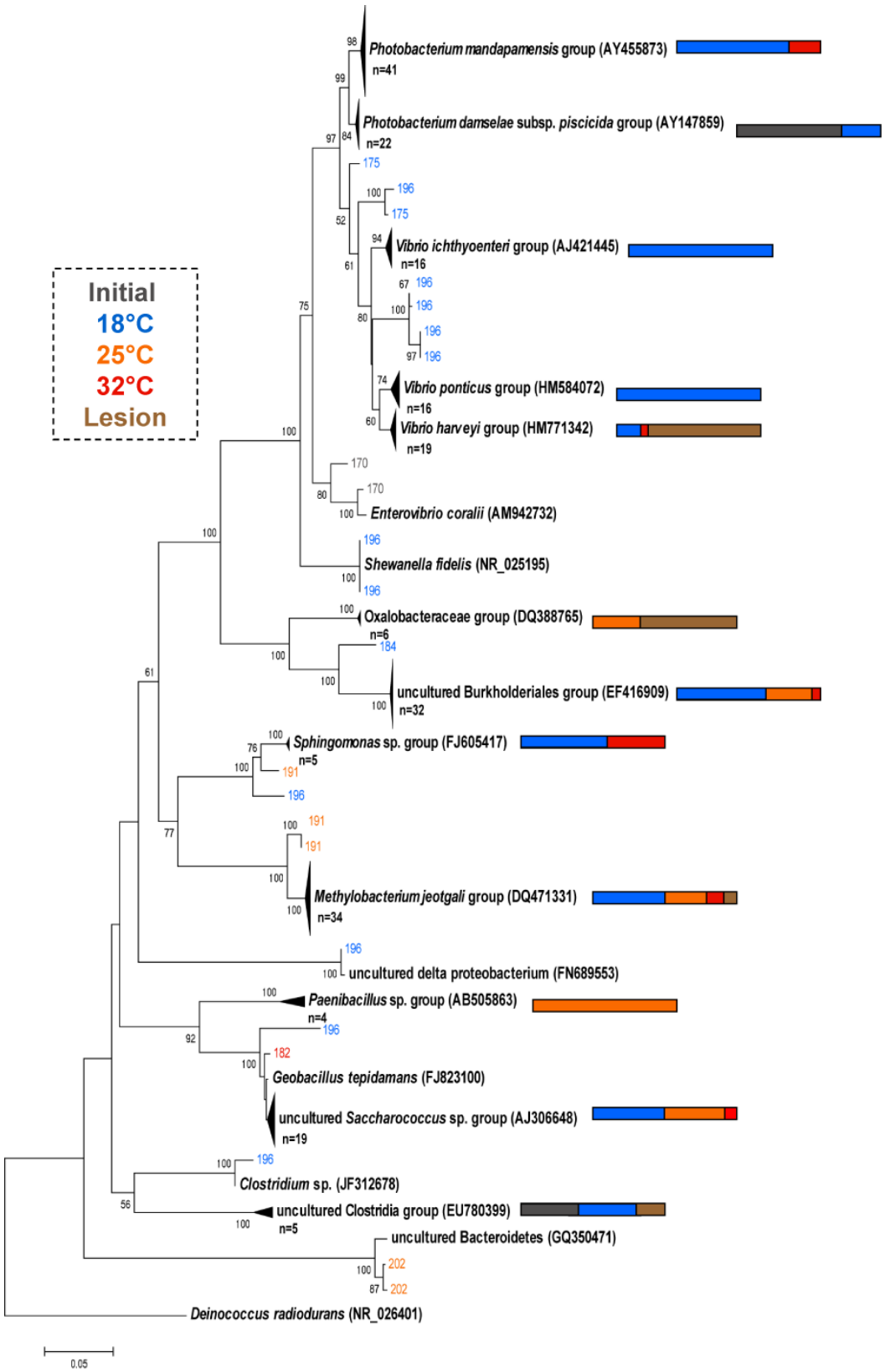


Figure 4.1: Phylogenetic analysis of Bacteria 16S rRNA gene sequences retrieved from the guts of *L. rhomboides* held at different temperatures. This is a Neighbor-

joining tree. The scale bar at bottom indicates Jukes-Cantor distances. Bootstrap values >50% (100 iterations) are shown. Numbers below the reference sequence identifiers give the number of sequences assigned to that clade while bars to right indicate the contribution of OTUs from different treatments to that clade.

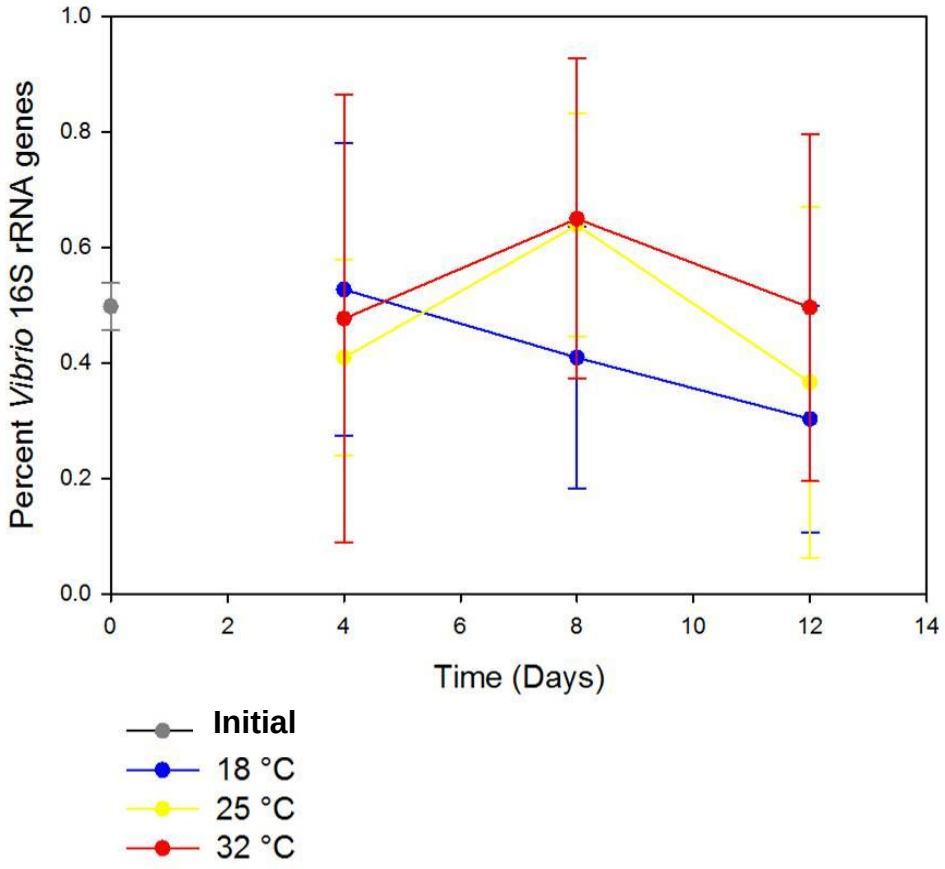


Figure 4.2: Changes in the relative abundance of *Vibrio* spp. ribotypes in guts of *L. rhomboides* fish held at different temperatures as a percent of all Bacteria ribotypes retrieved from each fish (n=3 for each temperature, bars indicate standard deviation).

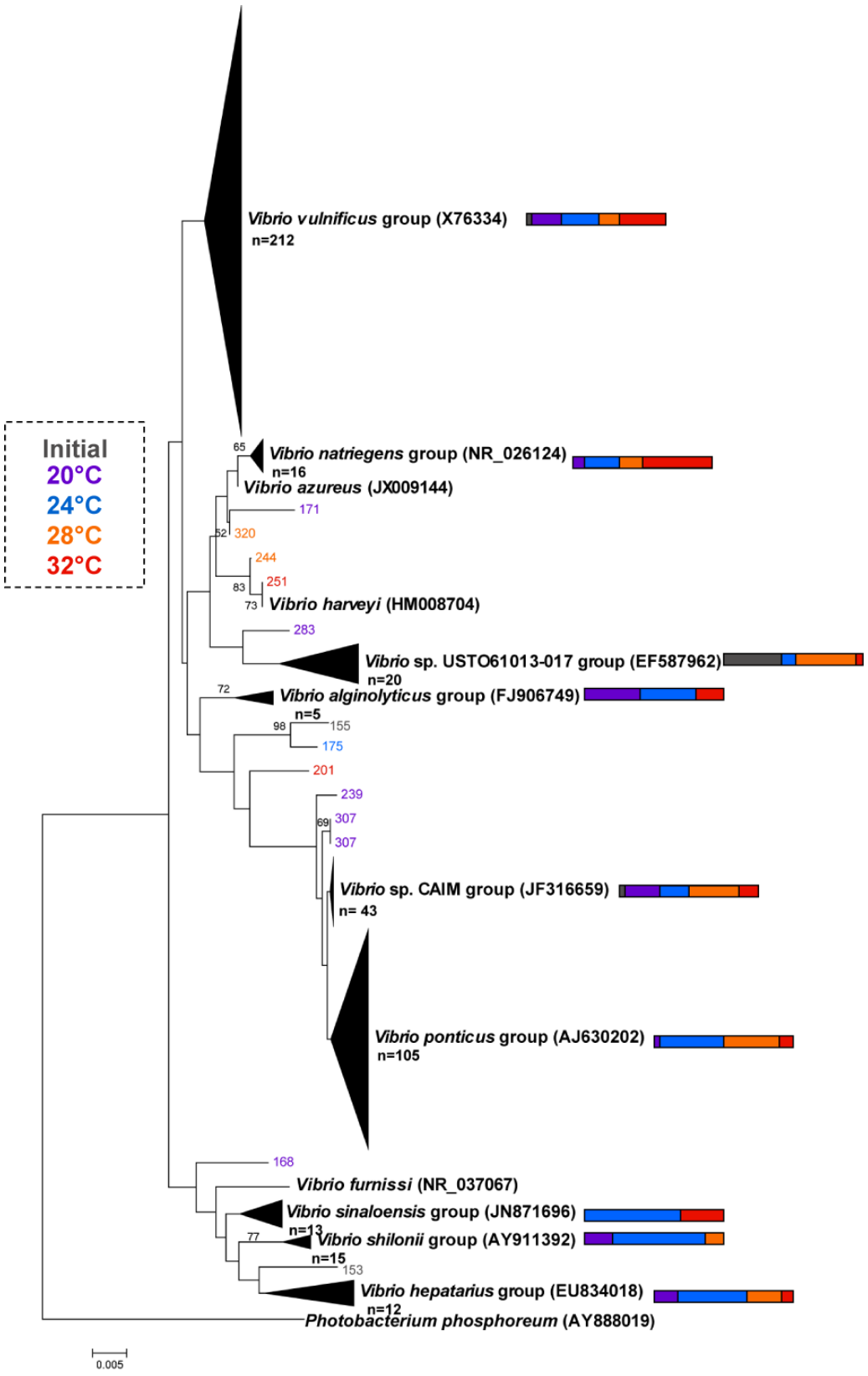
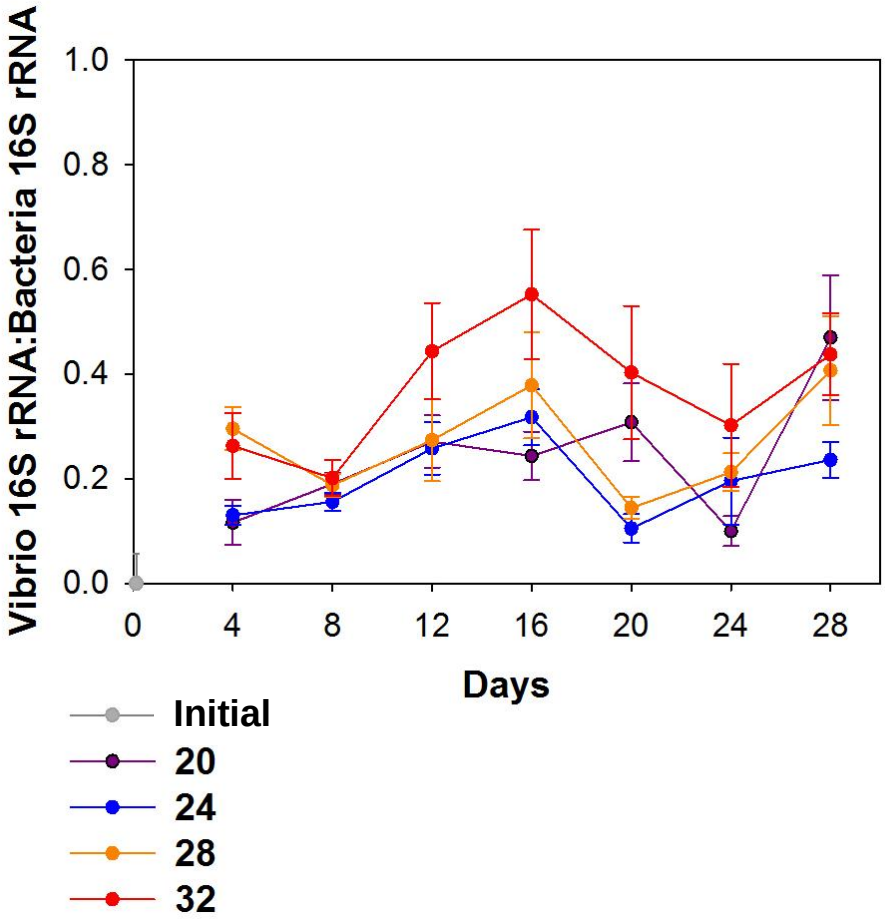

Initial
20°C
24°C
28°C
32°C
Vibrio vulnificus group (X76334)
n=212
65
Vibrio natriegens group (NR_026124)
n=16
Vibrio azureus (JX009144)
171
52
320
244
83
251
73
Vibrio harveyi (HM008704)
283
Vibrio sp. USTO61013-017 group (EF587962)
n=20
72
Vibrio alginolyticus group (FJ906749)
n=5
98
155
175
201
239
69
307
307
Vibrio sp. CAIM group (JF316659)
n= 43
Vibrio ponticus group (AJ630202)
n=105
168
Vibrio furnissi (NR_037067)
Vibrio sinaloensis group (JN871696)
n=13
77
Vibrio shilonii group (AY911392)
n=15
153
Vibrio hepatarius group (EU834018)
n=12
Photobacterium phosphoreum (AY888019)
0.005

Figure 4.3: Phylogenetic analysis of *Vibrio* 16S rRNA gene sequences retrieved from the guts of *F. heteroclitus* held at different temperatures. This is a Neighbor-joining tree. The scale bar at the bottom indicates Jukes-Cantor distances. Bootstrap values >50% (100 iterations) are shown. Numbers below the reference sequence identifiers give the number of sequences assigned to that clade while bars to right indicate the contribution of OTUs from different treatments to that clade.

a)


Vibrio 16S rRNA:Bacteria 16S rRNA
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
20
24
28
Days
Initial
20
24
28
32

]

b)

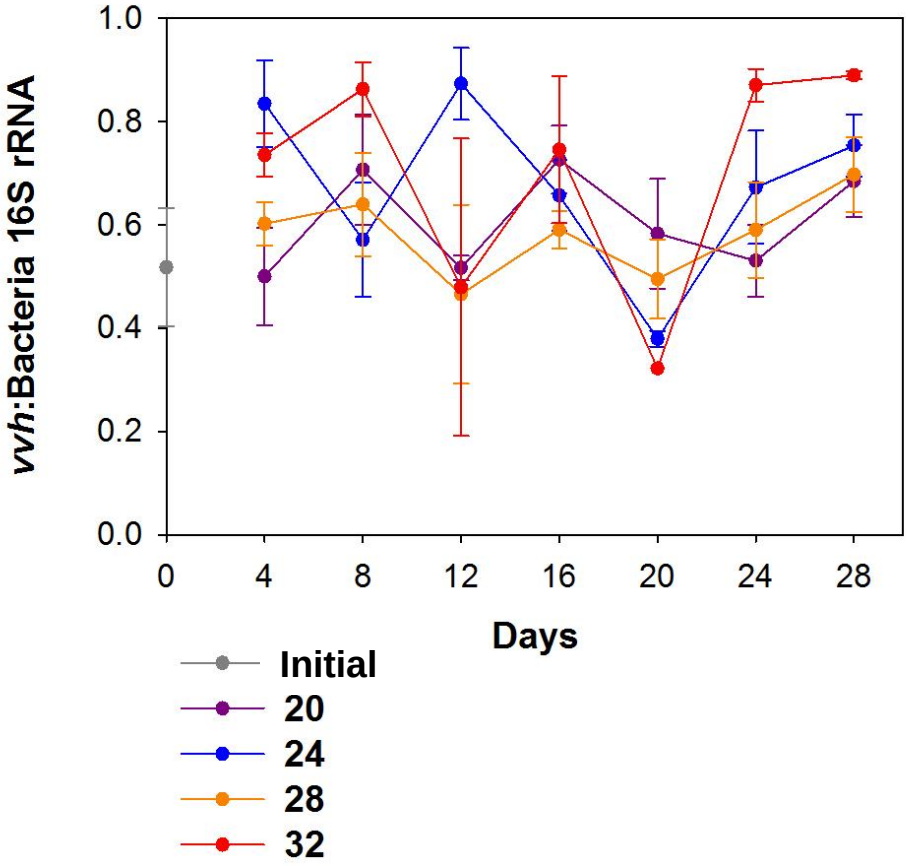


Figure 4.5: qPCR analysis of changes in the ratio of: a) *Vibrio* 16S rRNA:Bacteria 16S rRNA genes; and b) *V. vulnificus vvh*:Bacteria 16S rRNA genes in samples from intestines of *F. heteroclitus* held at different temperatures. Intestinal samples in assays targeting qPCR data have been corrected for average rRNA copy numbers for Bacteria (3.94) and *Vibrio* spp. (9.75). The ratio and standard error (error bars) have been plotted for n=6 fish at all temperatures and time points.

CHAPTER 5

INVESTIGATION OF FISH INTESTINES AND SEDIMENT AS POTENTIAL RESERVOIRS OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS*[1]

[1]Givens, C.E., J.C. Bowers, A. DePaola, J.T. Hollibaugh, and J.L. Jones. To be submitted to Applied and Environmental Microiology.

ABSTRACT

Vibrio vulnificus and *Vibrio parahaemolyticus* are Gram-negative bacteria native to estuaries in the Gulf of Mexico. These bacteria are also human pathogens, associated with the consumption of raw oysters in warm months. We examined fish intestines and sediment as potential reservoirs for *V. vulnificus* and *V. parahaemolyticus* during times of the year they are not detectable in oysters. We quantified these pathogens in fish intestine, sediment, oyster, and water samples from Mobile Bay, Alabama, USA. Vibrio densities were determined by quantitative PCR (qPCR) and direct plating-colony hybridization. The greatest densities of these bacteria were detected when water temperatures were 24.5°C and salinity was 8.6 psu. *V. vulnificus* and *V. parahaemolyticus* were detected by qPCR in 69 and 40% of fish intestine, 82 and 42% of water, 27% and 27% of sediment, 13 and 20% of oyster samples, respectively. Higher detection rates of both bacteria were obtained by colony hybridization due to the lower limit of detection (10 CFU/g vs. ~500 copies/g). Overall, *V. vulnificus* was detected at greater densities than *V. parahaemolyticus*. The data indicate that fish intestines and sediment may be reservoirs for *V. vulnificus* and *V. parahaemolyticus* during periods when temperatures and/or salinity are sub-optimal for these bacteria and few oyster-associated illnesses are reported.

INTRODUCTION

Vibrio parahaemolyticus and *Vibrio vulnificus* are Gram-negative marine bacteria that are naturally found in estuarine and marine waters (Kaneko and Colwell 1975). The abundance of *Vibrio* spp. increases seasonally with *V. parahaemolyticus* and *V. vulnificus*

densities increasing during warmer months when the water temperature is above 15 °C (Kaneko and Colwell 1974, 1975b; McLaughlin et al. 2005) and 20 °C, respectively (Kelly 1982; Tamplin et al. 1982; Kaysner et al. 1987). The highest densities of these cells occur when the water temperature ranges between 20 and 30 °C (Kelly, 1982, DePaola et al. 2003; Tantillo et al. 2004). During this period of increased temperatures, pathogenic *Vibrio* spp. can pose an increased risk of infections for humans (Lipp et al. 2002; McLaughlin et al. 2005). Approximately 75% of *V. vulnificus* infections occur between May and October when the water temperature is over 20 °C (Rippey 1994; CDC 2009). Additionally, higher densities of *V. parahaemolyticus* and *V. vulnificus* have been observed when salinities are between 15-25 psu (Anonymous 2010; Cook et al. 2002; DePaola et al. 2003; FAO/WHO 2011) and 5-15 psu, respectively (Kelly 1982; Wright et al. 1996; Motes et al. 1998, Lipp et al. 2001; Randa et al. 2004). Optimal environmental conditions for *V. vulnificus* and *V. parahaemolyticus* have been defined as >20°C (Kelly, 1982; Wright et al., 1996; Randa et al., 2004;) and 5-15 psu (Kelly, 1982; Wright et al., 1996; Motes et al., 1998; Lipp et al., 2001), and >15°C (Kaneko & Colwell, 1974; Kaneko & Colwell, 1975b; DePaola et al, 2003; McLaughlin et al., 2005) and 15-25 psu (Anonymous 2010; Cook et al., 2002; DePaola et al., 2003; FAO/WHO 2011), respectively.

Several members of the *Vibrio* genus are pathogenic to humans and marine animals, and *V. parahaemolyticus* and *V. vulnificus* are leading causes of seafood-associated bacterial illness and mortality (Iwamoto et al. 2010). Infection with these two species can cause gastroenteritis and septicemia through consumption of raw or undercooked seafood or wound infections (Constantin et al. 2009). A recent CDC report

on foodborne illnesses within the United States documented an annual incidence of *V. vulnificus* illnesses at 111 cases and that of *V. parahaemolyticus* at 287 cases (Scallan et al. 2011). However, these numbers do not account for under-reporting and misdiagnosis, with perhaps more accurate annual incidence estimates of 207 cases for *V. vulnificus* and 44,950 cases for *V. parahaemolyticus* (Scallan et al. 2011). Although *V. vulnificus* infections are rare, they are the leading cause of seafood-related deaths domestically and have one of the highest hospitalization (91.3%) and mortality (34.8%) rates of all foodborne pathogens (Scallan et al. 2011; Iwamoto et al. 2010). *V. parahaemolyticus* infections are common worldwide. In the United States, this bacterium is the leading cause of bacterial illness from seafood consumption (Iwamoto et al. 2010), but has a lower hospitalization (22.5%) and mortality (0.9%) rate than *V. vulnificus* (Scallan et al. 2011).

Not all *V. parahaemolyticus* or *V. vulnificus* strains are pathogenic and cause illness and infections. The genes *tdh* (thermostable direct hemolysin, TDH) and *trh* (TDH-related hemolysin, TRH) are generally associated with *V. parahaemolyticus* virulence (Honda and Iida 1993). The *tdh* and/or *trh* gene(s) are frequently present in clinical strains; they are less frequently recovered from environmental strains (Shirai et al. 1990, Nishibuchi and Kaper 1995). Although less frequent, *tdh* and *trh* genes have been found in environmental strains of *V. parahaemolyticus* (Wong et al. 2000; Cook et al. 2002, DePaola et al. 2000; Zimmerman et al. 2007; Johnson et al. 2010; Kirs et al. 2011; Jones et al. 2012). Aznar et al. (1994) found two main variations in 16S rRNA gene sequences for *V. vulnificus*, strains which were designated as Type A and B. The majority (76-94%) of *V. vulnificus* isolates originating from clinical fatalities linked with

oyster consumption were noted to be Type B, suggesting that this ribotype has increased virulence (Nilsson et al. 2003, Aznar et al. 1994).

Both of these bacteria are found not only in the water column, but also in shellfish, fish, plankton, and the sediment (Aznar et al. 1994, Zimmerman et al. 2007; Maugeri 2006; Martinez-Urtaza et al. 2012). *Vibrio* spp. are often considered to be the dominant cultivable bacteria found in and on marine fish and are common members of the gut microflora in both farmed and wild fish (MacFarlane et al. 1986; Sakata 1990; Martin-Antonio et al. 2007; Ward et al. 2009). DePaola et al. (1994; 1997) reported high densities of *V. vulnificus* in several finfish species collected from the Gulf of Mexico. De Paola et al. (1994) documented that *V. vulnificus* densities in sheepshead (*Archosargus probatocephalus*) intestines registered as 2-4 logs (log_{10}; mean *V. vulnificus* MPN/100 g or mL) greater than in corresponding oyster and sediment samples and 5 logs (log_{10}) greater than in seawater samples. Similarly, in a subsequent study, *V. vulnificus* densities in the fish gut were 2-3 logs (log_{10}) lower in March and December when water temperatures were 21.9 °C and 17.5 °C as compared to those recorded in May and September when water temperatures were 24.7 °C and 30.6 °C (DePaola et al. 1997).

The aim of this study was to determine if fish intestines and/or sediments are reservoirs of *V. vulnificus* or *V. parahaemolyticus* during periods of sub-optimal environmental conditions. Our objective was to quantify the abundance of both these bacteria in fish, oysters, sediment, and water using both culture-independent (qPCR) and culture-dependent (colony hybridization) techniques. All sample collections occurred during the spring (mid-March to May) when there was an expected increase in water temperature and thus, an expected increase in *V. parahaemolyticus* and *V. vulnificus*

abundance. Increased occurrence and abundance of these potentially pathogenic bacteria within the intestine may not only affect fish health, but also the surrounding environment (oysters, sediment, and water) once they are expelled from the gut with fecal matter. If pathogenic, these bacteria can impact public health through food transmissions and wound infections.

METHODS

Sample Collection

Fish, oyster, sediment, and water samples were collected from Mississippi Sound (30°15'N, 088°60'W) off Dauphin Island, Alabama from March 17 to May 2, 2011. Water temperature, salinity, and dissolved oxygen measurements were taken during each sampling using an YSI 85 meter (YSI; Yellow Springs, OH). Six oysters were collected at each sampling from a suspended oyster cage. Water was collected in sterile 500 mL wide-mouth polyethylene bottles. Sediment was also collected in sterile specimen cups. Fish samples were caught with hook and line or cast net and placed immediately on ice. A total of 10 fish species were used throughout the study including sheepshead (*Archosargus. probatocephalus*), sea catfish (*Aruis felis*), Atlantic croaker (*Micropogonias undulatus*), ground mullet (*Menticirrhus americanus*), spot (*Leiostomus xanthus*), black drum (*Pogonias cromis*), silver perch (*Bairdiella chrysoura*), pinfish (*Lagodon rhomboides*), southern flounder (*Paralichthys lethostigma*), and striped mullet (*Mugil cephalus*). These species were chosen because they could repeatedly be caught from the same pier from which oyster cages were suspended and water and sediment were sampled. Fish were weighed and then the external surfaces were sterilized with

ethanol (70%) to prevent potential contamination of the gut microflora during dissection. Fish were aseptically dissected, the mid to hind-gut regions removed, and digesta was squeezed into a sterile container and weighed. Multiple fish specimens of the same species were pooled when collected on the same sampling day.

DNA Extraction Control

Prior to extraction of each sample, an aliquot of an overnight alkaline peptone water (APW) culture of *ctx+ V. cholerae* was added. After addition of the *ctx* extraction control, the samples were vortexed briefly and incubated at room temperature for five minutes. Extractions were performed as described below. The amount of *ctx* recovered from the extraction and amount of *ctx* added were quantified by qPCR (Blackstone et al. 2007). These values were used to determine recovery of the extraction [amount recovered ÷ amount added = recovery]. The percent recovery was used to adjust qPCR data for Bacteria 16S rRNA, *Vibrio* spp. 16S rRNA, *V. vulnificus* and *V. parahaemolyticus*.

DNA Extractions

Approximately 1.0 gram of fish gut was transferred to an empty MoBio PowerMax Bead Beating tube (MoBio; Carlsbad, CA) and extraction control added. DNA extraction was completed using the MoBio PowerMax Soil DNA Extraction Kit following the manufacturer's instructions except that only 2 mL of Solution C6 was used for the final elution step, to increase DNA recovery.

Oysters were washed, shucked, and blended together for 90 seconds. Then 0.5 g was placed into a microfuge tube and extraction control added. DNA extraction for oyster samples was completed using the Qiagen Blood & Tissue Kit (Qiagen; Valencia, CA). As a modification to the manufacturer's kit instructions to enhance recovery, reagent volumes for Buffer ATL, Buffer AL, and ethanol were doubled and the final elution with Buffer AE was reduced to 100 µL.

The extraction control was added to the water samples (100 mL), filtered through a 0.22 µM nitrocellulose filter (Millipore; Billerica, MA), and extracted using the MoBio Power Water DNA Extraction Kit following the manufacturer's instructions.
A 1.0 g sample of sediment was mixed with the extraction control, and then DNA was extracted using the MoBio Power Soil DNA Extraction Kit following the manufacturer's kit instructions.

Culture-Dependent Methods

Colony Hybridization.

Serial 10-fold dilutions of each homogenized sample were made in phosphate buffered saline (PBS; 7.65 g of NaCl, 0.724 g of Na_2HPO_4 anhydrous, 0.21 g of KH_2PO_4 per liter, pH 7.4) and 100 µL was spread plated on T_1N_3 (1% tryptone, 3% NaCl, 2% agar, pH 7.6) and VVA (Kaysner and DePaola 2004) agars. Additionally, 0.1 g of oyster and sediment samples was spread plated onto T_1N_3 and VVA plates. For water samples, 100 mL and 10 mL aliquots were filtered through a 0.22 µM nitrocellulose filter (Millipore) and the filters plated on T_1N_3 and VVA. Additional water samples of 1 mL

and 100 µL volumes were spread directly onto T_1N_3 and VVA. All plates were incubated overnight at 35°C.

After overnight incubation, VVA and T_1N_3 plates with growth were used for colony lifts and hybridization using DNA probes (DNA Technology; Aarhus, Denmark) targeting the *V. vulnificus vvhA* gene and the *V. parahaemolyticus tlh* gene as previously described (Wright et al. 1993; McCarthy et al. 1999). Purple (probe-positive) spots were counted and *V. parahaemolyticus* and *V. vulnificus* levels reported as CFU/g or ml.

Colony Isolation.

For isolation of colonies, thiosulfate-citrate-bile salts sucrose (TCBS) and CPC+ (Warner and Oliver 2007) agars were spread plated as described above and incubated overnight at 35°C. A maximum of 30 colonies indicative of *V. parahaemolyticus* (from TCBS) or *V. vulnificus* (from CPC+) were picked for colony isolation and streaked onto TSA + 1% NaCl plates and incubated overnight at 35°C. Following overnight incubation all TSA + 1% NaCl plates were checked for colony growth. If the growth appeared to be mixed colony growth, colonies were once again isolated and each type was streaked onto separate TSA + 1% NaCl plates and incubated overnight at 35°C. If growth appeared to be pure culture growth, an isolated colony was transferred into a well of a 96-well microtiter plate containing 100 µL alkaline peptone water (APW; 1% peptone, 1% NaCl, pH 8.5 ± 0.2) per well. Microtiter plates were incubated overnight at 35°C. Following incubation, 100 µL TSB + 1% NaCl + 30% glycerol was added to all wells and then the plate was frozen for future isolate characterization. Isolated colonies were replicated to VVA and triplicate T_1N_3 plates, incubated overnight at 35°C, and then used for colony

lifts and hybridization as described below. Boiled preparations (10 min at 100°C) of the isolates were used as template in PCR to confirm whether they were positive for either *V. vulnificus* and 16S Type A or B genes (Kirs et al 2011; Vickery et al. 2007) or *V. parahaemolyticus* and *tdh/trh* genes (Nordstrom et al. 2007).

Culture Independent Enumeration

Quantitative PCR (qPCR) was completed using the Smart Cycler II (Cepheid; Sunnyvale, CA) and iCycler (BioRad Laboratories; Hercules, CA) systems and the primer and probe sequences listed in Table 5.1. All primers and probes were purchased from IDT (Coralville, IA) or Applied Biosystems (Foster City, CA). 16S and Vibrio 16S qPCR reactions were run in 25 μL volume with 1 X iQ SYBR Green Supermix (BioRad), forward and reverse primers, nuclease free water, and 3 μL of template DNA. For the 16S qPCR, the primers Bact1369F and Prok1492R (Suzuki et al. 2000) were used with cycling parameters previously described (Buchan et al. 2009). For *Vibrio*-specific 16S rRNA qPCR, primers 567F and 680R were used with published cycling parameters (Thompson et al. 2004). All reactions were run in triplicate with standards ranging from 10^1 to 10^7 copies per uL. Since many bacteria have more than one copy of the 16S rRNA gene, the number of gene copies per reaction (copies/reaction) was normalized to adjusted 16S rRNA genes/reaction by dividing by an average of 3.94 16S rRNA genes and 9.75 Vibrio 16S rRNA genes (Klappenbach et al. 2001; Lee et al. 2009).

The *V. parahaemolyticus* qPCR used the *tlh* primers and protocol as described previously (Nordstrom et al. 2007). The *V. vulnificus* PCR used the primers and protocols as previously published (Campbell and Wright 2003) with modifications for the

Smart Cycler II platform as previously described (Jones et al. 2012). Standard curves for each target were run in triplicate and all samples tested in duplicate for quantification.

Statistical Analysis

Correlation and Regression Tree (CART) analysis was performed on qPCR abundance data for total bacteria count (TBC, Bacteria 16S rRNA) and Vibrio 16S rRNA and also on colony hybridization data for *V. vulnificus* and *V. parahaemolyticus*. CART was not performed on qPCR data for *V. vulnificus* and *V.parahaemolyticus* because there were too many samples below the level of detection. We used CART to analyze abundance versus temperature and salinity in order to determine optimal and sub-optimal temperatures and salinity for our fish, oyster, sediment, and water samples. Additionally correlations between the abundance of bacteria ((TBC, Bacteria 16S rRNA), Vibrio 16S rRNA, *V. vulnificus*, and *V. parahaemolyticus*) among samples and also by method (culture dependent vs. culture-independent) were calculated using the non-parametric correlation, Kendall's tau.

RESULTS

Environmental Correlations

During the study, water temperature at the sampling site varied from 20.1-24.6 °C (average=22 °C), salinity ranged from 3.7-18.8 psu (average=10.5 psu), and dissolved oxygen ranged from 4.5-8.7 mg/L (average=6.9 mg/L). CART analysis defined optimal and sub-optimal environmental conditions for *V. vulnificus* and *V. parahaemolyticus* for our fish, oyster, sediment, samples (Table 5.1 and Table 5.2). For most samples,

temperature was the primary influence on abundance. Salinity was the primary indicator of optimal environmental conditions for *V. vulnificus* abundance in water samples and Bacteria 16S rRNA, Vibrio 16S rRNA, and *V. vulnificus* abundance in oysters. Optimal temperature for *V. vulnificus* abundance was >24.0 °C for the fish gut and >22.0 °C for sediment. The optimal temperature for *V. parahaemolyticus* was >23.5 °C for the fish gut, oysters, and water. Optimal salinity for *V. parahaemolyticus* was >8.3 psu in oyster and water samples and >11 psu in the fish gut. For *V. vulnificus*, optimal salinity was >3.8 psu in the fish gut, >12 psu in oyster samples, and <12 psu in the sediment, <10 psu in the water samples.

Bacteriological Levels

Figure 5.1 shows the abundance of all Bacteria (Bacteria 16S rRNA), all Vibrio (Vibrio 16S rRNA), *V. vulnificus*, and *V. parahaemolyticus* among each of the four sample types over the sampling period. By qPCR, *V. vulnificus* and *V. parahaemolyticus* were detected in 69 and 40% of fish intestine, 82 and 42% of water, 27 and 27% of sediment, and 13 and 20% of oyster samples, respectively. However, no direct correlation between levels in different sample types was observed.

Fish samples had the highest *V. vulnificus* densities and were, on average, 2 $\log_{10}$ greater than oyster samples, 3.4 $\log_{10}$ greater than sediment samples, and 4.5 $\log_{10}$ greater than water samples. Differences among sample type were less pronounced for *V. parahaemolyticus* densities with fish samples approximately 1.4 $\log_{10}$ greater than oyster samples, 1.7 $\log_{10}$ greater than sediment samples, and 3.4 $\log_{10}$ greater than water samples.

Isolate Virulence

Of the 123 *V. vulnificus* isolates tested for 16S rRNA type, 60% were Type A, 7.3% were Type B, 32% were Type AB, and 0.8% were untypable (Table 5.3). The 16S rRNA Type B (the more virulent genotype) isolates comprised 7.1%, 12.5%, 8.6%, and 3.4% of the total *V. vulnificus* isolates from fish, oysters, sediment, and water, respectively. The nine strains with the more virulent genotype were isolated on seven sampling occasions: four with optimal conditions (as defined in the published literature) and three with sub-optimal conditions for *V. vulnificus*.

A total of 24 *V. parahaemolyticus* isolates were tested for the presence of the *trh* and *tdh* genes (Table 5.4). Only one sediment sample (4%) yielded a virulent (*trh*+) *V. parahaemolyticus* isolate; no virulent strains were isolated from any other sample type.

Culture-Dependent versus Culture Independent Methods

Overall, there was a significant correlation ($p<0.05$) between levels observed by each method for *V. vulnificus* and *V. parahaemolyticus* (Table 5.5). There was also significant correlations between levels observed for Bacteria 16S rRNA (TBC) and Vibrio 16S rRNA (Table 5.6). qPCR abundances were consistently greater than those reported using colony hybridization targeting the same gene. However, there was more colony hybridization data for oyster, sediment, and water samples which had qPCR levels below the detection limitations. The largest discrepancy between colony hybridization and qPCR abundances was observed in the fish gut samples, with fish gut samples accounting for 18 of 31 (58%) and 13 of 22 (59%) of *V. vulnificus* and *V. parahaemolyticus* discrepancies (data not shown). Furthermore, these samples accounted

for 17 of 28 (61%) of *V. vulnificus* and 10 of 14 (71%) of *V. parahaemolyticus* discrepancies of greater than one order of magnitude. It is possible the discrepancies are more apparent within these samples since there are more independent samples of fish (n=34) versus oyster (n=13), sediment (n=12), or water (n=12). Fish accounted for 48% of total samples, but 58 and 59% of the discrepancies.

DISCUSSION

V. vulnificus and *V. parahaemolyticus* were consistently detected in the fish gut suggesting that the gut may be a reservoir for these pathogens during periods of sub-optimal temperatures and/or salinity when oyster-associated illnesses are infrequently reported. Both these bacteria were detected via qPCR and colony hybridization (Table 5.1; Figure 5.1) in fish intestine and sediment samples during the periods when salinity was sub-optimal (as defined in published literature) for either *V. vulnificus* and/or *V. parahaemolyticus*. In contrast, both of these bacteria were infrequently detected in oysters at low levels during these sub-optimal periods. However, the highest densities of *V. vulnificus* and *V. parahaemolyticus* were detected when water temperature was at 24.5 °C and salinity of 8.6 psu. These conditions would be considered optimal for *V. vulnificus*, but according to published studies this salinity would be considered less than optimal for *V. parahaemolyticus*. Densities of these bacteria did not significantly decrease during periods of sub-optimal salinity, suggesting that abundances are less dependent on salinity, within an optimal temperature range. This is in contrast to a previous study that noted that within a limited temperature range, salinity was the most correlated parameter to *V. parahaemolyticus* densities (Zimmerman et al., 2007).

Although *V. vulnificus* was consistently detected in both fish and water samples throughout the time course of the study, *V. parahaemolyticus* was less frequently detected in these samples. Additionally, *V. vulnificus* was detected at greater densities than *V. parahaemolyticus*. Overall the highest levels among all assays were consistently detected in fish, followed by oyster, sediment, and water samples. The differences among samples is similar to that reported by DePaola et al. 1994 who found highest *V. vulnificus* densities in the fish gut with a 2-4 $\log_{10}$ decrease in oyster and sediment samples and 5 log decrease in water samples. However, the *V. vulnificus* densities reported here are greater than those reported by DePaola et al. (1994), with 0.8 $\log_{10}$, 1.5 $\log_{10}$, and 2 $\log_{10}$ difference for water, sediment, and fish samples, respectively. DePaola et al. 1994 found that higher densities of *V. vulnificus* were found in the guts of inshore bottom fish (including *A. probatocephalus, P. cromis, A. felis, L. rhomboides, L. xanthus, M. undulatus*) with the highest density of 6.8 $\log_{10}$ MPN/gram in pigfish and sea catfish intestines. This study found the highest density of 8.05 $\log_{10}$ copies/gram in a striped mullet intestine. Some of these differences may be due to methodology as the values reported in this study are based on qPCR and the DePaola study used an MPN-colony hybridization method. This study's direct plating-colony hybridizations detection rates were similar to those determined previously by MPN.

Higher detection rates of *V. vulnificus* and *V. parahaemolyticus* were obtained by colony hybridization in this study as compared to qPCR, which is likely due to the lower limit of detection (10 CFU/g vs. ~500 copies/g). However, the qPCR assays reported higher abundances than colony hybridizations. This was particularly noticed in the case of *V. vulnificus*, perhaps due to inhibition of the plating media causing decreased

recovery of stressed cells. Approximately a 1 log decrease in recovery of *V. vulnificus* cells on VVA compared to a non-selective media such as TSA has been observed (Jones, unpublished data). The discrepancy between qPCR and colony hybridization levels was greatest in fish intestine samples, possibly due to a higher number of stressed cells than in other sample types. This difference could also be a result of aggregated or attached cells in the fish intestine, generating one colony on a plate, but being recognized as multiple individuals by qPCR. Although possible, it is unlikely dead cells are being detected by the qPCR as the extraction methods utilized did not allow unprotected DNA to survive the process. However, further analysis is needed to determine the definitive cause(s) behind these differences.

This is the first study to date that not only reports the densities of Bacteria 16S rRNA, Vibrio 16S rRNA, *V. vulnificus*, and *V. parahaemolyticus* in fish intestine, oyster, sediment, and water samples, but also compares these values through culture-dependent and culture-independent methodology. These data demonstrate strong evidence for fish intestinal tracts and sediment acting as reservoirs for *V. parahaemolyticus* and *V. vulnificus,* which may help explain the annual resurgence of these organisms in oysters. Through expulsion with fecal matter, fish may be a link in the Vibrio cycling between the fish gut, water column, and oysters. Abundance of these pathogens in the environment can potentially affect human health, and subsequently, the commercial fishing and aquaculture industry.

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Table 5.1: Optimal temperatures and sub-optimal temperatures for Bacteria 16S rRNA, Vibrio 16S rRNA, *V. vulnificus*, and *V. parahaemolyticus* abundance defined by CART analysis

Sample	Optimal Temperature	Abundance	Sub-optimal Temperature	Abundance
Fish				
Bacteria 16S	>20.5°C, <24.6 °C	9.50 $\log_{10}$	<20.5°C, >24.6 °C	8.14 $\log_{10}$, 8.82 log10
Vibrio 16S	<20.5 °C**	7.85 $\log_{10}$	>20.5 °C	7.04 $\log_{10}$
V. parahaemolyticus	>23.5 °C**	4.50 $\log_{10}$	<23.5 °C**	3.12 $\log_{10}$
V. vulnificus	>24.1 °C, <24.6 °C	4.94 $\log_{10}$	<24.1 °C**	3.03 $\log_{10}$
Oyster				
Bacteria 16S	N/A*	N/A*	N/A*	N/A*
Vibrio 16S	N/A*	N/A*	N/A*	N/A*
V. parahaemolyticus	>23.5 °C	3.32 $\log_{10}$	<23.5 °C**	2.16 $\log_{10}$
V. vulnificus	N/A*	N/A*	N/A*	N/A*
Sediment				
Bacteria 16S	>20.5 °C, <22.8 °	9.3 $\log_{10}$	>22.8 °C	7.95 $\log_{10}$
Vibrio 16S	<23.0 °C	4.70 $\log_{10}$	>23.0 °C	3.41 $\log_{10}$
V. parahaemolyticus	>22.0 °C	2.88 $\log_{10}$	<22.0 °C	1.70 $\log_{10}$
V. vulnificus	>22.0 °C*	3.01 $\log_{10}$	<22.0 °C*	2.33 $\log_{10}$
Water				
Bacteria 16S	<22.0 °C*	6.08 $\log_{10}$	>22.0 °C*	5.09 $\log_{10}$
Vibrio16S	<22.0 °C*	2.56 $\log_{10}$	>22.0 °C*	1.54 $\log_{10}$
V. parahaemolyticus	>23.5 °C	1.08 $\log_{10}$	<23.5 °C**	0.12 $\log_{10}$
V. vulnificus	N/A*	N/A*	N/A*	N/A*

*For this assay, salinity was of primary importance in the regression analysis. **For this assay, temperature was of primary importance and salinity was of secondary importance.

Table 5.2: Optimal temperatures and sub-optimal temperatures for Bacteria 16S rRNA, Vibrio 16S rRNA, *V. vulnificus*, and *V. parahaemolyticus* abundance defined by CART analysis

Sample	Optimal Salinity	Abundance	Sub-optimal Salinity	Abundance
Fish				
Bacteria 16S	N/A	N/A	N/A	N/A
Vibrio 16S	<3.9 psu**	9.24 $\log_{10}$	>3.9 psu**	8.46 $\log_{10}$
V. parahaemolyticus	<16 psu, >11 psu**	4.50 $\log_{10}$	<11 psu**	2.32 $\log_{10}$
V. vulnificus	>3.8 psu**	3.03 $\log_{10}$	<3.8 psu**	2.15 $\log_{10}$
Oyster				
Bacteria 16S	>12 psu	8.91 $\log_{10}$	>7.4 psu <12 psu	5.86 $\log_{10}$
Vibrio 16S	>10 psu	5.20 $\log_{10}$	>4.6 psu, <10 psu	3.99 $\log_{10}$
V. parahaemolyticus	>8.3 psu**	2.16 $\log_{10}$	<8.3 psu**	1.32 $\log_{10}$
V. vulnificus	>12 psu	3.14 $\log_{10}$	<6.9 psu	1.62 $\log_{10}$
Sediment				
Bacteria 16S	N/A*	N/A*	N/A*	N/A*
Vibrio 16S	N/A*	N/A*	N/A*	N/A*
V. parahaemolyticus	N/A*	N/A*	N/A*	N/A*
V. vulnificus	<12 psu	3.10 $\log_{10}$	>12 psu	1.93 $\log_{10}$
Water				
Bacteria 16S	>12.1 psu	6.58 $\log_{10}$	<12.1 psu	6.01 $\log_{10}$
Vibrio16S	>12 psu	3.24 $\log_{10}$	<12 psu	2.56 $\log_{10}$
V. parahaemolyticus	>8.3 psu**	0.12 $\log_{10}$	<8.3 psu**	-0.48 $\log_{10}$
V. vulnificus	<10 psu	0.66 $\log_{10}$	>12 psu	0.11 $\log_{10}$

**For this assay, temperature was of primary importance and salinity was of secondary importance.

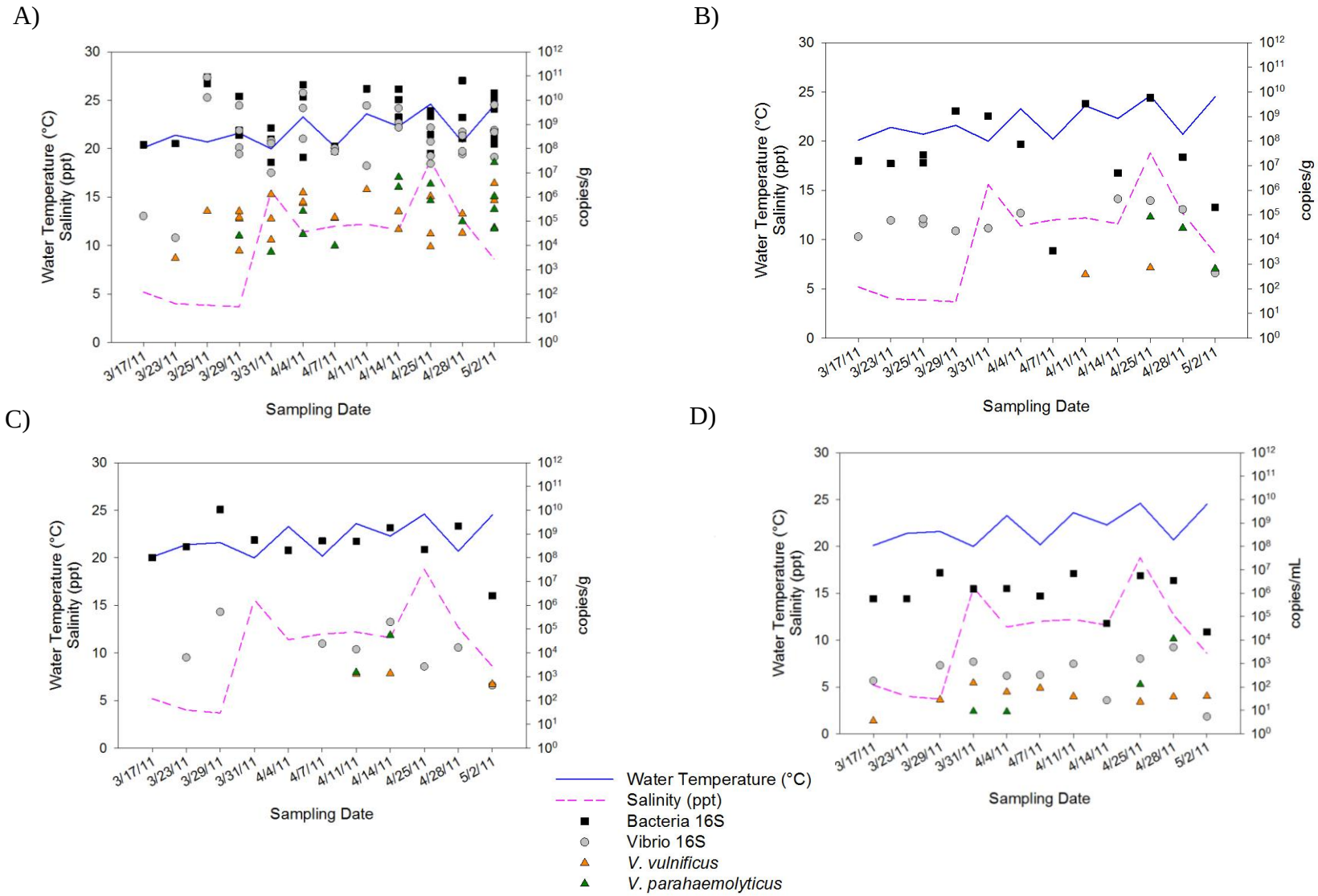

Figure 5.1: qPCR results for A) Fish Intestine, B) Oyster, C) Sediment, and D) Water samples

Table 5.3: Summary of *V. vulnificus* 16S rRNA Types for sample isolates

Sample	Type A	Type B	Type AB	ND	Total Tested
Fish	**36**	**5**	**17**	**0**	**58**
Oyster	**21**	**2**	**4**	**1**	**28**
Sediment	**4**	**1**	**3**	**0**	**8**
Water	**13**	**1**	**15**	**0**	**29**
Total	**74**	**9**	**39**	**1**	**123**

***ND-not determined; untypable**

Table 5.4: Summary of *V. parahaemolyticus tdh*$^+$/ *trh*$^+$ for sample isolates

Sample	*tdh*$^+$	*trh*$^+$	Total Tested
Fish	**0**	**0**	**7**
Oyster	**0**	**0**	**1**
Sediment	**0**	**1**	**3**
Water	**0**	**0**	**13**
Total	**0**	**1**	**24**

Table 5.5: Correlations between Colony Hybridization (CH) vs. qPCR for *V. vulnificus* and *V. parahaemolyticus*

Sample Type	Comparison	Slope	Intercept	tau	p
All	Vp CH vs. qPCR	1.12	Null	0.27	0.001*
Fish	Vp CH vs. qPCR	0.84	1.90	0.26	0.026*
Oyster	Vp CH vs. qPCR	0.80	1.80	0.25	0.245
Sediment	Vp CH vs. qPCR	1.54	-1.40	0.20	0.040*
Water	Vp CH vs. qPCR	0.77	Null	0.13	0.061*
All	Vv CH vs. qPCR	0.98	1.36	0.24	0.004*
Fish	Vv CH vs. qPCR	0.46	4.77	0.27	0.028*
Oyster	Vv CH vs. qPCR	3.59	-6.74	0.16	0.474
Sediment	Vv CH vs. qPCR	0.52	-0.63	0.16	0.538
Water	Vv CH vs. qPCR	-0.80	2.33	-0.29	0.212

*Indicates statistical significance. Vp=*V. parahaemolyticus*, Vv=*V. vulnificus*, CH=colony hybridization, qPCR=quantitative PCR

Table 5.6: Correlations between Colony Hybridization (CH) vs. qPCR for *V. vulnificus* and *V. parahaemolyticus*

Sample Type	Comparison	Slope	Intercept	tau	p
All	TBC vs Vibrio	1.36	-4.86	0.62	<0.001*
Fish	TBC vs Vibrio	0.83	0.94	0.59	<0.001*
Oyster	TBC vs Vibrio	0.17	3.60	0.06	0.837
Sediment	TBC vs Vibrio	1.08	-5.25	0.86	0.004*
Water	TBC vs Vibrio	0.81	-2.44	0.56	0.032*
All	TBC vs Vv (CH)	0.47	-1.40	0.30	<0.001*
Fish	TBC vs Vv (CH)	0.22	1.16	0.12	0.335
Oyster	TBC vs Vv (CH)	0.02	2.87	0.05	0.876
Sediment	TBC vs Vv (CH)	-0.32	5.11	-0.24	0.371
Water	TBC vs Vv (CH)	-0.12	1.18	-0.22	0.375
All	TBC vs. Vp (CH)	0.56	-2.42	0.30	<0.001*
Fish	TBC vs. Vp (CH)	0.36	-0.39	0.10	0.440
Oyster	TBC vs. Vp (CH)	0.16	1.03	0.18	0.482
Sediment	TBC vs. Vp (CH)	0.05	1.60	0.04	0.928
Water	TBC vs. Vp (CH)	0.13	-0.40	0.09	0.754
All	Total Vibrio vs. Vv (CH)	0.31	0.47	0.34	<0.001*
Fish	Total Vibrio vs. Vv (CH)	0.12	2.16	0.06	0.640
Oyster	Total Vibrio vs. Vv (CH)	0.22	1.30	0.22	0.466
Sediment	Total Vibrio vs. Vv (CH)	0.07	2.39	0.05	1.000
Water	Total Vibrio vs. Vv (CH)	-0.11	0.64	-0.24	0.350
All	Total Vibrio vs. Vp (CH)	0.35	0.30	0.34	<0.001*
Fish	Total Vibrio vs. Vp (CH)	0.25	1.17	0.07	0.594
Oyster	Total Vibrio vs. Vp (CH)	0.97	-2.62	0.31	0.295
Sediment	Total Vibrio vs. Vp (CH)	-0.02	3.05	-0.05	1.00
Water	Total Vibrio vs. Vp (CH)	0.00	0.30	0.00	1.00
All	Vv vs Vp (CH)	0.90	0.13	0.42	<0.001*
Fish	Vv vs Vp (CH)	0.59	1.11	0.26	0.029*
Oyster	Vv vs Vp (CH)	1.23	-1.12	0.62	0.010*
Sediment	Vv vs Vp (CH)	0.87	0.26	0.36	0.178
Water	Vv vs Vp (CH)	-0.61	0.18	-0.20	0.421

*Indicates statistical significance. Vp=*V. parahaemolyticus*, Vv=*V. vulnificus*, CH=colony hybridization, qPCR=quantitative PCR

CHAPTER 6

MICROBIAL COMMUNITIES OF THE CARAPCE AND GUT AS POTENTIAL SOURCES OF HEMOLYMPH INFECTIONS IN *CALLINECTES SAPIDUS*[1]

[1] Givens, C.E., K.G. Burnett, L.E. Burnett, and J.T. Hollibaugh. To be submitted to Marine Biology.

ABSTRACT

The Atlantic blue crab, *Callinectes sapidus*, is an important fisheries resource. Previous studies have reported potentially pathogenic bacteria in the hemolymph of *C. sapidus* that may cause disease and mortality. We used culture-independent methods based on analysis of 16S rRNA genes to characterize and quantify the microflora community of carapace, gut and hemolymph samples from 7 *C. sapidus* specimens to identify potential sources of pathogens and pathways of hemolymph infection. We found that the carapace, gut, and hemolymph microflora have a core Proteobacteria community with additional contributions from other phyla including Bacteriodetes, Firmicutes, Spirochaetes, and Tenericutes. Within this Proteobacteria core, γ-Proteobacteria (including members of the Vibrionaceae that are closely related to potential pathogens) dominate. Bacteria closely related to hemolymph pathogens were found on the carapace, supporting the hypothesis that punctures or broken dactyls are potential causes of hemolymph infections. These results provide some of the first data on the blue crab microbiome obtained with culture-independent techniques and offer insights to routes of infection and potential bacterial pathogens associated with blue crabs.

INTRODUCTION

The Atlantic blue crab, *Callinectes sapidus*, is an important marine resource (Phillips and Peeler 1972). As such, diseases of blue crabs are of commercial importance and factors that affect the risk to humans of handling and consuming blue crabs are of public health interest. Previous culture-based studies of the microbiome have identified potential pathogens including *Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio*

vulnificus (Sizemore et al. 1975; Davis and Sizemore 1982; Welsh and Sizemore 1985) associated with *C. sapidus* (Krantz et al. 1969). These bacteria have been found within gills, in viscera, in processed meat taken from healthy crabs and within the hemolymph of diseased crabs held in commercial tanks (Tubiash et al. 1975). The presence of these pathogens is of concern for human health as pathogenic *Vibrio* spp. in crab meat have been implicated in incidences of foodborne illness (Anonymous 1971; Molenda et al. 1972; Anonymous 1976; Anonymous 1999), and *V. vulnificus* has been linked to septicemia cases in humans handling and ingesting crabs (Blake et al. 1979).

Based on studies of vertebrates, it has been assumed that hemolymphs of healthy invertebrates are sterile. However, previous studies have indicated that healthy *C. sapidus* naturally harbor low-level populations of bacteria in their hemolymphs that may be capable of causing infections (Davis and Sizemore 1982; Welsh and Sizemore 1985). Counts of bacteria within the hemolymph are higher in crabs that are missing appendages, or that have been injured or stressed during capture and holding (Tubiash et al. 1975; Welsh and Sizemore 1985). The abundance of bacterial cells in samples of hemolymph fluids from infected crabs vary widely, from 1.8×10^3 to 6.7×10^5 CFU/mL (Davis and Sizemore 1982; Welsh and Sizemore 1985). Davis and Sizemore (1982) evaluated 81 crabs and divided the crab population into four categories based on the level of bacterial infection. Ten percent had light hemolymph infections ($<10^3$ CFU/mL), 52% had moderate infections (10^3-10^5 CFU/mL), 25% had heavy infections ($>10^5$ CFU/mL) and only 12% were found to have sterile hemolymphs. A study of bacteria associated with freshly captured *Cancer magister* (Dungeness crabs) also reported low levels of bacteria in hemolymph fluid ($<10^2$ CFU/mL; ref (Faghri et al. 1984)).

The relative abundance of *Vibrio* spp. in crab hemolymph bacterial populations is also highly variable. Monthly mean relative abundance of *Vibrio* spp. in *C. sapidus* hemolymph fluids ranged from 6-64% of total CFU (Welsh and Sizemore 1985). Incidence of *Vibrio* spp. within the hemolymph appeared higher in crabs subjected to commercial handling, in crabs from warmer water, and in those that already had hemolymph infections (defined as $>10^2$ CFU/mL; ref (Welsh and Sizemore 1985)). Sizemore et al. (1975) reported an average of 21% *Vibrio parahaemolyticus* within the hemolymph bacterial community of crabs in Chesapeake Bay during the months of May, June, and July when concentrations of this bacterium increased in the water. Another study using crabs from Galveston Bay, Texas reported *V. parahaemolyticus* in 23% of hemolymph samples. *V. cholerae* and *V. vulnificus* were detected at lower incidences: 2% and 7%, respectively (Davis and Sizemore 1982). In addition to *Vibrio* spp., these studies documented the presence of *Pseudomonas* sp., *Acinetobacter* sp., *Aeromonas* sp., *Bacillus* sp., and *Flavobacterium* sp. in crab hemolymphs (Sizemore et al. 1975).

Most previous studies of *C. sapidus* microflora, including those discussed above, relied on culture-based techniques to enumerate and identify bacteria. Culture-based studies have provided valuable insights into the composition of microbial communities and yielded isolates for detailed physiological investigation; however, they are known to provide biased assessments of the microbial community, as typically <1% of the cells known to be present by direct microscopic enumeration produce colonies on solid media (Ferguson et al. 1984b; Head et al. 1998a). This bias applies to Vibrio species (Thompson et al. 2004a), leading to questions of the role of these "viable but non-culturable" bacteria in the epidemiology of cholera outbreaks (Huq et al. 1990). The goal of the present study

was to characterize and quantify the *C. sapidus* microbiome using culture-independent analysis and to evaluate the potential for microbiome populations associated with the carapace or the gut to serve as inocula for hemolymph infections. Samples were taken from crab guts (G), hemolymphs (H), pieces of carapaces (carapace clips – CP) and of the biofilms found on the crab's integument (carapace swab - CS). We determined the abundance of Bacteria in samples from crabs by quantitative PCR (qPCR) and analyzed microbial community composition by cloning and sequencing 16S rRNA genes. This study also provides insight into the distribution of blue crab-associated bacterial pathogens that could affect human health via consumption or through wounds that become infected.

METHODS

Sample Collection and DNA Extraction.

Male *C. sapidus* (n=7; wet weight range=78.5-207 grams) were caught in a crab pot (Crabs 1; 4-7) and by trawl (Crabs 2 and 3) in Charleston Harbor, SC during June 2010. The crabs were examined visually and any injuries identified were noted and categorized as old versus new based on appearance. Crabs were banded, weighed, measured, and placed in individual holding tanks of well-aerated, static saltwater (30 psu; 24-26 °C), then sampled after holding them in quarantine for 24 hours to allow expulsion of diet-associated bacteria. Hemolymph samples were taken by sterilizing the carapace around the pericardial sinus with Betadine (povidone-iodine solution USP, 10%) and isopropanol, then inserting a 23-gauge needle attached to a 1-mL syringe through the carapace into the pericardial sinus and collecting 500 µL of hemolymph. Hemolymph samples were placed in PowerBead tubes (MoBio Laboratories), immediately vortexed,

and placed on ice. Sterile swabs of the carapace and 10 mm^2 clips of the carapace were collected, put in PowerBead tubes, and processed as above. The same region was swabbed and clipped for all samples. Once the carapace was removed, the gut (mid to hindgut) was excised aseptically and placed in PowerBead tubes and processed as above. DNA extractions were completed using the MoBio PowerSoil DNA Extraction Kit per kit instructions.

Sequence Analysis.

DNA was amplified using Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare) with the Bacteria-specific 16S rRNA primers 27F/1492R (Table 6.1; ref (Lane 1991b)) with the following PCR conditions: initial denaturation at 95 °C for 5 minutes; 35 cycles of: denaturation at 95 °C for 45 seconds, annealing at 62 °C for 30 s, and extension at 72 °C for 1 minute; finishing with a final extension at 72 °C for 45 minutes. Amplified DNA was electrophoresed on a 1% agarose gel, bands of the expected product size were excised, then the DNA in them was extracted and purified using QIAGEN QIAquick gel extraction kits. DNA extracted from the gel was cloned with TOPO TA cloning kits (Invitrogen) using the pCR 4.0-TOPO TA vector and competent *E. coli* cells. Clones were selected randomly and sequenced using the 27F primer by Georgia Genomics (Athens, GA) or Genewiz (South Plainfield, NJ). All sequences were checked for chimeras using the Bellerophon server (Huber et al. 2004). Taxonomic identities were assigned to each sequence using both RDP SeqMatch (Cole et al. 2007b; Cole et al. 2009b) and BLAST against the non-redundant nucleotide database (NCBI GenBank), then grouped phylogenetically. Sequences were assigned to a genus if

there was >95% sequence similarity (Tindall et al. 2010) and to a species if there was >97% sequence similarity (Stackebrandt and Goebel 1994; Tindall et al. 2010).

Of a total of 846 sequences (combined libraries for gut, carapace clip, carapace swab, and hemolymph samples), only 26 sequences (~3%) were discarded because they were poor quality or chimeric. A total of 239 sequences (415-1118 bp; median=992) was retrieved from gut libraries; 201 sequences (366-1242 bp; median=827) from the carapace clip libraries; 189 sequences (693-1129 bp; median =973) from the carapace swab libraries and 193 sequences (362-1267 bp; median=927) from hemolymph libraries. Cyanobacteria and chloroplast 16S rRNA sequences contributed 27%, 41%, 0.84%, and 4.2% to the 16S rRNA clone libraries from the carapace clip, carapace swab, gut, and hemolymph communities, respectively. These sequences were excluded from further analysis.

Quantitative PCR.

Quantitative PCR (qPCR) was done with a BioRad iCycler and the primers given in Table 6.1. qPCR cycling conditions followed those published in Buchan et al. (2009b). qPCR reactions were run in 25 μL with 1x iQ SYBR Green Supermix (BioRad Laboratories), forward and reverse primers, nuclease free water, and 3 μL of template DNA. All reactions were run in triplicate with standards ranging from 10^1 to 10^7 copies per uL^{-1}. Because there was no robust way to normalize across the different sample types used in this study, we took care to be consistent from crab to crab in our sampling and extraction protocols and qPCR data are reported as copies of 16S rRNA genes mL^{-1} of final, purified template DNA extract.

*Statistical Analysis***.**

The software package PRIMER (v.6; ref (Clarke and Gorley 2006b)) was used for non-metric multidimensional scaling analysis (NMDS) of ribotype distributions and to compare the composition of clone libraries from crab samples at both phylum and genus levels of phylogenetic discrimination. Multiresponse permutation procedures (MRPP) were performed in R (R Core Team 2009) using the vegan statistical package (Oksanen et al. 2009) to test whether there was a significant difference between clustered groups. MRPP was run with the Bray-Curtis distance matrix with 999 permutations.

RESULTS

Crab condition.

During pre-quarantine physical inspection, Crab 1 (C1) was found to be missing part of the tip of a cheliped, Crab 2 (C2) and Crab 4 (C4) were both missing an entire cheliped, and Crab 4 (C4) had extensive algal growth on his carapace. All injuries appeared to be pre-entrapment and had healed externally. At the time of the initial examination, Crab 3 (C3) appeared physically healthy with no injuries; however, after 24 hours in quarantine, C3 became extremely lethargic and moribund. All appendages were intact on the rest of the specimens and they were outwardly healthy in appearance both pre-and post-quarantine.

Gut community.

As evident in Figure 6.1a, the composition of the gut microflora varied among crabs. We detected a total of 8 different bacterial phyla in the 239 sequences retrieved

from gut samples. Forty seven percent of these ribotypes were assigned to the Proteobacteria, which was the most frequently encountered taxon. Ribotypes assigned to Spirochaetes, Bacteriodetes, Fusobacteria, and Firmicutes were found in most gut samples with relative abundances (all samples) of 10 to 12%. The Fusobacteria sequences retrieved from specimens C1, C2, C5, and C7 were >97% similar to *Propionigenum maris*. C1, C3, and C6 all contained sequences most closely related to the phylum Tenericutes, which were 90-93% similar to uncultured Mycoplasmataceae.

γ-Proteobacteria were the most abundant class of Proteobacteria, accounting for 71% of all Proteobacteria sequences retrieved (Figure 6.2a). Within the γ-Proteobacteria 46% were most closely related to *Photobacterium* spp., 26% to *Marinobacter* sp., 23% to *Vibrio* spp., 2.5% to *Escherichia* spp., and 2.5% to *Thalassomonas* sp (Figure 6.2b). The *Photobacterium* spp. clones could be further assigned at >97% sequence similarity to either *P. damselae* subsp. *damselae* or *P. damselae* subsp. *piscida.* Some of the *Vibrio* spp. clones could be further assigned at >97% sequence similarity to *V. gallicus, V. harveyi, V. tubiashii,* and *V. xuii*. ε-Proteobacteria were also important, contributing to 27% of the Proteobacteria community. All of the ε-Proteobacteria sequences were >97% similar to *Arcobacter* sp.

Carapace community.

Proteobacteria dominated the microbial assemblage found in carapace clip samples, comprising 59% of all phyla detected (Figure 6.1b). Carapace clip samples included the bacterial community on the external carapace and that within the layers of the carapace. As with the gut samples, libraries from crabs C3 and C6 both contained

sequences representative of the Tenericutes (Mycoplasmataceae). The Proteobacteria were represented by γ- (54%) and α-Proteobacteria (43%) (Figure 6.2a). Most of the α-Proteobacteria sequences were >97% similar to either *Erythrobacter* sp. or to members of the family Rhodobacteraceae (*Oceanicola* sp., *Roseobacter* sp., *Roseovarius* sp. and *Ruegeria* sp.). Thirty seven percent of the γ-Proteobacteria sequences were most similar to *Alteromonas* sp., 18% to *Pseudoalteromonas* sp., and 14% to *Vibrio* spp. Of the *Vibrio* spp. sequences, 25% were most similar to *V. harveyi* (Figure 6.2b). The ribotypes found in the carapace swab samples (Figure 6.1c) were similar to those reported for the carapace clip, with 55% of the ribotypes identified as Proteobacteria. The Proteobacteria in these samples were comprised of 81% γ-Proteobacteria and 15% α-Proteobacteria (Figure 6.2a). Fifty-four percent of the γ-Proteobacteria ribotypes were most similar to *Alteromonas* sp. with additional smaller contributions from *Pseudoalteromonas* sp. (12%), *Thalassomonas* sp. (10%) and *Vibrio* spp. (3%) (Figure 6.2b).

Hemolymph community.

Seventy-two percent of the 193 sequences retrieved from hemolymph samples were identified as belonging to the phylum Proteobacteria (Figure 6.1d). Ribotypes associated with the Proteobacteria almost completely dominated the hemolymph assemblages of all crabs except C2 and C6. The library from crab C2 contained primarily Firmicutes (85% *Bacillus* sp.) with only a small contribution from Proteobacteria. In contrast, ribotypes retrieved from the hemolymph of crab C6 were a combination of Proteobacteria (60%) and Bacteroidetes (32%). A few (~3%) Tenericutes (Mycoplasmataceae) were found in hemolymph samples from crabs C1 and C7, and

Mycoplasmataceae ribotypes accounted for 10% of the ribotypes retrieved from the gut of crab C1. However, no Tenericutes were found in the hemolymph samples of crabs C3 or C6, despite the presence of Tenericute ribotypes in both gut and carapace clip samples from these crabs. The Proteobacteria assemblage in these hemolymph samples was comprised of 86% γ-Proteobacteria, 7.8% β-Proteobacteria, and 6.5% α-Proteobacteria (Figure 6.2a). The majority of the γ-Proteobacteria were *Acinetobacter* sp. (43%), *Vibrio* spp. (24%), and *Alteromonas* sp. (10%) (Figure 6.2b). The *Acinetobacter* sp. sequences were >97% similar to *A. junii*. Most of the *Vibrio* spp. ribotypes, including all of those from crab C3, were >97% similar to *V. harveyi.*

Statistical Analysis.

We compared the composition of libraries from our samples using NMDS. When we compared composition at the level of bacterial phylum (Figure 6.3), all gut samples clustered together and were at least 60% similar to each other. Most of the carapace clip and carapace swab samples also clustered together with at least 60% similarity. The libraries from C5 hemolymph, C2 and C7 carapace clip, and C1, C3, and C4 carapace swab were largely dominated (>75%) by Proteobacteria with slight contributions from Bacteroidetes (<25%) and clustered together with 80% similarity at the phylum level. The carapace clip from C6 was the only sample with similar contributions (~42%) of Bacteroidetes and Proteobacteria and thus did not cluster with any of the other samples. MRPP indicates that clusters defined at 60% and 80% similarity are significantly different ($p=0.002$ and $p=0.001$, respectively).

We also compared the composition of γ-Proteobacteria ribotypes in the libraries from our samples (Figure 6.4). This analysis showed that libraries from hemolymph samples are distinct from those obtained from carapace and gut samples of the same crab. Hemolymph samples of crabs C3 and C4 clustered with either gut or carapace samples of other crabs, but not with the gut or carapace samples from C3 or C4. Carapace clip and carapace swab samples from crabs C1, C2 and C3 clustered together with at least 60% similarity. These samples all contained elevated abundances of *Alteromonas* sp. ribotypes. Gut samples from crabs C1, C4, C5, and C6 all had higher incidence of both *Photobacterium* sp. and *Vibrio* spp. than other crabs and clustered together with 60% similarity. The hemolymph sample from crab C3 was dominated by *Vibrio* spp. related to *V. harveyi* and clustered with the gut samples from C1, C4, C5, and C6 with 40% similarity. MRPP indicates that clusters separated at the 20% 40%, 60%, and 80% similarity level were all significantly different at p=0.001.

qPCR analysis.

All gut samples had similar bacterial abundances, ranging between 2.1×10^8 and 4.3×10^9 copies of 16S rRNA genes mL^{-1} of template (Figure 6.5). Abundances in carapace swab samples were between 3.8×10^6 and 2.1×10^8 copies of 16S rRNA genes mL^{-1} of template. Carapace clip samples had bacterial abundances between 2.4×10^6 and 6.5×10^7 copies of 16S rRNA genes mL^{-1} of template. Hemolymph samples ranged from 5.8×10^4 to 1.5×10^9 copies of 16S rRNA genes mL^{-1} of template. These same hemolymph samples plated on marine agar yielded counts ranging from 0 to 1.5×10^4 CFU mL^{-1} of hemolymph fluid (Burnett, unpublished data). The hemolymph sample

from crab C3 had the highest bacterial abundance detected by both qPCR (1.5×10^9 copies of 16S rRNA genes mL^{-1} of template) and CFU counts (1.5×10^4 CFU mL^{-1} of hemolymph fluid). Bacterial abundances (qPCR) in carapace clip, carapace swab, and gut samples from crab C3 were similar to those reported in the other crabs.

DISCUSSION

Data from this study show that the microflora of *C. sapidus* is more diverse than previously reported (Table 6.2). The carapace, gut, and hemolymph all have a core Proteobacteria community (47-72% of the ribotypes detected) that is dominated by γ-Proteobacteria (54-86%). However, other phyla including Acidobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Lentisphaerae, Planctomycetes, Spirochaetes, Tenericutes, Verrucomicrobia all contribute to the blue crab microbiome. Of the four sample types, the gut microbiome was most diverse (Figure 6.1a).

Some of these bacteria, such as Mycoplasmataceae, are notoriously difficult to culture and so were not found in previous studies using solely culture-based methods. Although some Mycoplasmataceae have been classified as pathogenic, others have been observed to be commensal and natural components of bacterial communities (Giebel et al. 1990b). *Mycoplasma* sp. have been associated with the gut microflora of a variety of terrestrial and marine hosts including rats (Giebel et al. 1990b), termites (Hongoh et al. 2003), fish (Holben et al. 2002a; Bano et al. 2007; Ward et al. 2009b), abalone (Tanaka et al. 2004b; Huang et al. 2010), lobsters (Meziti et al. 2010b), and the marsh fiddler crab (Gulmann 2004b). None of the Mycoplasmataceae sequences we retrieved clustered with the *Mycoplasma* spp. found in the termite (Hungoh et al., 2003) or fish gut studies

(Holben et al. 2002a; Bano et al. 2007; Ward et al. 2009b). Our sequences did, however, cluster with uncultured Mycoplasmataceae from guts of the mud crab (*Scylla paramamosain*) (Accession Number HE610322) and with symbionts from isopod midguts (EU646198) (Frane and Zimmer 2008).

We did not retrieve *V. cholerae, V. parahaemolyticus,* or *V. vulnificus* from any part of any of the crabs we sampled; however, we retrieved many sequences that were similar to other potential pathogens. *A. junii, Alteromonas* sp., *Bacillus* sp., *E. coli, P. damselae subsp. damselae, P. damselae subsp. piscida, Pseudoalteromonas* sp., and *V. harveyi* are all potentially pathogenic and sequences assigned (>97% similarity) to these species were associated with blue crabs in our study. *V. harveyi* and both subspecies of *P. damselae* are known to be opportunistic pathogens of both finfish and shellfish (Thyssen et al. 1998b; Fouz et al. 2000a; Austin and Zhang 2006). *P. damselae* subsp. *damselae* is also documented as a human pathogen with 3 cases reported in 2010 and an incidence of 0.01 per 100,000 persons (Anonymous 2011). *A. junii* has been documented to be an occasional, opportunistic human pathogen (Kappstein et al. 2000; Linde et al. 2002).

Previous studies reported that crabs with physical injuries had increased levels of hemolymph infections. Of the crabs sampled in this study, C1, C2, and C4 had sustained injuries prior to capture that resulted in partial (dactyl) or complete (chelipeds) loss of appendages. The hemolymph sample from crab C1 had the second highest abundance of bacteria in these samples, with 1.5×10^6 copies of 16S rRNA genes mL^{-1} of template. Crabs C2 and C4 had much lower concentrations, in the range of 10^4-10^5 copies of 16S rRNA genes mL^{-1} of template. Crab C3 had no injuries, but at the time of dissection had

the highest concentration of bacteria in its hemolymph (10^9 copies of 16S rRNA genes mL^{-1} of template), with all clones having >97% sequence similarity to the opportunistic pathogen *V. harveyi*. When we assessed the abundance of bacteria in crab hemolymph samples using published classifications based on plating (Davis and Sizemore 1982), 29% had sterile hemolymphs, 42% had light infections, 29% had moderate infections. No crabs had high level ($>10^5$ CFU mL^{-1} of hemolymph fluid) infections. In contrast if we convert our qPCR data (copies of 16S rRNA genes mL^{-1} of template) to estimates of genome (cell) abundance by dividing by an average copy number of 3.3 16S rRNA genes/genome (the average of the ribosomal gene copy numbers for genera present in this study's clone libraries; refs (Klappenbach et al. 2001b; Lee et al. 2009a), we can estimate abundance as genomes (cells) mL^{-1} of template. We then use the categories proposed by (Davis and Sizemore 1982) to classify crab hemolymph samples by qPCR assay: 86% had moderate infections and 14% had high level infections. Although the previously-injured crabs C1, C2, and C4 only had light infections and were apparently healthy, their hemolymph communities were dominated by potential pathogens: *A. junii* (C1); *Bacillus* sp. (C2); and *Alteromomas* sp., *Bacillus* sp., *P. damselae,* and *Vibrio* spp. (C4).

NMDS suggests the gut microflora community is similar among sampled crabs and is different from that found in either carapace or hemolymph bacteria communities. The hemolymph microflora community is not the same as that found on the carapace or in the gut. However, sequences representing many of the same phyla (Bacteroidetes, Firmicutes, and Proteobacteria) and even ribotypes (i.e. *Alteromonas* sp., *Escherichia* sp., and *Vibrio* sp.) that were found in the carapace and gut samples were also found in the hemolymph samples.

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Table 6.1: Primers used in this study

Primers	Gene	Use	Primer Sequences (5' to 3')	Reference
27F	16S rRNA	Sequencing	AGAGTTTGATCMTGGCTCAG	(Lane 1991b
1492R	16S rRNA	Sequencing	GGTTACCTTGTTACGACTT	(Lane 1991b
BACT1369F	16S rRNA	qPCR	CGGTGAATACGTTCYCGG	(Suzuki et al 2000a)
PROK1492R	16S rRNA	qPCR	GGWTACCTTGTTACGACTT	(Suzuki et al 2000a)

Table 6.2: Bacteria taxa and concentration identified in the blue crab microbiome in previous studies using culture-based methods compared with the ribotypes identified by culture-independent analysis in this study.

Sample Type and Assemblage Composition	Concentration	Reference
Carapace Clip		
Alteromonas sp., *Pseudoalteromonas* sp., *Erythrobacter* sp., Verrucomicrobia, *Vibrio* spp. (*V. harveyi*), Rhodobacteraceae (*Oceanicola* sp., *Roseobacter* sp., *Roseovarius* sp., and *Ruegeria* sp.), Bacteroidetes, Mycoplasmataceae	2.4 X 10^6- 6.5 x 10^7 copies mL^{-1}	This Study
Carapace Swab		
A*lteromonas* sp., *Pseudoalteromonas* sp., *Thalassomonas* sp., *Aestuariibacter* sp., Rhodobacteraceae (*Roseobacter* sp., *Roseovarius* sp., *Loktanella* sp), Bacteriodetes, *Vibrio* spp.	3.7 x 10^6- 2.1 x 10^8 copies mL^{-1}	This Study
Vibrio sp., *Pseudomonas* sp.	NR*	(Cook and Lofton 1973)
Vibrio spp.; *V. cholerae, V. parahaemolyticus, V. vulnificus*	NR*	(Davis and Sizemore 1982)
Vibrio sp., *Pseudomonas* ssp., *Aeromonas* sp., *Plesiomonas* sp.	NR*	(Noga et al. 1994)
Achromobacter spp., *Acinetobacter* spp., *Aeromonas* spp., *Plesiomonas* spp., *Pseudomonas* spp., *E. coli, Serratia* sp., *Vibrio* spp. including *V. alginolyticus, V. mimicus, V. parahaemolyticus, V. vulnificus*	NR*	(Noga et al. 2000)
Gut		
P. damselae (subsp. *damselae, piscida*), *Arcobacter* sp., *Spirochaeta* sp., Bacteroidetes, *P. maris*, Firmicutes (*Bacillus* sp., *Paenibacillus* sp.) M*arinobacter* sp., *Vibrio* spp. (*V. gallicus, V. harveyi, V. tubiashii, V. xuii*), Mycoplasmataceae, *Escherichia* sp., *Thalassomonas* sp	2.1 x 10^8- 4.3 x 10^9 copies mL^{-1}	This Study
Hemolymph		
A. junii, Bacillus sp., *Vibrio* spp. (*V. harveyi*), *Alteromonas* sp., M*arinobacter* sp., *Escherichia* sp., Bacteroidetes, *Methylobacterium* sp., *Comamonas* sp., *Diaphrobacter* sp., *Paenibacillus* sp., *Thalassomonas* sp., Mycoplasmataceae	5.8 x 10^4- 1.5 x 10^9 copies mL^{-1}	This Study
Clostridium botuilinum type F	NR*	(Williams-Walls 1968)
V. parahaemolyticus	NR*	(Krantz et al. 1969)
Vibrio spp., *V. parahaemolyticus*	NR*	(Colwell et al. 1975)
Vibrio spp. (especially *V. parahaemolyticus*), *Pseudomonas* sp., *Acinetobacter* sp., *Aeromonas* sp., *Bacillus* sp., *Flavobacterium* sp., coliforms	NR*	(Sizemore et al. 1975)
Vibrio spp.; *V. cholerae, V. parahaemolyticus, V. vulnificus*	8.6 x 10^1 – 3.0 x 10^7 bacteria mL^{-1}	(Davis and Sizemore 1982)
Vibrio spp.	0-9.5 x 10^4 CFU mL^{-1}	(Welsh and Sizemore 1985)

*NR-not reported

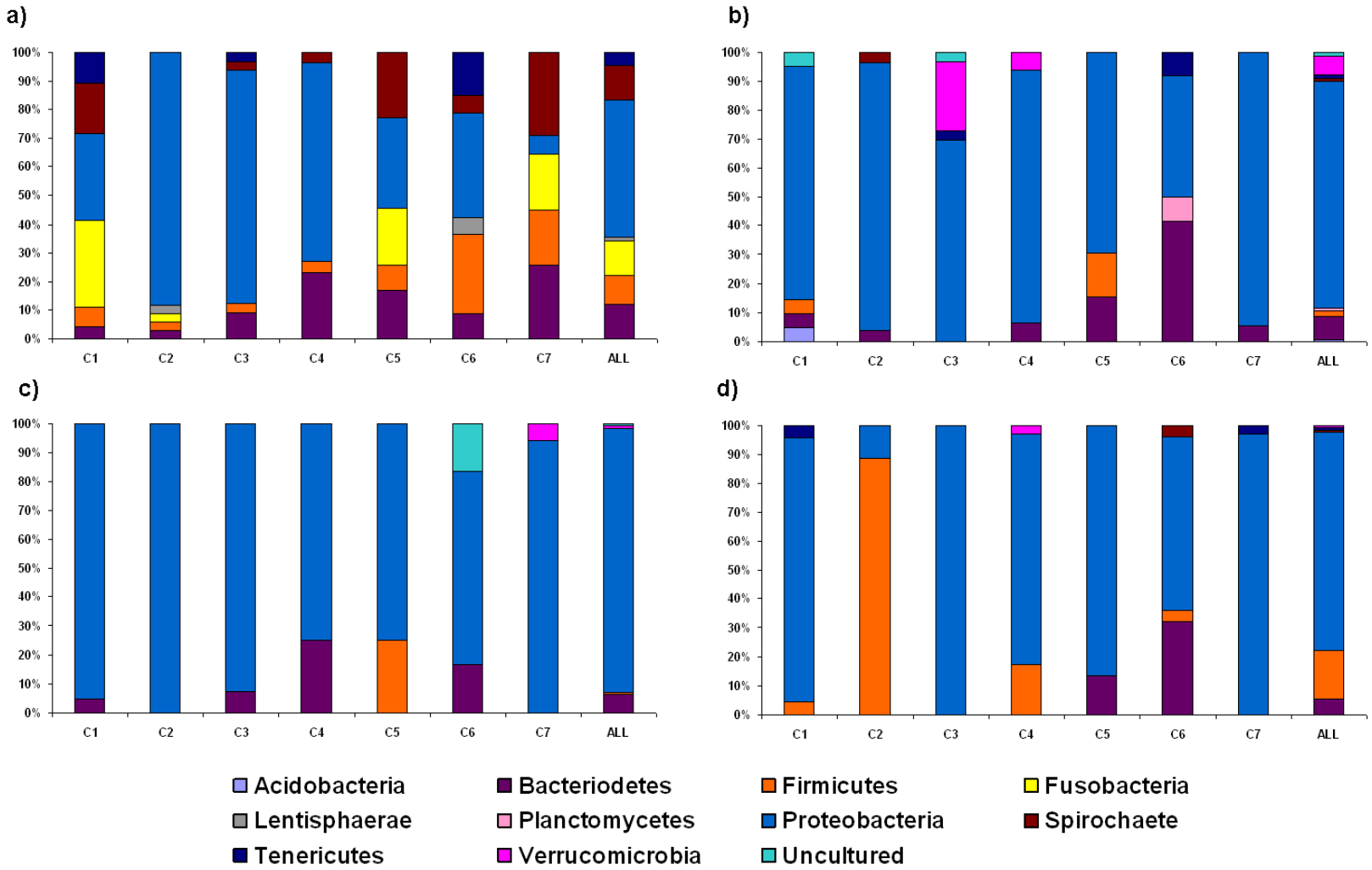


Figure 6.1: Bacteria phyla (% of ribotypes retrieved) detected in a) Gut; b) Carapace Clip; c) Carapace Swab; and d) Hemolymph samples.

C1-C7 refer to seven different sampled crabs. ALL corresponds to combined results from all sampled crabs.

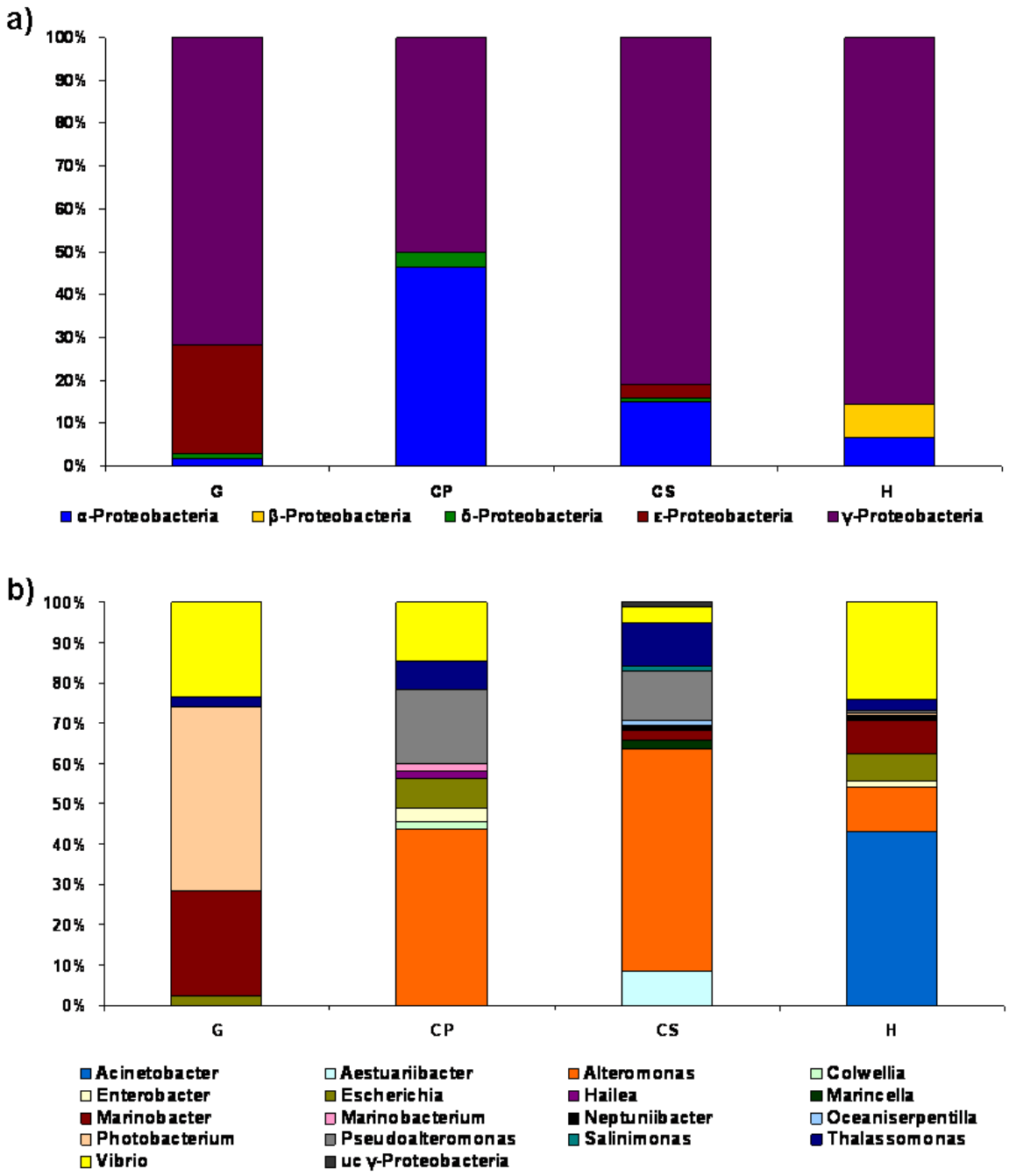


Figure 6.2: Contribution of a) Proteobacteria and b) γ-Proteobacteria ribotypes to libraries of sequences retrieved from Gut (G), Carapace Clip (CP), Carapace Swab (CS), and Hemolymph (H) samples from all of the crabs that were sampled.

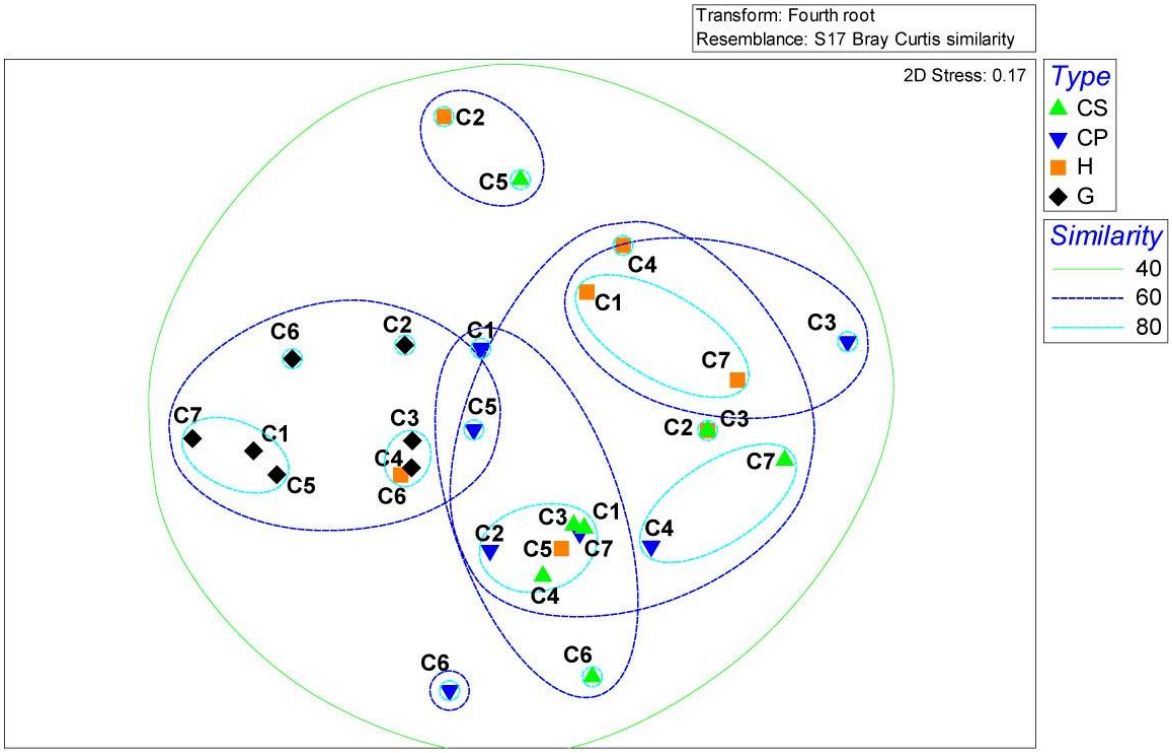


Figure 6.3: Non-Metric Multidimensional Scaling analysis of the distribution of Bacteria phyla found in carapace clip (CP), carapace swab (CS), gut (G), and hemolymph (H) samples.
Samples from crabs 1-7 (C1-C7) are displayed in a two-dimensional space and clustered according to percent similarity of the Bacterial assemblages they contain. Note that in many instances, the 80% similarity cutoff only included one sample. The C2/CS point overlaps that of the C3/H sample. Samples not present in plot were below the 40% similarity cutoff. MRPP indicates that clusters defined at 60% and 80% similarity are significantly different (p=0.002 and p=0.001, respectively).

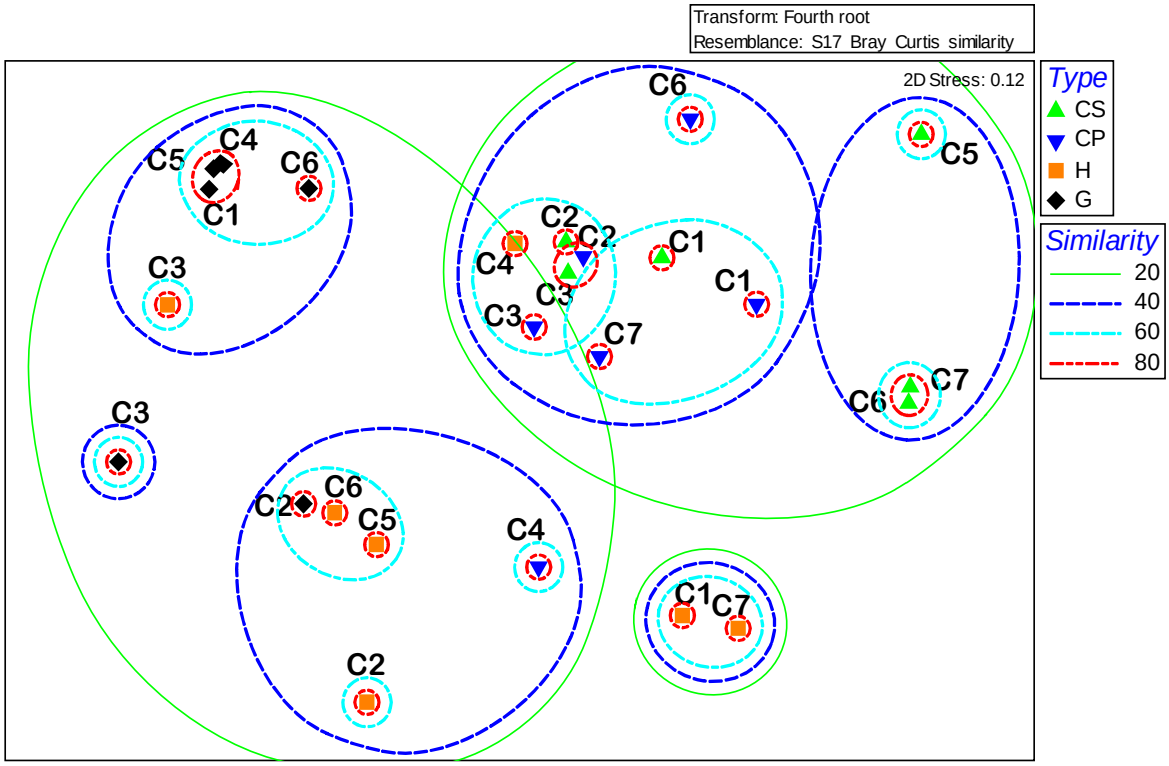


Figure 6.4: Non-Metric Multidimensional Scaling analysis of γ-Proteobacteria ribotypes retrieved from carapace clip (CP), carapace swab (CS), gut (G), and hemolymph (H) samples.
Samples are displayed in a two-dimensional space and clustered according to percent similarity. Note that in many instances, the similarity cutoff only included one sample. Samples not present in plot were below the 20% similarity cutoff. MRPP indicates that clusters separated at the 20% 40%, 60%, and 80% similarity level were all significantly different at p=0.001.

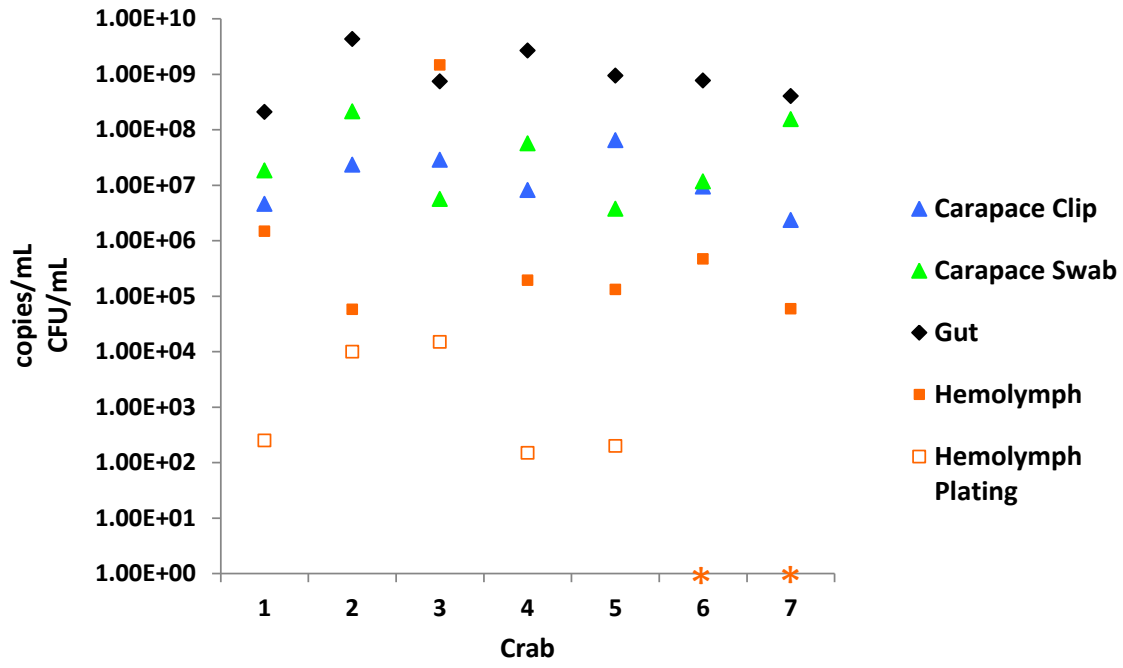


Figure 6.5: qPCR analysis of the abundance of Bacteria in Carapace Clip, Carapace Swab, Gut, and Hemolymph Samples.
Abundance is reported as copies of 16S rRNA genes/mL of DNA extract from each sample and thus are comparable across sample types but not between sample types. * = Hemolymph plates for C6 and C7 had no colony growth.

CHAPTER SEVEN

CONCLUSIONS

This dissertation used the molecular methods of 454-pyrosequencing, 16S rRNA clone libraries, and qPCR to 1) determine and compare the gut microflora of 12 finfish and 3 shark species, 2) assess the effects of food quality and diet-associated bacteria on gut microflora, 3) gauge the effects of increased water temperature on the abundance *Vibrio* spp. within fish guts, and 4) assess whether the fish gut is a reservoir of *Vibrio* spp. when growth condition are less favorable and also a vector for *Vibrio* spp. distribution. I also determined the composition of blue crab (*Callinectes sapidus*) microbiome as an example an invertebrate prey item. Additionally, blue crabs are of commercial importance and the crab-associated microbiome may affect crab, fish, and human health.

I found that Proteobacteria ribotypes dominated (>50%) the gut microbiomes of most (67%) of the 15 fish species examined (reported in Chapter 2). However, Firmicutes, Fusobacteria, Spirochaetes, and Tenericutes, not Proteobacteria, were the dominant ribotypes found in the guts of 5 fish species. Our data also suggested that fish-to-fish variability in composition of the gut microbiome was significant in some species, suggesting that the composition of the gut microflora community responds to external factors such as habitat and diet. We did not find a core microbial assemblage that encompassed all of the fish species. However, many of the OTUs present in one species'

core group were also found in the core groups of other species. The gut microbiome of piscivorous fish was less diverse than that of our omnivorous fish, suggesting that a more varied diet results in increased gut bacterial diversity.

This observation led to experiments designed to test the variability of the composition of the gut microbiome, and the potential influence of food-borne bacteria on the apparent composition of the gut microbiome (Chapter 3). I found that diet and diet-associated bacteria contributed ribotypes to the fish gut microflora, potentially complicating interpretation of gut microflora samples. However, I found that fish retain a core group of ribotypes that consistently constitute the bulk of the respective fish's gut microbiome. Diet had a greater effect on the composition of the gut microbiome of *F. heteroclitus*, which has a simple gut, than for *L. rhomboides* which has a more complex and differentiated gut. This may explain why some fish with simple gut tracts (i.e. the great barracuda) had high fish-to-fish variability of gut microbiome composition in my comparison study (Chapter 2). Diversity of the gut microflora community was lowest among *L. rhomboides* fed a strict carnivorous diet, greater among *L. rhomboides* fed an omnivorous diet, and greatest among those with herbivorous diet. Since the composition of the gut microflora community in *F. heteroclitus* was more directly related to diet-associated bacteria, diversity of their gut microflora was directly related to the diversity of the diet.

Chapter 4 attempted to assess if and how elevated temperature regimes affected the relative abundance of *Vibrio* spp. in fish gut microbiomes. *Vibrio* spp. ribotypes, including several that are documented fish and human pathogens, are found within fish guts. Our results from this study were inconclusive. We did not see a correlation

between the relative abundance of *Vibrio* spp. 16S rRNA genes and increased temperature in the *L. rhomboides* study, in part due to the rapid onset of sepsis in these fish once tank temperature was raised. In contrast, there was a statistically significant difference between the relative abundance of both *Vibrio* 16S rRNA and *V. vulnificus vvh* genes in the *F. heteroclitus* gut from the 20 °C and 32 °C treatments. Chapter 5 used field data to explore this same problem and provided strong evidence that the fish gut is a reservoir for *V. vulnificus* and *V. parahaemolyticus* during periods of sub-optimal temperature and/or salinity when these bacteria are apparently absent from oyster or water column samples. I concluded that fish are a link in the Vibrio life cycle, with viable cells passing between fish guts, the water column, and oysters through expulsion with fecal matter.

Lastly, Chapter 6 provided evidence that the crab microbiome was more diverse than reported previously in culture-based studies. Proteobacteria ribotypes dominated the crab microbiome; however, there were distinct differences between the microflora assemblages collected in carapace, gut, and hemolymph samples.

There is a diverse and abundant gut microbiome associated with finfish, sharks, and blue crabs. These gut microbiomes all have a core Proteobacteria community, which is in contrast to the human and terrestrial mammalian gut microbiome which is dominated by Firmicutes and Bacteroidetes ribotypes. This difference may partly be attributed to the fact that some of these Proteobacteria (such as *Vibrio* spp. and *Photobacterium* spp.) are typically associated with aquatic environments. Further research is needed to better the underlying mechanisms shaping the gut microbiome and resulting in differences in these gut microbiomes of fish and mammals. Thus, we need to

next address the functionality of the gut microflora so we can better understand how they contribute to fish physiology and health.

APPENDIX A

CHAPTER 2 SUPPLEMENTARY MATERIAL[1]

[1] Supporting Material for:
Givens, C.E. and J.T. Hollibaugh. To be submitted to The International Society for Microbial Ecology Journal.

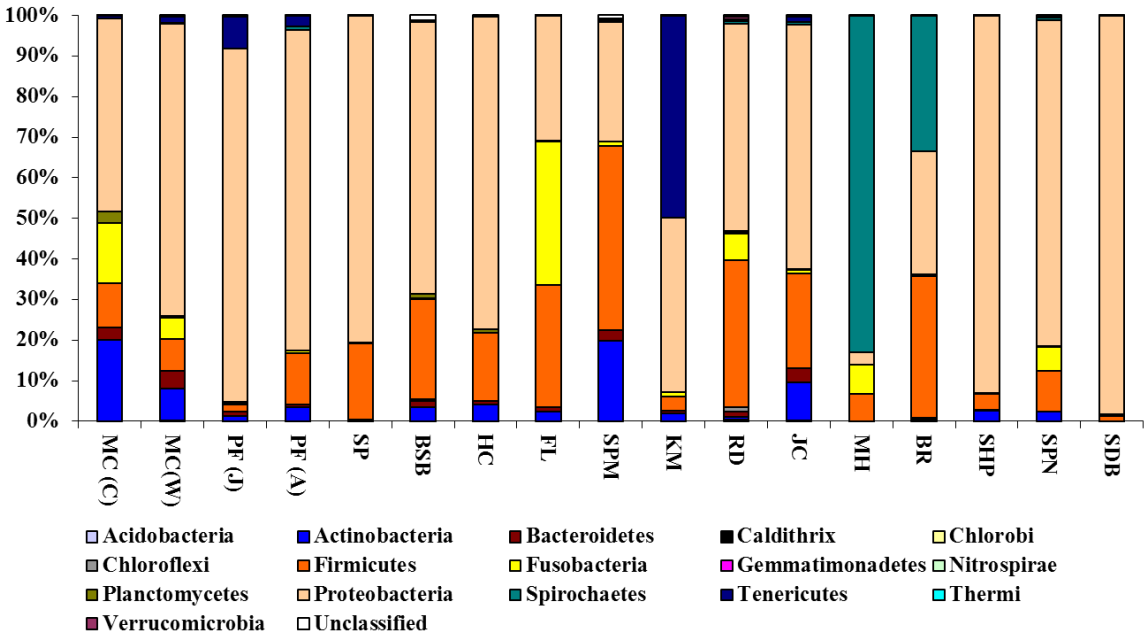
Supplementary Table 2.1: Number of sequences retrieved for all finfish and shark samples.

Sequences post-quality control refer to those remaining after the Qiime split_library.py workflow which filters reads based on length, primer mismatches, and quality score (<25). Number of chimeras refer to those sequences identified with the Chimera Slayer program in Qiime. Number of unrooted sequences refer to those sequences not binned as Bacteria. The last column refers to the total number of sequences used for our analyses and to calculate alpha and beta diversity metrics.

Sample	Sequences Post Quality Control	# of Chimeras	# of unrooted sequences	# of Chloroplast Sequences	Total Used in Analyses
MC1-C	30498	322	3314	1149	25713
MC2-C	14591	22	745	8556	5268
MC3-C	41963	32	83	40066	1782
MC4-C	35876	104	283	23910	11579
MC5-W	10317	75	622	187	9433
MC6-W	25639	992	2168	1247	21232
MC7-W	18079	134	9523	219	8203
MC8-W	32637	1317	2003	317	29000
PF1-J	16645	42	34	154	16415
PF2-J	13177	159	416	831	11771
PF3-J	57130	583	2812	1190	52545
PF4-J	23316	108	28	581	22599
PF1-A	34671	327	545	323	33476
PF2-A	17660	39	163	323	17135
PF3-A	15694	145	4	4	15541
PF4-A	10031	191	1379	409	8052
SP1	14348	193	748	17	13390
SP2	19381	103	1342	15	17921
SP3	42339	165	31471	445	10258
BSB1	22207	11563	1664	128	8852

BSB2	13359	14	11959	328	1058
BSB3	18138	32	54	447	17605
HC1	12571	67	222	331	11951
HC2	15050	198	1112	657	13083
HC3	21626	80	5950	328	15268
FL1	44908	100	15081	200	29527
FL2	33274	74	27972	161	5067
FL3	47845	116	38002	29	9698
SPM1	7921	10	7023	51	837
SPM2	18233	25	15610	256	2342
KM1	19880	29	221	0	19630
KM2	8057	61	1321	227	6448
RD1	22746	2691	5003	1154	13898
RD2	22887	2	19970	102	2813
RD3	21740	3044	730	53	17913
JC1	18093	60	14426	223	3384
JC2	8703	117	1008	236	7342
JC3	16052	428	51	18	15555
MH1	26288	22	310	0	25956
MH2	26689	1	116	0	26572
MH3	25337	5	223	1	25108
BR1	21861	3	21001	1	856
BR2	13548	0	17	1	13530
BR3	15302	365	28	0	14909
SHP1	18008	152	121	1	17734
SHP2	7563	54	482	68	6959
SPN1	8447	30	1648	43	6726

SPN2	11784	148	24	9	14449
SDB1	21498	6	9	0	11769
SDB2	30498	347	25	62	21064



Supplementary Figure 2.1: Phylum composition (%) of 12 finfish and 3 shark species with 454-pyrosequencing.

Percent compositions are averaged from 2-4 fish samples depending on species and do not reflect the sample-to-sample variability.

APPENDIX B

CHAPTER 6 SUPPLEMENTARY MATERIAL[1]

[1] Givens, C.E., K.G. Burnett, L.E. Burnett, and J.T. Hollibaugh. To be submitted to Marine Biology.

Supplementary Table 6.1: Percent (%) Composition of Proteobacteria ribotypes in Gut, Carapace Clip, Carapace Swab, and Hemolymph sequence libraries

Sample Type	Composition
Gut	71.7% γ-Proteobacteria, 25.7% ε-Proteobacteria, 1.77% α-Proteobacteria, 0.89% δ-Proteobacteria
Carapace Clip	50.0% γ-Proteobacteria, 46.4% α-Proteobacteria, 3.64% δ-Proteobacteria
Carapace Swab	81.2% γ-Proteobacteria, 14.9% α-Proteobacteria, 2.97% ε-Proteobacteria, 0.99% δ-Proteobacteria
Hemolymph	85.7% γ-Proteobacteria, 6.42% α-Proteobacteria, 7.86 β-Proteobacteria

Supplementary Table 6.2: Percent (%) Composition of γ-Proteobacteria ribotypes to Gut, Carapace Clip, Carapace Swab, and Hemolymph sequence libraries

Sample Type	Composition
Gut	45.6% *Photobacterium* sp., 25.9% *Marinobacter* sp., 23.4% *Vibrio* sp., 2.47% *Escherichia* sp., 2.47% *Thalassomonas* sp.
Carapace Clip	43.6% *Alteromonas* sp., 14.5% *Vibrio* sp., 7.27% *Thalassomonas* sp., 7.27% *Escherichia* sp., 3.63% *Enterobacter* sp., 1.82% *Colwellia* sp., 1.82% *Hailea* sp., 1.82% *Marinobacterium* sp., 1.82% *Pseudoalteromonas* sp.
Carapace Swab	54.9% *Alteromonas* sp., 12.2% *Pseudoalteromonas* sp., 10.9% *Thalassomonas* sp., 8.54% *Aestuariibacter* sp., 3.66% *Vibrio* sp., 2.44% *Hailea* sp., 2.44% *Marinobacter* sp., 1.22% *Neptuniibacter* sp., 1.22% *Oceaniserpentilla* sp., 1.22% *Salimonas* sp., 1.22 % uncultured γ-Proteobacteria
Hemolymph	43.3% *Acinetobacter* sp., 24.2% *Vibrio* sp., 10.8% *Alteromonas* sp., 8.33% *Marinobacter* sp., 6.67% *Escherichia* sp., 2.5% *Thalassomonas* sp., 1.67% *Enterobacter* sp., 0.83% *Neptuniibacter* sp., 0.83% *Photobacterium* sp., 0.83% *Pseudoalteromonas* sp.