Title

An Examination of Intestinal Microbiota of Mesopelagic Fish Reveals Microbial Community Diversity Across Fish Families

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Publication Date

2019

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA SAN DIEGO

An Examination of Intestinal Microbiota of Mesopelagic Fish Reveals Microbial Community Diversity Across Fish Families

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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2019

The Thesis of Caroline Iacuaniello is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2019

Dedication

In recognition of all they have done to help me along my path, this thesis is dedicated to my family, Frank, Margaret, Juli, Jeffrey, and my wonderful son, Kai.

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Acknowledgements

I am very grateful to Eric Allen for being a wonderful and supportive thesis advisor. I appreciate that he was always available to provide advice, inspiration and enthusiasm. I am also incredibly thankful for Jessica Blanton who taught me the ropes and provided insight, assistance, and advice every step of the way.

I would also like to thank Brice Semmens and Carolyn Kurle for both their assistance as my committee members, and the advice and lab space each provided during the isotope pilot study.

For assistance during the collection of samples I would like to thank Dr. Brice Semmens, Dr. Lisa Levin, and Dr. Ana Sirovik for allowing me to collect fish during their class cruises, Phil Zerovski and Dr. Natalya Gallo for providing assistance with fish taxonomy and general teleost information, and the entire crew of the R/V Robert Gordon Sproul.

For a crash course on mesopelagic fish, what to expect in the California Current, and how to dissect said fish, I would like to thank Cynthia Klepadlo. For further information on mesopelagic fish and assistance collecting, I would like to also thank Ben Frable.

I would like to extend a huge thank you to all the members of the Allen Lab; Marco Allemann, Sheila Podell, Alex Neu, Jeremiah Minich, they all made me feel very welcome in the lab. Also, for all her hard work assisting me while I cut fish, I would like to thank Quartz Clark.

Lastly, I would like to thank my incredible family. My parents, Frank and Margaret Iacuaniello, my siblings Juli and Jeffrey Iacuaniello, their families, and my amazing son Kai. They have all been incredibly supportive of me over the last many years and I cannot express enough how grateful I am!

Abstract of the Thesis

An Examination of Intestinal Microbiota of Mesopelagic Fish Reveals Microbial Community Diversity Across Fish Families

by

Caroline Iacuaniello

Master of Science in Biology

University of California San Diego, 2019

Professor Eric Allen, Chair

Mesopelagic fishes are of utmost importance to the health of global oceanic ecosystems. These fishes comprise the largest known marine biomass, and are a vital source of food many economically important fish and marine mammals. Further, they serve as a major component of the biological carbon pump, moving food items through the water column via diel migration. Though significant effort has gone into understanding species diversity, and positioning in marine food webs, the microbial component of these animals remains poorly characterized. Symbiotic microbial populations associated with the

gastrointestinal (GI) tract assist the host with nutrient uptake, digestion, defense against pathogenic microbes, but can also be detrimental to host health as parasites or pathogens. This study investigates the normal intestinal microbiota of wild mesopelagic fish to determine community diversity and distribution across multiple host families with varying migratory and dietary lifestyles. The results presented here indicate that mid-water fish have unique microbial communities from fish of other pelagic zones, where, for example, mesopelagic hosts show enrichment in Betaproteobacteria and Gammaproteobacteria microbial classes. Within mesopelagic fish families, major differences were seen: Gonostomatidae are enriched in three Mycoplasma taxa, Stomiidae had highest alpha diversity, Melamphaidae had the highest abundance of Planctomycetes, and Myctophidae gut communities were enriched in Betaproteobacteria. This study brings new understanding to the microbial ecology of the mesopelagic, and demonstrates that despite sharing space in the water column, mesopelagic fishes contain different microbial communities. Clearly, life history traits must be considered in addition to environment in order to better understand the mesopelagic ecosystem.

Chapter 1 Introduction

In the ocean, the mesopelagic zone is an area of the water column that extends from 200 meters down to 1000 meters. In recent years, interest in the mesopelagic region of the ocean has grown as it has become increasingly apparent that understanding this ecosystem is key to better management of the ocean. Fish that live in the mesopelagic zone are thought to be the most abundant fishes by biomass[1], as current studies place this biomass globally at upwards of ten billion metric tons[1,2]. Thus, mesopelagic fishes are an important component of pelagic ecosystems, especially as they serve as a main food source for larger fish and mammals. Among the many animals that prey on mesopelagic fish are many species of economic and ecological importance to humans including tuna[3,4], sharks[5], and swordfish[6,7] as well as a host of mammals like dolphins[8] and elephant seals[9]. Often referred to as the 'greatest migration of animals on earth', many mesopelagic fish, make diel vertical migrations[10,11], following their prey up to the surface during the night and returning to depths by day to escape predation. This was first discovered through the use of sonar when a 'deep scattering layer' was observed to move through depth and time. This diel migration is important to the biological carbon pump, a cycle where surface carbon is transported down to depth through biological processes[28,29]. In this case, the feeding, migrating fish bring fixed carbon in the form of prey items and fecal matter from the surface waters down to depth[12]. Because of this biological carbon cycle, the health of these fish becomes very important to the health of the ocean.

It has been suggested that this region might be a rich source of exploitation in the years to come, possibly utilizing these fish as a source of fishmeal for fisheries as well as nutraceuticals in the form of omega-3 oils[13]. It is thus imperative to more fully understand the mesopelagic ecosystem as a whole. Knowing the place of each species in the food web would allow for better management of the mesopelagic region so as to not lead to catastrophic overfishing. Further, understanding the role of these fish in the carbon cycle will be important as climate conditions change.

In recent years it has been repeatedly demonstrated that the health of an organism is heavily reliant upon its resident microbial community[14–17]. The gut (gastrointestinal tract) microbiota have been shown to be involved with a host's digestion and nutrient uptake, immune system development, and the ability to overcome potential pathogens[18]. With regards to marine animals, the majority of studies examining intestinal microbial communities have largely focused on aquaculture species or those that carry clear economic value[15]. A major objective of any microbiome study is to determine a core microbiome with the goal of possibly learning how to alter it for optimal host health, as is seen in research aiming to identify probiotic strains for aquaculture[15,18]. An argument for examining mesopelagic microbiomes is that mesopelagic fish make up the largest fish biomass in the ocean, it stands to reason that mesopelagic microbial communities are in turn the most plentiful microbes in the ocean. Up until now, nothing has been known about the structure or composition of these communities.

While there has long been interest in understanding the health of an animal in relation to its gut microbial community, surveying the full breath of microbial diversity has proven difficult through culture-dependent techniques alone. With the development of next generation DNA sequencing technologies, however, it has become routine to conduct whole community analysis on gut microbiota[19].

The first step in better understanding a microbial diversity within a host is to ascertain taxonomic composition through community genetic analysis. The current study represents the first analysis to date to catalogue the microbial populations associated with the gastrointestinal tract of multiple mesopelagic fish species using high-throughput 16S rRNA gene amplicon sequencing. This study will provide a baseline analysis to which future research may compare to determine how, if at all, microbial mesopelagic communities change over time or in response to changing oceanic conditions. Results from this study show that the mesopelagic fish gut microbiome is unique when

compared to the microbiomes of fish from other oceanic regions. This further emphasizes the unique biology and ecology of these globally abundant and ecologically important marine animals.

Chapter 2 Results and Discussion

Mid-water fish are distinct when compared to fish from other pelagic zones

Representative samples of five mesopelagic fish families, Gonostomatidae, Stomiidae, Myctophidae, Melamphaidae, and Sternoptychidae were collected, intestines were removed and processed for whole community microbial sequencing (Figure 1, Table 1). Data was compared between these mesopelagic fish and that of fish specimens from other pelagic zones (hadal, coastal pelagic, coastal demersal, bathydemersal, and bathypelagic) that had been generated by Jessica Blanton in the Allen lab. When comparisons were made at a phylum level, the mesopelagic fish communities showed little divergence from communities within fish from all other pelagic zones, however at the class level distinct divergences became apparent. The mesopelagic fish clustered with some hadal demersal samples and some coastal pelagic samples. Family level analysis showed similar patterns. When assessed at genus level, some overlap was seen between mesopelagic fish and coastal pelagic fish, as well as coastal benthopelagic elasmobranchs. Bathydemersal snailfish and hadal demersal fish occupied close space to the mesopelagic samples yet still cluster apart, according to fish family.

At the OTU level, using Shannon-Jensen Divergence metric ordinated on a NMDS plot, the microbial communities of mesopelagic fish were divergent from the majority of the other pelagic zones and seawater (Figure 2). While it was noted that the stress on the NMDS plot at the OTU level was 0.215, slightly higher than the accepted 0.2 limit[20], the patterns seen merited a closer look and further analysis lend insight to observed differences. In the OTU-level ordination, the mesopelagic

communities overlap with the coastal pelagics, and bathydemersal snailfish. Further analysis of differential abundance (Figure 3) showed enrichment and relative abundance in mesopelagic fish for the microbial classes of Betaproteobacteria and Cyanobacteria in comparison to fish from other pelagic depths. Interestingly, mesopelagic fish were comparatively depleted in Mollicutes, thought they still contained a high relative abundance of this group. Highest enrichment (Log2 change) was seen of the Sphingobacteria, however this class did not show high relative abundance. The fish groups most similar in microbial communities to mesopelagic fish at a class level were benthopelagic elasmobranchs and coastal pelagic fish. Least similar at the class level were bathydemersal elasmobranchs, bathydemersal myxinii, and coastal demersal myxinii. These results make sense as they suggest more similarity in the microbial communities between pelagic fish of differing depths than between pelagic and demersal fish. This suggests that placement in the water column might be an important driver of fish microbiota, well worth considering in a future study. A heatmap comparing fish microbiomes from across the water column at the genus level suggested that mesopelagic fish had only one genus, *Actinobacter*, in common with the majority of other fish, however a core microbiome between mesopelagic fish emerged including genera *Mycoplasma, Vibrio, Synechococcus, Burkholderia-Paraburkholderia, Pseudomonas, Mythylotenera,* and *Sphingomonas* (Figure 4).

Microbial communities in mesopelagic fish are driven by biological factors such as diet or migratory status, or abiotic factors such as season or water conditions

It is known that biotic and abiotic factors are instrumental in determining microbial intestinal composition[40]. Consideration was given to the idea that perhaps diet rather than family is a primary driver of microbial diversity. To evaluate this, families were assigned diet status as per Drazen et. al 2017, (summarized in table 1). Fish families were assigned to one of three diets. Gonostomatidae, Myctophidae, and Sternoptychidae were grouped together as zooplanktivores, consuming copepods, euphausiids, ostracods, nauplii, and other small zooplankton[21]. Melamphaidae were classified as

gelativores, encompassing a diet of various jellies including medusae, ctenophores, salps, pyrosomes, and micronekton, with the caveat that they are likely opportunistic and might veer into pelagic micronektonivore territory[21]. The last assignment was made for the family Stomiidae, as pelagic micronektonivores, a diet that includes fish, shrimp, large mysids, and occasional cephalopods[21]. Migratory status, as linked to prey items, is another possible driver of microbial diversity. In order to assign migratory or non-migratory status, a literature search was performed. Families Stomiidae and Myctophidae were assigned migratory status[21] while Sternoptychidae, Gonostomatidae, and Melamphaidae were grouped as non-migratory[21–23].

Microbial diversity within each sample (alpha diversity) was examined using seven different metrics (observed, Chao1, ACE, Shannon, Simpson, InvSimpson, Fisher, (plot_richness, phyloseq with ggplot2 in R)). Results were consistent across all metrics showing highest diversity within fish in family Stomiidae, and lowest diversity within fish in family Gonostomatidae. Families Myctophidae, Sternoptychidae, and Melamphaidae all also had low diversity (Figure 5). However, each family had a surprisingly large spread (variation) among samples. Seawater samples were seen to have the highest diversity of all, but had the least variation between samples. Alpha diversity was high in pelagic micronektonivores (Stomiidae) and low between gelativores (Melamphaidae) and zooplanktivores (Myctophidae, Sternoptychidae, Gonostomatidae). Alpha diversity of migratory fish showed higher diversity than that of non-migratory fish (likely driven by family Stomiidae). On both migratory and non-migratory the variability within groups is large. Of relevance to the study of wild populations, no significant difference in alpha diversity was seen across cruise samplings.

Diversity between fish family microbiome communities (beta diversity) was evaluated using the Jenson-Shannon divergence with NMDS ordinations (ordinate, phyloseq, R, figures). At high taxonomic levels, there is little divergence between mesopelagic microbial communities. However, when examined at the OTU level, it is evident that samples within a family were less divergent than then between families (Figure 6). The Gonostomatidae, Melamphaidae, and Stomiidae fishes were

further from the center of the ordination, with less overlap between one another. Families Myctophidae and Sternoptychidae were more central in this ordination, with high overlap with other families. The seawater samples were the most divergent and did not cluster at all with the fish families (Figure 6).

The zooplanktivory (represented by the Myctophidae, Sternoptychidae, and Gonostomatidae) ordinate to the left on this plot, whereas the gelativores (Melamphaidae) and the pelagic micronektonivores (Stomiidae) cluster by diet and family, on the right side of the ordination. Differential analysis of gelativores and pelagic micronektonivores was not done as both groups consisted of only a single family (Melamphaidae and Stomiidae respectively), however zooplanktivores were examined more closely. At a class level, zooplanktivores were seen to be enriched in Mollicutes (likely driven by patterns in the Gonostomatidae and Myctophidae) as well as Betaproteobacteria. At an OTU level, 17 OTUs showed enrichment in zooplanktivores, including four *Mycoplasmas*, a *Vibrio* an unassigned Proteobacteria, and an *Actinobacter,* while 20 OTUs were depleted (Figure 7A).

Migratory fish, Myctophidae and Stomiidae, ordinated together to the bottom of the aforementioned NMDS (Figure 6), while non-migratory Gonostomatidae, Melamphaidae, and Sternoptychidae clustered to the top half of the ordination. Differential analysis revealed the migratory fish to be both enriched and abundant in one *Mycoplasma,* (due to family Myctophidae), and the non-migratory to be enriched in three *Mycoplasmas* (family Gonostomatidae) (Figure 7B).

Nothing was seen to imply that different cruises had significant impact on the sampled fish microbiomes (Figure 6).

Overall it was noted that while both the diet and migratory lifestyle analysis suggest that there is some community divergence due to each factor, there is so much divergence apparent within the lifestyles that at this point it seems like fish family better predicts the observed microbial community

diversity. That said, it should be noted that this study was not explicitly designed to address diet or migratory status, future study design would do well to consider these and other fish lifestyles.

Common bacterial phyla shared in mesopelagic fishes

Certain bacterial phyla that were prevalent throughout the majority of the samples were the Proteobacteria, Bacteroides, Cyanobacteria, Tenericutes, Actinobacteria, and Planctomycetes. Proteobacteria were seen to be especially abundant within two classes, Gammaproteobacteria and Betaproteobacteria (Figure 8). Highest diversity in Gammaproteobacteria was present in Stomiidae fishes, while the most diversity in Betaproteobacteria was present in Myctophidae. Alphaproteobacteria and Deltaproteobacteria, were present across the fish families, thought at lower abundances. Overall, Alphaproteobacteria had the greatest abundance within mid-water fish in genera *Sphingomonas* and, a bit more sporadically, *Methylobacterium*. The *Methylobacterium* while showing spread across all fish families, were not present in all samples. They were most prevalent in family Gonostomatidae. Families Myctophidae, Stomiidae, and Sternoptychidae had an abundance of *Bradyrhizobium* in addition. A group of uncultured bacteriums, along with Aegean_169 marine group was abundant in both seawater and Stomiidae. A core microbiome at the class level was determined to include seven main taxa that were seen in varying degrees between fish families. Those taxa were Gammaproteobacteria, Mollicutes, Betaproteobacteria, Cyanobacteria, Alphaproteobacteria, Planctomycetacia, and Flavobacteria.

When phylum Bacteroides was examined, the classes Sphingobacteria and Flavobacteria had the highest abundance of OTUs, with classes Cytophagia and Bacteroidia represented in lower abundances. Most common in the class Sphingobacteria was the genus Sediminibacterium, present across all families, but most abundant in family Myctophidae. Flavobacteria showed no such dominant genera. Overall a wide diversity of genera was predominantly seen in family Stomiidae. Within the phylum Cyanobacteria, the most abundant genus across all fish was by far *Synechococcus*. Within

phylum Actinobacteria, the majority of the OTUs were assigned to what is most likely the class Actinomycetes. Class Acidmicrobia was present to a lesser degree (table 2).

Higher microbial diversity is present within family Stomiidae

The family Stomiidae was observed to have the highest microbial diversity of any of the five families across all metrics. The highest abundance was seen in the class Gammaproteobacteria which was seen across all mid-water fish, dominated by the genera *Acinetobacter, Pseudomonos*, *Photobacterium,* and *Vibrio* (Figure 9B). This is not surprising as these genera are prevalent across marine teleosts[15]. However some differences arise by fish family. Stomiidae, a pelagic micronektonivore, has the highest diversity and abundance of Gammaproteobacteria. All of the aforementioned genera are seen in Stomiidae, as well as *Psychrobacter, Pseudoalteromonas*, and *Colwellia*. Seawater samples are richest in *Vibrio*, *Colwellia*, and *Pseudoalteromonas*.

As well as showing much diversity in Gammaproteobacteria, Stomiidae were rich in a multitude of bacteria from classes Alphaproteobacteria, Betaproteobacteria, Flavobacteria, Planctomycetes, and Cyanobacteria (Figure 9A). Two classes with particular abundance within the phylum Planctomycetes were Phycisphaerae and OM190. Both were slightly more abundant in family Stomiidae but did have some presence across other fish families. Flavobacteria were fourth most abundant in Stomiidae samples. In Stomiidae, Betaproteobacteria are comparatively depleted along with five other classes, but these fish are enriched in eleven classes of microbes.

Deltaproteobacteria did not appear to be abundant in the majority of sampled mid-water fish, however some abundance across this class was apparent in Stomiidae. Family Stomiidae showed abundance in the OM27_clade, as well as numerous uncultured members of delta proteobacteria.

Assessment with differential analysis showed Stomiidae are both enriched and depleted in 17 different OTUs. One OTU that is enriched in Stomiidae but depleted in all other families is a *Caedibacter* (Figure 10). The *Caedibacter* is present in only 9 of 25 Stomiidae samples

(36%) but is of note in that in those 9 fish it is abundant enough to be the third most abundant microbe in the collective Stomiidae samples. *Caedibacter* is of interest in that it is typically known to be a endosymbiont in Paramecium[26,27]. Perhaps this is indicative of a higher parasite load in those specific animals? It should be mentioned that *Caedibacter* is also present in a few samples across other fish families. Interestingly, wherever present it is, as in Stomiidae, present in high abundance.

Overall, Stomiidae had the greatest microbial diversity of any of the fish families studied. It is worth considering that this might be driven by diet, as this family is the only one surveyed that fell into the category of pelagic micronektonivores. Further, the migratory behavior of these fish might introduce a greater variety of microbes throughout the water column. It is possible that these animals, feeding on whatever small fish they might manage to hunt, and possibly catching less food on a daily average, might have a greater range of intestinal microbes as a result.

Family Gonostomatidae showed strong enrichment in three Tenericute OTUs

Tenericutes in mesopelagic fish were almost entirely *Mycoplasmas,* bacteria that are known to be parasitic or commensal with hosts, as well as often pathogenic[35]. They were most abundant in family Gonostomatidae, a non-migratory zooplanktivore. Interestingly, Gonostomatidae showed strong enrichment in three specific *Mycoplasma* OTUs, with high relative abundance of those OTUs throughout the samples. Also of note was that Gonostomatidae had little diversity overall, especially when compared to the diversity seen in family Stomiidae. At the class level, Gonostomatidae was most heavily abundant in Tenericutes, followed by Gammaproteobacteria, Betaproteobacteria, Cyanobacteria, and Alphaproteobacteria (Figure 11A). Top genera include *Mycoplasma, Acinetobacter, Photbacterium* and *Vibrio* (Figure 11B).

Differential analysis in R (DESeq2) revealed Gonostomatidae to be enriched in Mollicutes, and depleted in another ten classes. Specifically, Gonostomatidae shows enrichment and abundance in three OTUs, all *Mycoplasmas* (Figure 12). A *Vibrio* OTU that is present in Melamphaidae,

Sternoptychidae, and Stomiidae is depleted in Gonostomatidae, as is a different *Mycoplasma* OTU that is quite enriched in family Myctophidae. As already seen with the alpha diversity metrics, Gonostomatidae remain the fish family with least diverse microbial communities. These further analyses suggest a possible reason for the low diversity is that there is such high relative abundance of *Mycoplasmas* in their microbiome. From a biological standpoint, this is not entirely surprising. These are tiny fish that are non-migratory and are feeding exclusively on zooplankton. It stands to reason that they would have a less diverse microbiome than a Stomiidae or Melamphaidae.

Melamphaidae comparatively enriched in Planctomycetes

Melamphaidae, a non-migratory gelativore, is, like all mid-water fish, most enriched in Gammaproteobacteria. This family is especially enriched in *Photobacterium*, and has stronger abundance of genera *Coxiella* and BD1-7 clade when compared to Myctophidae, Gonostomatidae, and Sternoptychidae (all zooplanktivores).

Plancktomycetacia is the next most abundant class present in Melamphaidae (Figure 14A). Within the class Planctomycetacia, the greatest abundance was seen in family Melamphaidae, with Sternoptychidae and Stomiidae also having strong abundance. Genera *Blastpirellula*, *Rubripirellula*, *Planctomyces*, and Pir4 lineage were most abundant across Melamphaidae. The least abundance of Planctomycetacia was seen in family Gonostomatidae. Interestingly, families Myctophidae and Gonostomatidae were seen to have entire samples lacking in Planctomycetacia when said samples were particularly enriched in Betaproteobacteria, Tenericutes, or Alphaproteobacteria. The presence of Gammaproteobacteria did not appear to visibly impact the abundance of Planctomycetacia.

Top genera in Melamphaidae include *Photobacterium*, an uncultured Planctomycetia, *Vibrio* and *Mycoplasma*. Typically these genera are known to be pathogenic, the *Photobacterium*, in addition to being pathogenic, are often bioluminescent and symbiotic with their hosts[30]. It is interesting that these *Photobacterium* are so enriched in Melamphaidae, as this family, unlike the other four fish

families, does not have any photophores or other bioluminescent organs[36,37]. Perhaps the enrichment is instead due to the Melamphaidae being a gelativore and feeding on jellies and other animals that are themselves in symbiosis with these *Photobacterium*[31].

Differential analysis revealed that Melamphaidae is enriched only in Planctomycetes and depleted in three classes. At the OTU level, Melamphaidae had the greatest enrichment in two *Photobacterium* (enriched in 4 OTUs, depleted in 2) and was depleted in two *Mycoplasmas* (Figure 13).

Myctophids contain distinct Betaproteobacteria groups

Within Myctophidae, Gammaproteobacteria, Mollicutes, and Betaproteobacteria were the top three classes (Figure 14). Betaproteobacteria were not abundant in seawater samples overall, only two genera were present (OM43_clade and MWH_UniPi aquatic group). The genera that were the most prevalent across all fish families were *Burkhoderia-Paraburkhoderia* (less represented in Myctophidae) and one uncultured genera. Specifically, those samples of Myctophidae that showed less abundance of *Burkholderia-Paraburkholderia* were seen to be instead rich in genera that were not seen as frequently in other fish families. This genus, *Burkholderia-Paraburkholderia,* includes known pathogens (*Burkholderia*) and environmental microbes (*Paraburkholderia*)[34]. At the class level Myctophids show enrichment in Betaproteobacteria but depletion in seven other classes (Figure 16A). Genus *Methylotenera* was heavily represented in the Myctophidae as well as present across samples of Family Sternoptychidae, both zooplanktivores. Top genera in Myctophidae include *Ancinetobacter, Mycoplasma, Vibrio, Synechococcus* (Figure 16B).

Of note in Myctophidae was the fact that there was high abundance and diversity of Betaproteobacteria. In this class genera *Burkholderia-Parabukholderia, Methylophilus, Hydrogenophaga*, and *Vogosella*, as well as an uncultured Betaproteobacteria, were present and

abundant across myctophid samples (Figure 17). The genus *Mythylophilus* is known to be a methanol-utilizing bacteria[32], *Hydrogenophaga* is a hydrogen-oxidizing bacteria[33]. The presence of these bacteria in mesopelagic fish, and the heightened presence in Myctophidae, is curious and certainly worth further examination into whether it is due to some host-specific function, connected to diet, or to some external factor. These genera also often appear in the same samples of Sternoptychidae that *Methylotenera* does. It is worth noting that the enrichment in these unusual bacteria is not well explained by diet, as the Gonostomatidae, also zooplanktivores, do not show the same trend. It is further not well described by migratory status-Myctophids are migratory where Sternoptychidae are not.

Myctophidae, migratory zooplanktivores, also showed some enrichment and great abundance in one specific *Mycoplasma*, however these were different OTUs than the three *Mycoplasmas* that were abundant within the non-migratory Gonostomatidae. With the exception of a few samples, families Sternoptychidae, Stomiidae, and Melamphaidae appear to have less *Mycoplasma* abundance. Myctophidae had were enriched in 11 OTUs and depleted in 26. (R package DEseq2) (Figure 17).

Sternoptychidae lack enriched or depleted taxa

Sternoptychidae had the same top three classes in their microbiome as had been seen the other fish families, Gammaproteobacteria, Mollicutes, and Betaproteobacteria (Figure 18A). Top genera include *Acinetobacter, Vibrio, Photobacterium,* and *Synechococcus* (Figure 18B).

However, using differential analysis, Sternoptychidae was found to neither be enriched nor depleted in any classes. Overall this family showed the least difference from the other families, it was enriched comparatively in no OTUs and was depleted in only one *Mycoplasma*. This remained the case across all analysis on family Sternoptychidae. This is not unexpected when earlier results are considered, in the NMDS (Figure 6), family Sternoptychidae was seen to occupy a very central space on the ordination, overlapping with all fish families and diverging very little from family

Myctophidae. It may be that this was because there was so much species diversity within the sampled Sternoptychidae. Within this family samples spanned two genera and 5-6 species of fish, whereas Gonostomatidae, Stomiidae, and Myctophidae only spanned two species and Melamphaidae 3-4.

Conclusions

In summation, it was seen that mesopelagic fish were in possession of a unique microbial composition when compared to fish from other pelagic zones. Of note, mesopelagics were comparatively both enriched and abundant in Cyanobacteria and Betaproteobacteria. Overall, similarities were seen between microbial communities of mesopelagic fish, with a core microbiome that includes Gammaproteobacteria, Planctomycetacia, Mollicutes, Cyanobacteria, Betaproteobacteria, Flavobacteria, and Alphaproteobacteria. There was however wide divergence between fish families due to abundance and genera within these groups. Further, host traits such as diet and migration status show some indications of being linked to microbial composition. These findings are important because they showcase how different these fish families really are, and how little is still known about them. It has been shown that mesopelagic fish have a unique microbial environment from other fishes, this implies that conclusions drawn from studies of fish from other pelagic zones are not necessarily applicable when considering mesopelagic fish. Further, when seeking to understand the linkage between host and microbiome within mid-water fishes, each family should be considered independently. Lastly, because host diet and migration status are likely linked to intestinal microbial composition, deeper sampling would be beneficial for future studies. Understanding as much as possible about the health and ecology of these fish is imperative to better understanding how to best manage their populations in order to keep the oceanic ecosystem healthy. This first study outlining the core microbiome in mesopelagic fish, and highlighting family-specific differences therein, will provide a reference point for future studies seeking to ascertain how ecological differences may affect these unique teleosts.

Future work

Future work on mesopelagic fish and their microbial communities should include examining correlations between diet, migratory status, and the microbiome in greater depth. Other biological factors that could be considered include animal gender, gravidity (if female), parasite load, and stomach fullness (how recently fed). Abiotic factors that might influence fish microbiota and merit a closer look include placement in the water column (benthic versus demersal or pelagic, etc.), water temperature, and oxygen levels.

Chapter 3 Materials and Methods

Materials and Methods

Sample collection

Representative samples of mid-water fish families were collected from aboard the Scripps ship R/V Robert Gordon Sproul (Table 3, Table 4). Collections for this study were taken on cruises SP1728, SP1729, and SP1733 (Table 5). Cruises SP1728 and SP1729 were for undergraduate marine laboratory class SIO_136. SP1728 was a one-day cruise on October 14, 2017 with Chief Scientist Ana Sirovic; SP1729 was the following day, October 15, 2017 with Chief Scientist Brice Semmens. SP1733 was a one-day cruise for graduate course Deep Sea Biology, SIO_277 with Chief Scientist Lisa Levin. All three cruises were funded by grants through SHIP funds.

Fish were harvested using an Isaacs-Kidd Midwater Trawl (IKMT), at depths of 550-820 meters. Once on board the ship, fish were bagged by family and immediately frozen on dry ice. Identifications were done visually using Pete Davison's key[24]. Upon returning to the lab all fish specimens were stored at -80^0 C until dissection.

Dissection

Before and after every dissection all dissection tools were sterilized with 75% ethanol and flamed. The dissection tray was covered with a paper towel and over that was a sheet of stretch-wrap. This was discarded after every fish and replaced with a clean sheet for the next fish. Fish were defrosted 3-4 at a time on ice. This minimized the time that a fish was thawing without losing too much time waiting for them to thaw. Each fish was weighed, measured, and photographed before dissection. Measurements were taken for standard length and, where possible, for fork length. Anything notable about the fish, for example the gravidity, or the presence of obvious parasites, was recorded and where possible, photographed. Dissections were done to obtain the intestine, stomach, and two muscle tissue samples (Figure 19). The intestine, stomach, and one muscle sample were rinsed three times with 500ul 75% ethanol and three times with 500ul sterile MilliQ water. The second tissue sample was washed only one time with sterile MilliQ water so as to not throw off later isotopic testing. While every effort was made to get muscle tissue only, many fish were so small that some skin remained on the sample. Bones were far to small to remove and are likely in all tissue samples. In extreme cases, due to the small size of the fish, whole fish bodies or heads were kept as the tissue sample. Samples were placed in pre-weighed eppendorf tubes directly after washing, weighed and recorded. Immediately after weighing, 500ul of Chaos Buffer (50mL: GSN: 26.59g (4.5M), 2% Sarkosyl: 5mL 20% soln of 50mM EDTA: 5mL 0.5M soln, dissolve in 25mL H2O @ 65C) was added to the stomach and intestine samples. Tissue samples were not stored in any buffer. Samples were placed on ice for the remainder of the dissection, than stored at -80 C until DNA extraction.

Phenol chloro-isoamyl alcohol extraction

Chemical lysis

Samples already having been stored in 500ul Chaos Buffer were transferred to a sterilized (autoclaved) tube filled with glass disruption beads (Research Products International, 0.1mm). To each

sample, 10ul Proteinase K (10mg/ml) and 50ul Beta-Mercaptoethanal (C2H6OS) were added. Samples were next incubated in a heat block, in water, at 55C for 60 minutes, inverted ~every 15 minutes.

Mechanical lysis

Samples were removed from the heat block and homogenized in a Biospec Products Mini-beadbeater at high speed for 20 seconds. After homogenizing they were briefly centrifuged. Organic extraction: An amount of Phenol chloro-isoamyl alcohol that was equivalent to the total volume of the liquid inside each sample tube was added. For most samples this was around 750ul. Samples were mixed using a Fisher Scientific rotating mixer on '2' for 5 minutes, then centrifuged at maximum for 5 minutes. Supernatant was removed and transferred to a clean new tube.

Zymo cleanup

For DNA cleanup Zymo Research *Quick*-gDNA Miniprep Kit (Cat. No. D3024) was used. To the supernatant a volume of isopropanol equal to one third the volume of supernatant was added. This entire liquid was than loaded onto a macro column (except in the case of 16 samples on 4/12/18 which were loaded onto micro columns) and centrifuged at max for one minute. The outside of the column was rinsed with 500ul of 100% EtOH and the column was moved to a clean tube. Next 200ul PreWash Buffer was added, spun 1 minute at max, and column was moved to a clean tube. A mastermix of 5ul RNaseA and 95ul Wash Buffer per sample was mixed and 100ul of this mix was added to each column. This was sealed with parafilm and transferred to a water bath (heat block) to incubate one hour at 37C. After incubation the tubes were spun for 1 minute. 400ul Wash Buffer was added and spun through for one minute. After a final two-minute spin, columns were allowed to air dry for five minutes before eluting in 100ul of elution buffer. Most samples were eluted in Zymo DNA elution buffer, some were eluted in Mo Bio PowerSoil DNA Isolation Kit Solution C6 (cat # 12888-50-6). Following cleanup, samples were quantified using Qubit and a PCR check to verify that no inhibitions to amplification remained was done.

DNA cleanup

Samples that required extra cleanup were treated using MoBio Powerclean DNA Cleanup Kit Catalog # 12877-50 following the manufacturer's protocol. For each cleanup, 50ul of sample was used (except for two samples that were cleaned a second time as the first round was used up- in this case only 25ul of sample were used because so little remained) and nuclease free water was used to bring the quantity to the required 150ul. Samples were eluted in 20ul of Zymo DNA elution buffer instead of the Solution 7 from the kit.

Water filter extraction

Microbial seawater communities were extracted from three filters. The filters were collected on different cruises than the fish, two were collected on cruise SP1608, at depths of 50m and 340m. The last was collected on cruise SP1527 at a depth of 832m.

Extraction was done using MoBio PowerWater Sterivex DNA Isolation Kit (catalog number 14600-50-NF). All three filters were extracted as per the protocol,with the exception of two steps. In step 11, due to the vortex bouncing, the unit was vortexed at 7 rather than max. In step 25, when solution ST4 was added to supernatant, because solution needed to be kept warm so as not to clog the binding column, each sample was broken into five aliquots 1mL and kept on the heating block until loading on column. Final volumes of 100uL were eluted in PowerWater Sterivex Solution ST7.

Qubit

DNA assays were done using a Qubit 2.0 flourometer, High Sensitivity buffer, High Sensitivity DNA dye. (Standards: 10ul standard 1, 2, 90ul HS buffer. DNA: 2ul sample, 98ul HS buffer. Dye mastermix: 1ul dye in 99ul HS buffer, 100ul mastermix/tube)

PCR amplification

16S rRNA

After DNA was quantified, PCR amplifications were done in 24uL reactions to ascertain what size of DNA was present. One microliter of template was added to each tube. Primers for the 16S rRNA V4 region were used (ill515F and ill806rb) at a quantity of 0.5uL each per reaction. The

polymerase (Hot Start 2X NEB Taq (MO496S)) was added in the amount of 12.5uL per reaction, and ultra pure water was added to bring the entire reaction to 25uL. Reactions were processed for a cycle consisting of an initial denature of 1 minute at 94C. Following that, a repeat cycle starting with a denature of 30 seconds at 94C, an annealing step at 50C for 30 seconds, and an extension at 68C of 1 minute was performed a total of 35 times. After the last repeat, a final extension of 68C for 5 minutes was done once and then the temperature was held at 4C.

Gel electrophoresis

Gel electrophoresis was used to determine whether amplification of the 16S rRNA gene was successful. One percent agarose gels (125 ml 1X TAE buffer, 1.25g agarose, 7.5ul Syber Safe) were covered with 1X TAE running buffer (10X consisting of 48.4 g Tris base, 11.42 mL glacial acetic acid, 7.44 g Na2EDTA-2H2O, filled to 1L with milliQ H2O). Five microliters of each sample was combined with two microliters of loading dye. The gel electrophoresis was set at 100V for 35 minutes. Resulting gels were examined under ultraviolet light (Bio-Rad, photographed, and viewed with Quantity One 4.6.9 1-D Analysis Software.)

Troubleshooting

12S rRNA contamination

It was seen during PCR amplification that incredibly low amounts of 16S gene fragments were present in the samples compared to host DNA in the form of 18S and mitochondrial 12S. Initially the small band 12S was mistaken for 16S bands on gels, however it was eventually determined that they were 12S via Sanger sequencing. At this point many samples had little to no visible band after 35 cycles of PCR amplification using 16S amplification primers. For this reason, the majority of samples required further work in the form of gel extraction. Fortunately, most samples were salvageable. After final analysis only six samples sent to Next Generation Sequencing were dropped.

Gel extraction

Gel extractions were done to obtain bands at the expected 16S rRNA length of ~ 360bp while excluding the larger 18S band (~550bp). Gels used were 1% agarose gel (125 ml 1X TAE buffer, 1.25g agarose, 7.5ul Syber Safe) and were placed in fresh 1X TAE running buffer (10X consisting of 48.4 g Tris base, 11.42 mL glacial acetic acid, 7.44 g Na2EDTA-2H2O, filled to 1L with milliQ H2O). Samples were PCR amplified in triplicate, combined with 3ul loading dye, and loaded onto gels. The gel electorophoresis was set at 80V for 100 minutes. After doing the electorophoresis, samples were carefully excised with sterile (ethanol/flame sterilized before every sample) blades under UV light. Gel extractions were done using a Zymoclean Gel DNA Recovery Kit. Each sample was transferred to a sterile (autoclaved) pre-weighed 2mL tube. Tubes were weighed again and the gel weight calculated. For each volume of agarose gel, three volumes of ADB were added. Samples were briefly vortexed and incubated in a water bath (heat block) for 15 minutes until agarose was fully dissolved. Next samples were transferred to micro spin columns and centrifuged 1 min at 10,000 x g. This step was repeated until all liquid had been passed through the column. Liquid was discarded. After the column was loaded, 200ul DNA wash buffer was added, centrifuged 30s at 10,000 x g, and discarded. The wash step was performed twice, than sample was eluted in 20ul zymo DNA elution buffer (10,000 x g, 1 min). Qubit readings were taken after all gel extractions.

Sequencing and analysis

Bacterial community profiling with 16S rRNA gene sequences

Sequencing barcodes were added to each sample's amplicon as described in Illumina's guide to "16S Metagenomic Sequencing Library Preparation". Barcoded amplicons were cleaned, pooled in equimolar concentrations, and multiplexed on a single run of 2×300 bp sequencing on Illumina's MiSeq platform at the UC Davis Genome Center sequencing core.

Reads were trimmed with Trimmomatic version 0.339, and merged using FLASH version 1.2.1110. Successfully merged sequences from all samples were combined into a single file, and filtered to a minimum average quality score of q20 using scripts in Qiime version 2.6. Primers were

removed with Cutadapt version 1.9.112, and were filtered again with Qiime scripts to exclude reads outside 200-600 bp in length or containing homopolymer runs greater than 6 bp. Sequences were checked for chimeras against the Ribosomal Database Project gold database (training database v9) using vsearch version 1.1.1 (https://github.com/torognes/vsearch). Sequences were denoised using the DADA2 version 1.9.1 algorithm and, and taxonomy was assigned using a the SILVA v123 database. Eukaryotic, chloroplast, and mitochondrial sequences were removed from the dataset. Singletons were discarded, and samples were normalized using the cumulative-sum scaling method in the R package MetagenomeSeq version 1.1216.

Further analysis was done in R. Differential analysis was done with DESeq2. Heatmaps of microbial abundance and NMDS plots were generated in phyloseq with ggplot2, cowplot, and gridextra. Packages magrittr, dplyr, and plyr, were also used to run R script.

Figures and Tables

Figure 1. Representative fish from five families. From left to right: Family Sternoptichydae-*Argyropelicus aculeatus, Sternoptix* sp., Myctophidae-*Triphoturus mexicanus*, Gonostomatidae-*Cyclothone pacifica*, *Cyclothone signata*, Stomiidae-*Stomias atriventer*, and Melamphaidae-*Scopelogadus bispinosus*.

Table 1. Five fish families with migratory and diet assignments.

Fish Family	Fish Species	Migratory status	Diet
Myctophidae	*Triphoturus mexicanus* *Nannobrachium regale*	Migratory[25]	Zooplanktivore[21]
Sternoptychidae	*Argyropelicus spp.* *Sternoptyx spp.*	Non-migratory[25]	Zooplanktivore[21]
Gonostomatidae	*Cyclothone spp.*	Non-migratory[25]	Zooplanktivore[21]
Stomiidae	*Stomias atriventer* *Idiacanthus antrostomus*	Migratory[21]	Pelagic Micronectonivore[21]
Melamphaidae	*Scopelagudus bispinosus*	Non-migratory[25]	Gelativores[21]

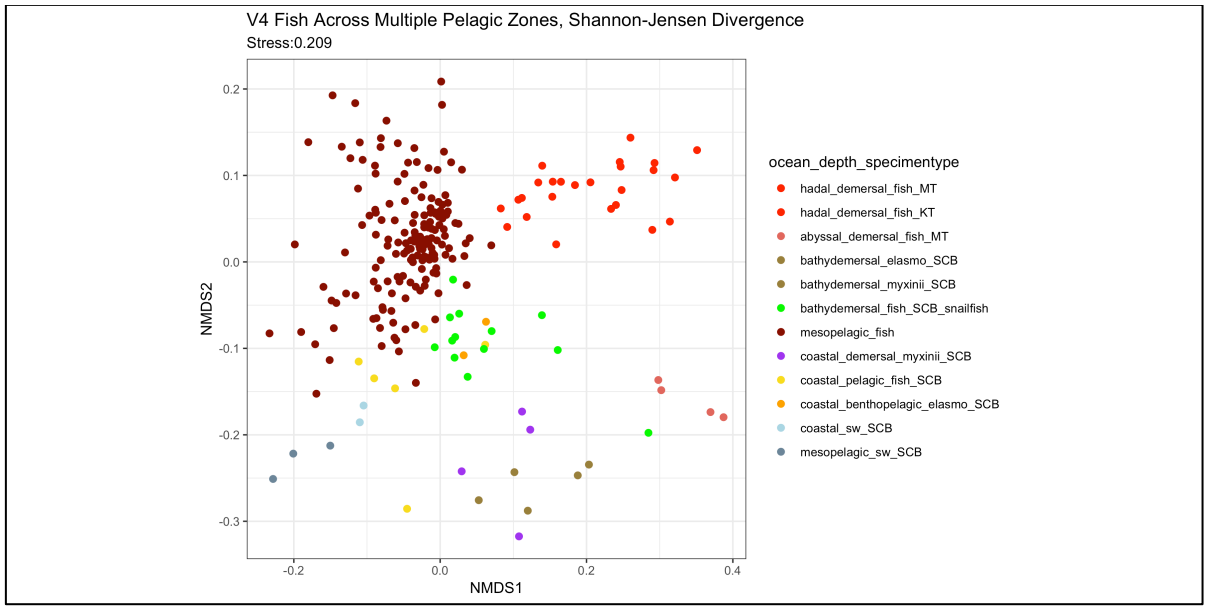


Figure 2. Fish from varying pelagic depths NMDS ordination of Shannon-Jensen divergence at the OTU level. Mesopelagic fish show divergent microbial communities from fish in hadal and bathydemersal zones, while showing less divergence from coastal pelagic and demersal waters

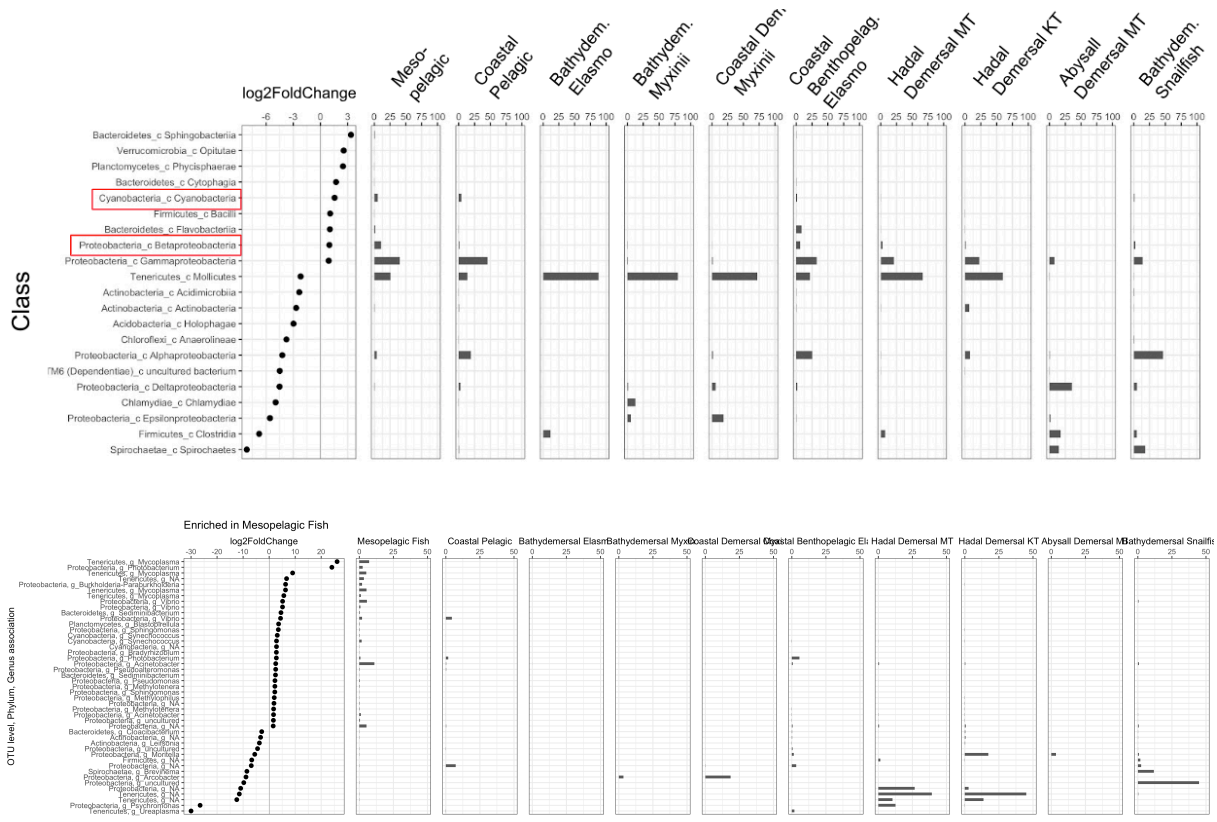


Figure 3. Top: Differential analysis of enrichment in mesopelagic fish compared to other pelagic fish at class level. Mesopelagic fish are enriched and show abundance in Cyanobacteria and Betaproteobacteria. They are depleted comparatively but show high abundance in Mollicutes. Bottom: OTU level analysis shows enrichment in 30 OTUs and deplete in 15. Relative abundance in enriched OTUs was seen in a few Mycoplasmas, Burkholderia-Paraburkholderia, and some Vibrios.

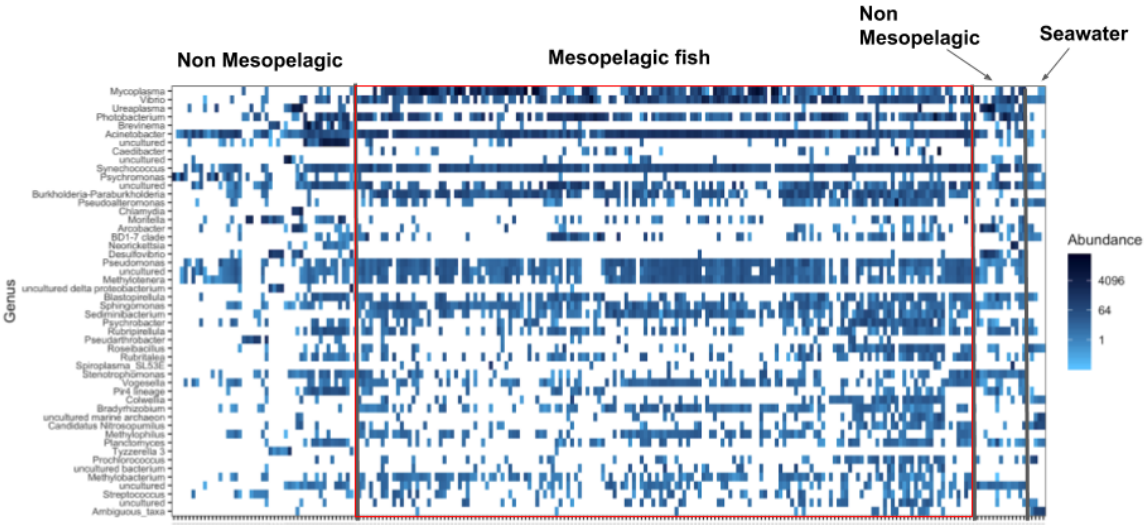


Figure 4. Heatmap at genus level of mesopelagic fish and non-mesopelagic fish

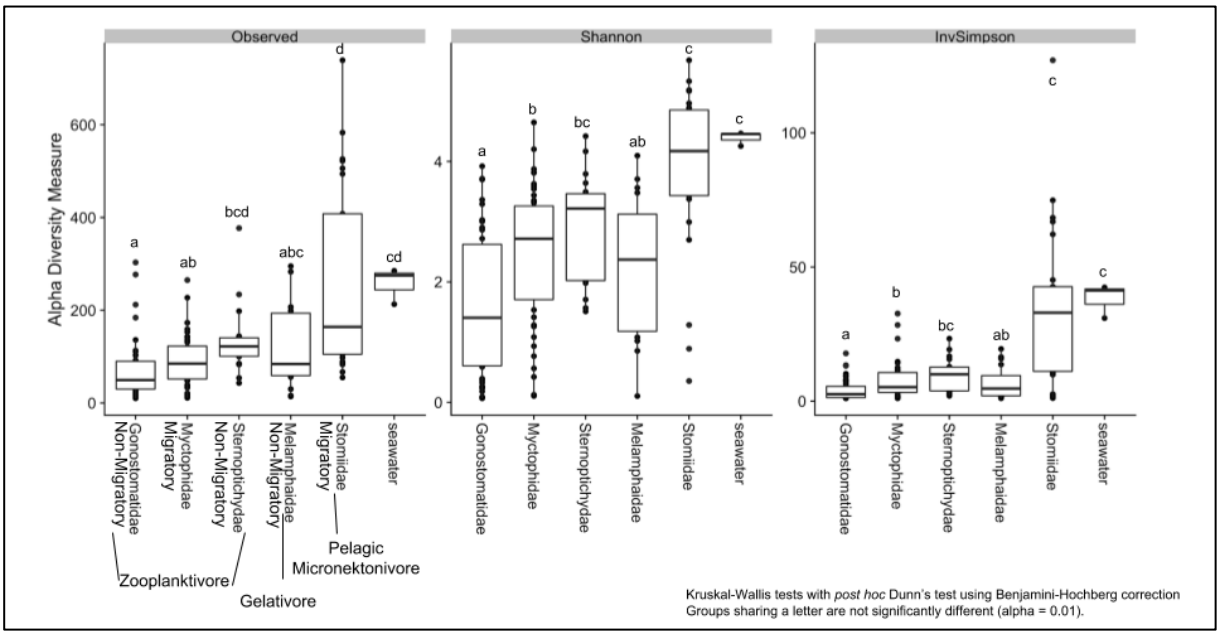


Figure 5. Alpha diversity plotted by three metrics. Kruskal-Wallis tests with *post hoc* Dunn's test using Benjamini-Hochberg correction. Groups sharing a letter are not significantly different (alpha = 0.01).

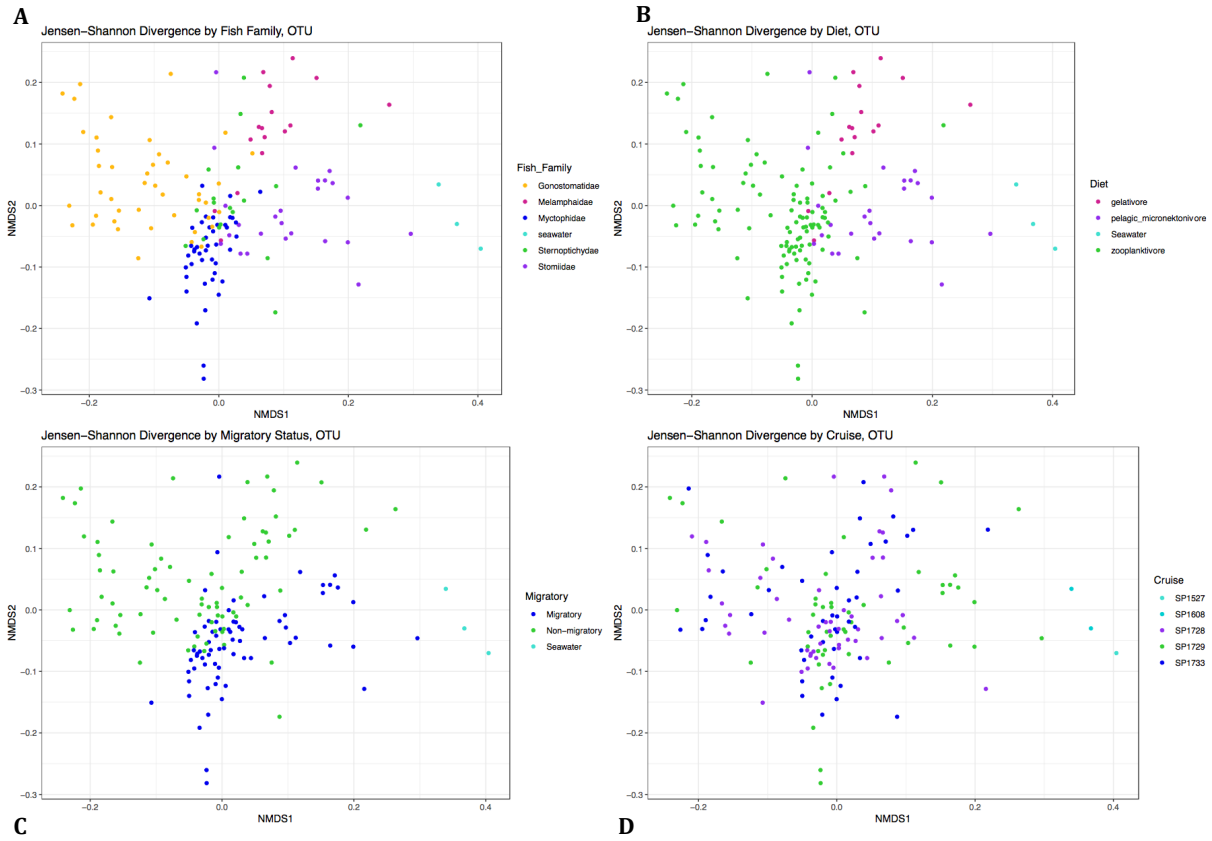
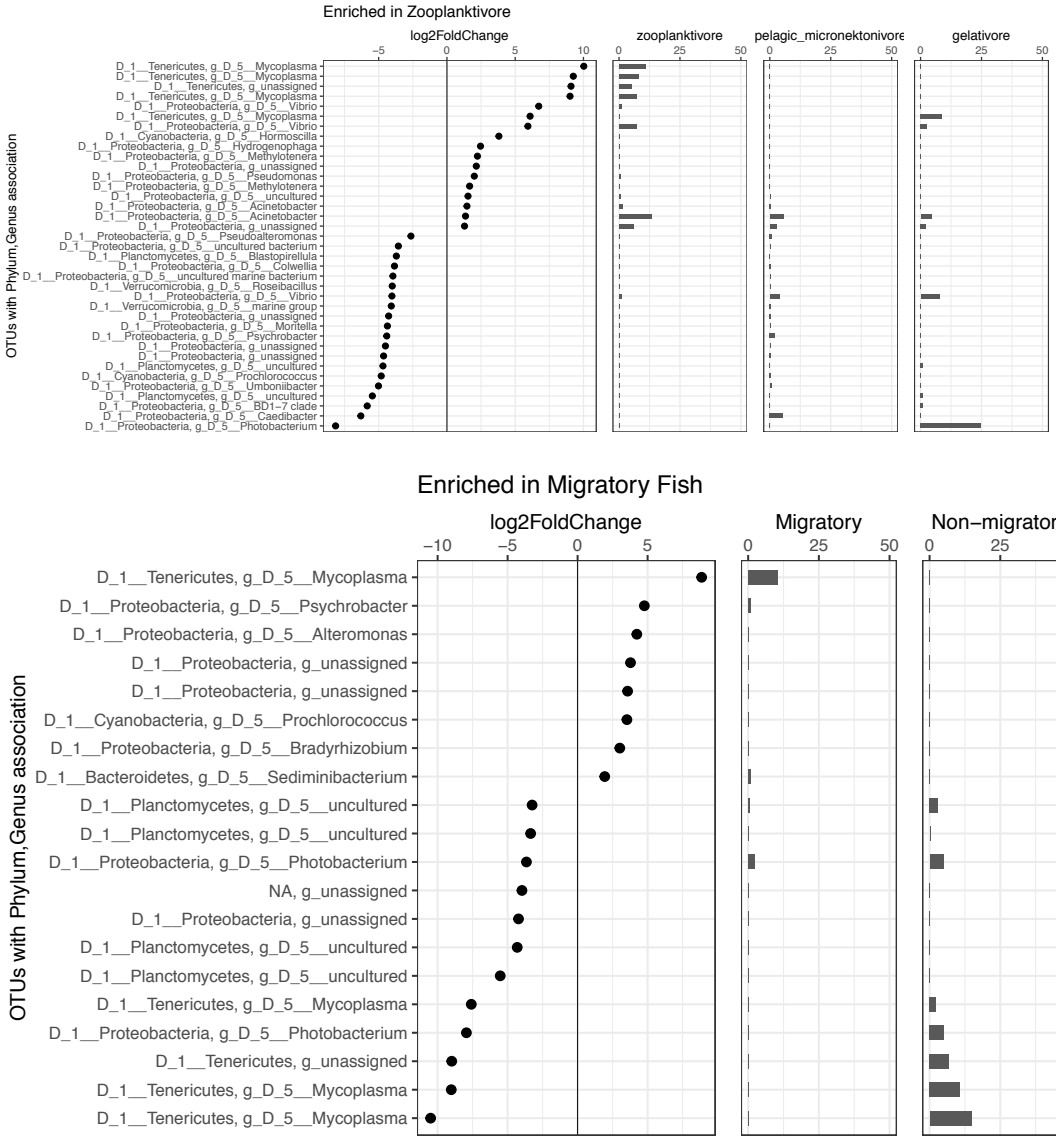


Figure 6. Mesopelagic fish ordinated on NMDS, recolored according to family, diet, migratory status, and cruise. OTU level Jensen-Shannon Divergence ordinated on NMDS. Four panels colored by: A) Fish family, divided into microbial communities of five mesopelagic families and seawater. Shows some divergence in families Gonostomatidae, Melamphaidae, and Stomiidae. Shows divergence from seawater. B) Diet divided into gelativores, pelagic micronektonivores, zooplanktivores, and seawater. C) Migratory status, divided into migratory, non-migratory, and seawater. D) Divided into three collection cruises, SP1728, SP1729, SP1733, and two seawater cruises, SP1527 and SP1608.



A

B

Figure 7. A. Differential abundance in zooplanktivores. Zooplanktivores include 3 families, 9-10 species, and 106 fish. Pelagic micronektonivores include 1 family, 2 species, 25 fish. Gelativores include 1 family, 3-4 species, 16 fish. B. Differential abundance in migratory fish. Migratory fish span 2 families, 4 species, and 69 fish, non-migratory fish span 3 families, 9-11 species, and 78 fish.

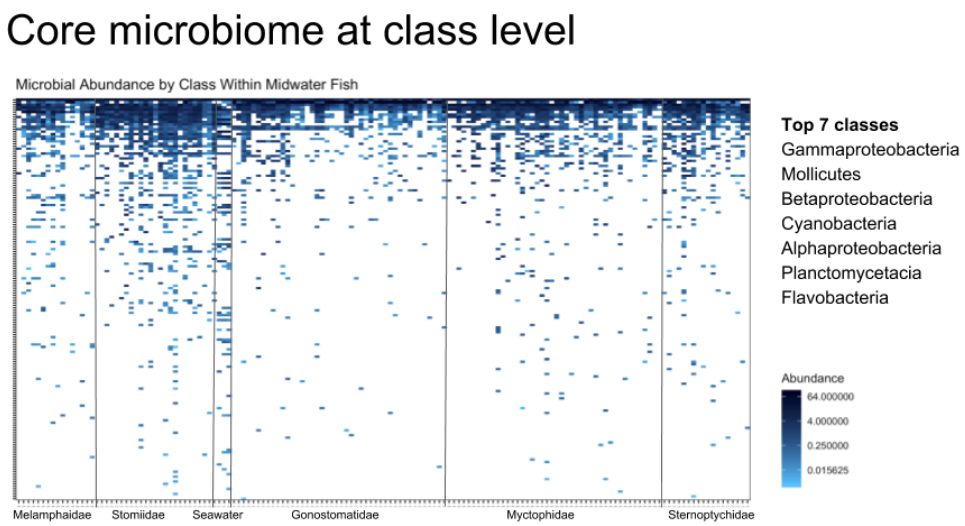


Figure 8. Heatmap of microbial abundance by class across five mid-water fish families and seawater.

Table 2. Top 10 bacteria by fish family

Class	Midwater fish	Myctophidae	Melamphaidae	Sternoptychidae	Stomitidae	Gonostomatidae	Seawater
Sphingobacteria	x	x		x	x	x	
Flavobacteriia	x	x	x	x	x	x	
Planctomycetacia	x	x	x	x	x	x	
Mollicutes	x	x	x	x	x	x	
Cyanobacteria	x	x	x	x	x	x	x
Verrucomicrobiae	x		x	x	x		
Alphaproteobacteria	x	x	x	x	x	x	x
Gammaproteobacteria	x	x	x	x	x	x	x
Betaproteobacteria	x	x	x	x	x	x	
Deltaproteobacteria	x		x		x		x
Actinobacteria		x	x			x	
Bacilli		x		x		x	
Marine Group I							x
Thermoplasmata							x
Arctic97B-4 marine group							x
uncultured bacterium							x
SAR202 clade							x
Acidimicrobiia							x

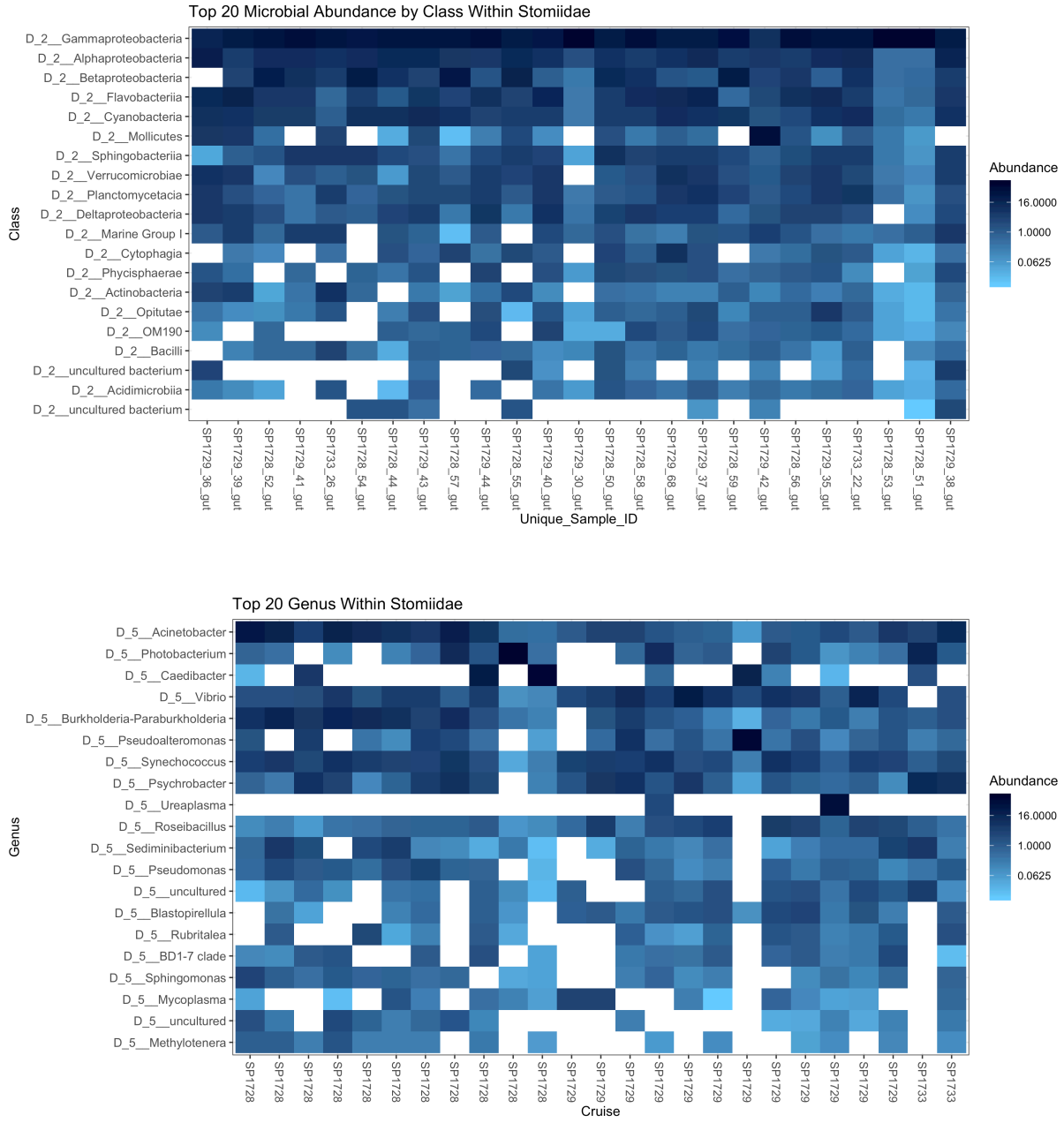


Figure 9. A. Heatmap of Microbial Abundance within Stomiidae, class level. Gammaproteobacteria are most abundant, followed by Alphaproteobacteria, Betaproteobacteria, Flavobacteria, and Cyanobacteria. B. Top 20 genus in Stomiidae. Most abundant are *Acinetobacter, Photobacterium, Caedibacter, Vibrio,* and *Burholderia-Paraburkholderia.*

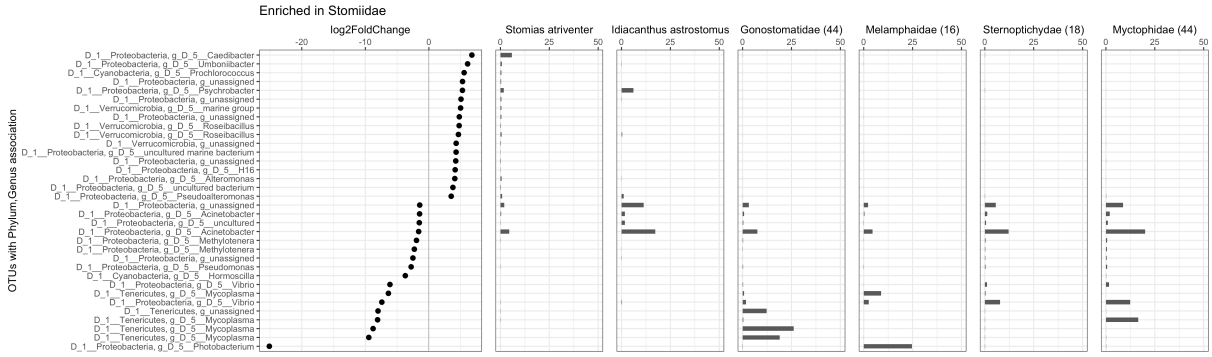


Figure 10. Differential analysis of Stomiidae compared to other mesopelagic fish.

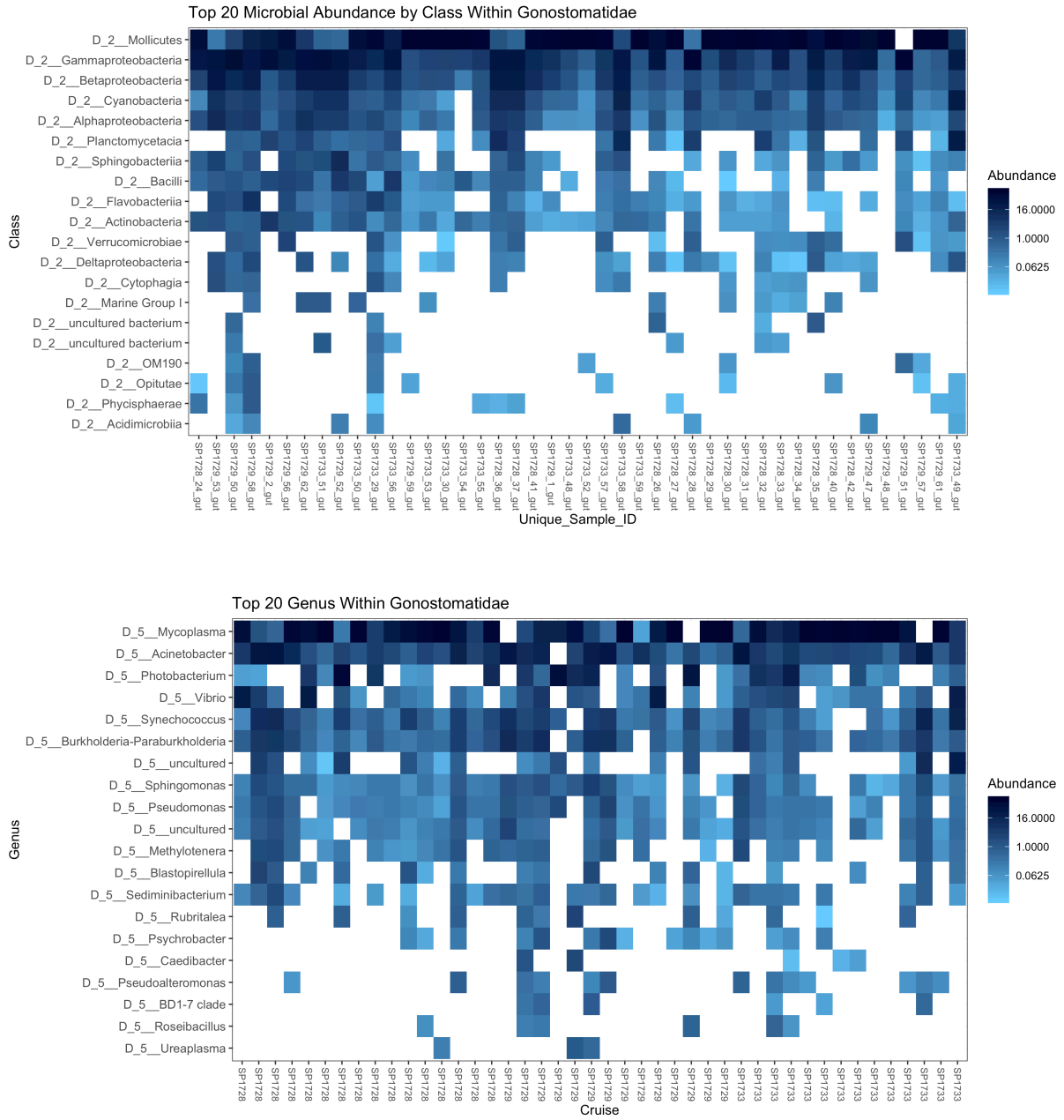


Figure 11. A. Heatmap of Microbial Abundance within Gonostomatidae, class level. Mollicutes are most abundant, followed by Gammaproteobacteria and Betaproteobacteria. B. Genus level, top genera include *Mycoplasma, Acinetobacter, Photbacterium* and *Vibrio*.

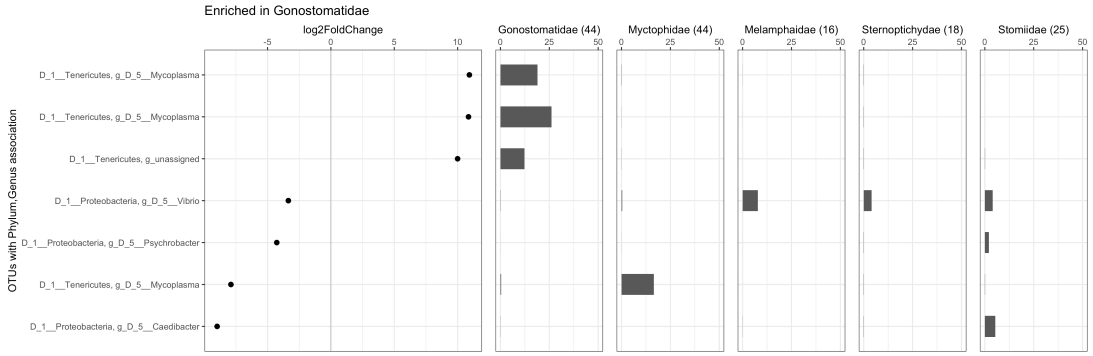


Figure 12. Differential analysis OTU level, Gonostomatidae. Three *Mycoplasma* are both enriched and relatively abundant.

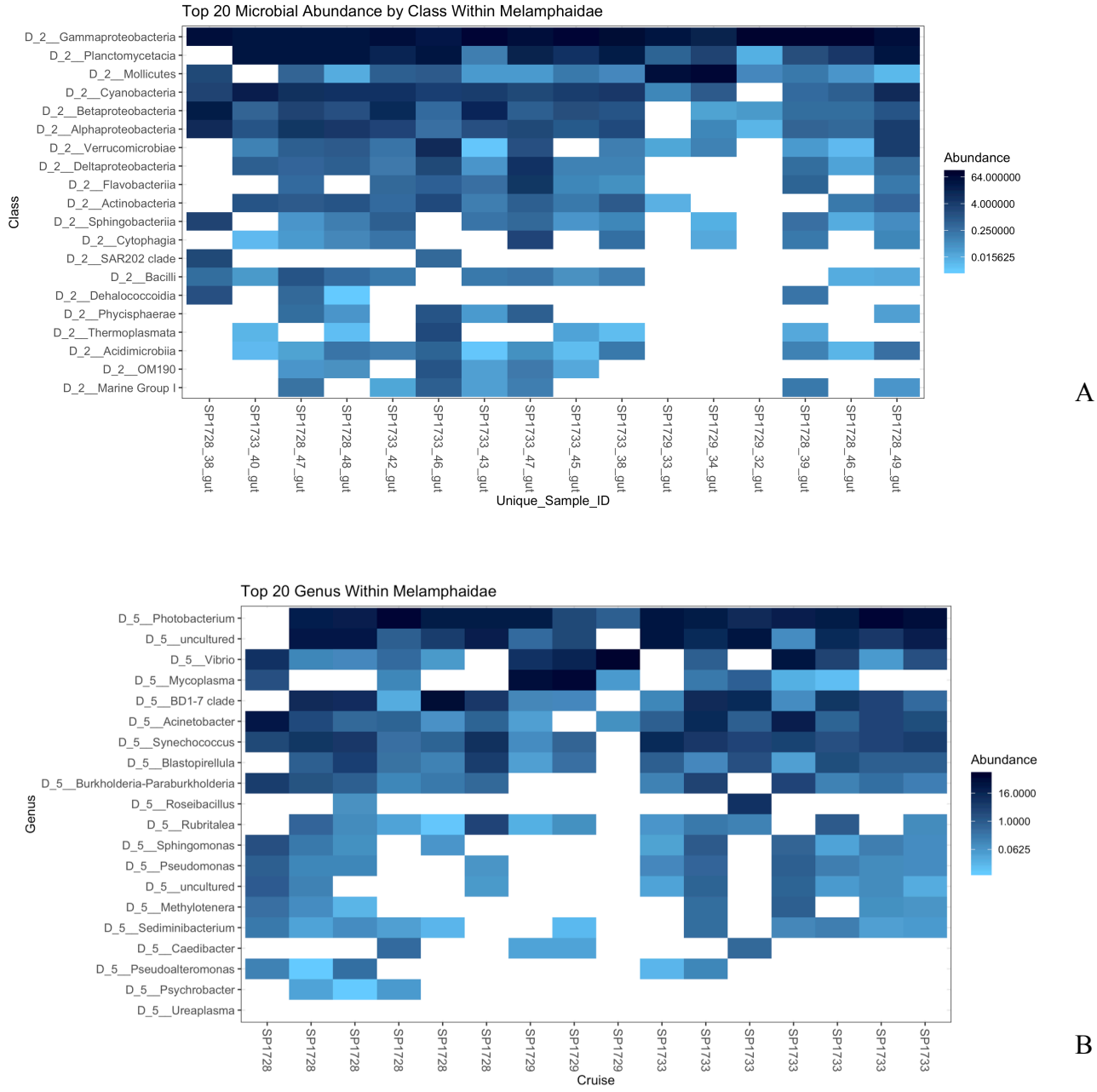


Figure 13. A. Heatmap of Microbial Abundance within Melamphaidae, by class. Most abundant class is Gammaproteobacteria, followed by Planctomycetacia. B. Top genera include *Photobacterium*, an uncultured Planctomycetia, *Vibrio* and *Mycoplasma*.

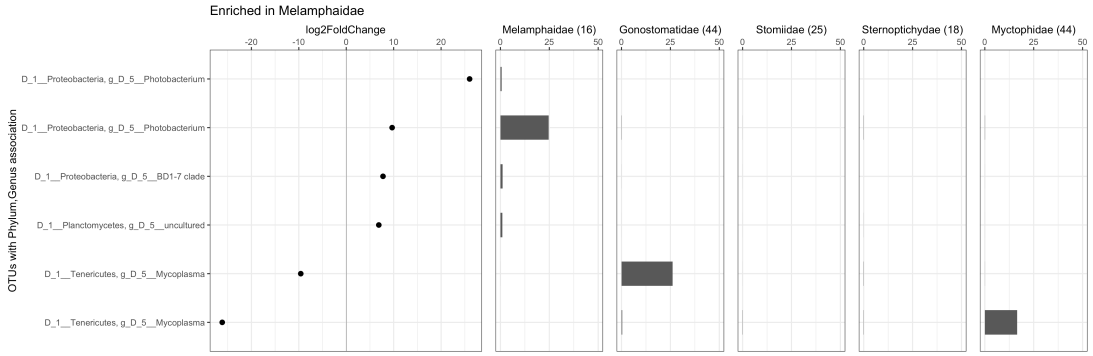


Figure 14. Differential analysis of Melamphaidae. Enriched and relatively abundant in one Photobacterium.

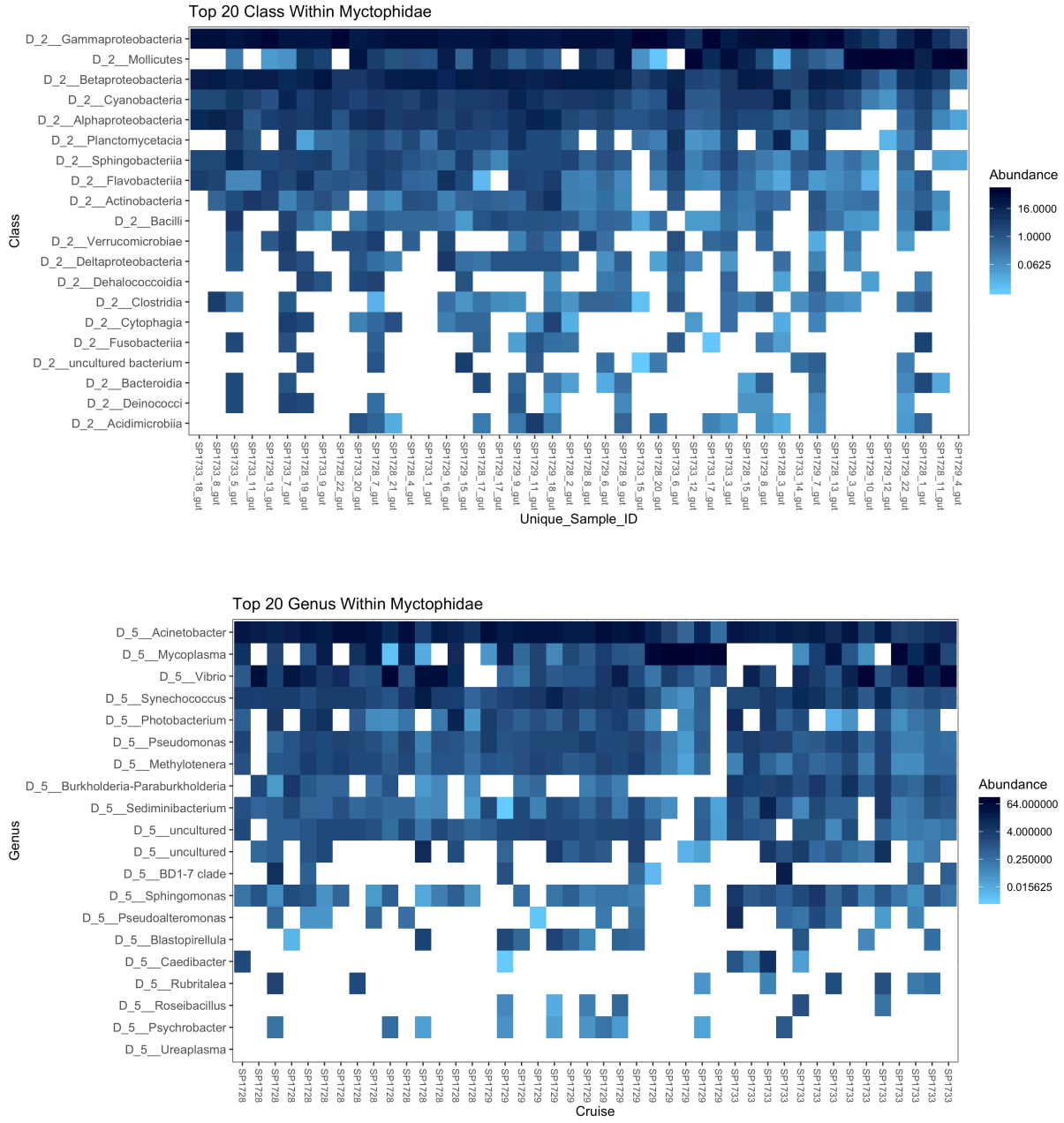


Figure 15. A. Microbial abundance within Myctophidae, top 20 classes. Most abundant is Gammaproteobacteria, followed by Mollicutes and Betaproteobacteria. B. Top genera include *Ancinetobacter, Mycoplasma, Vibrio, Synechococcus*.

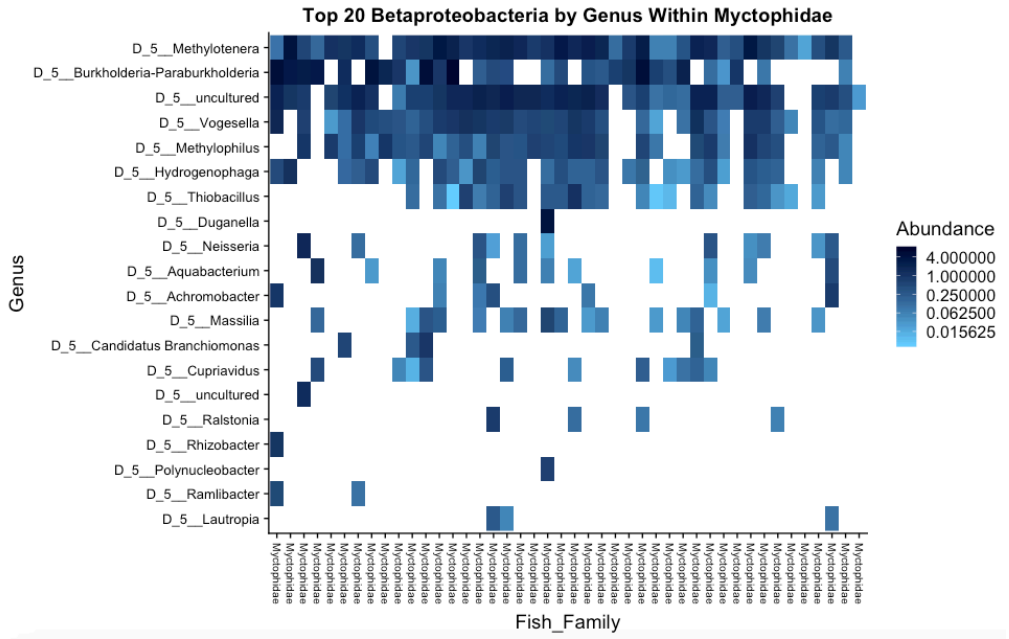


Figure 16. Top 20 Betaproteobacteria by genus in Myctophidae

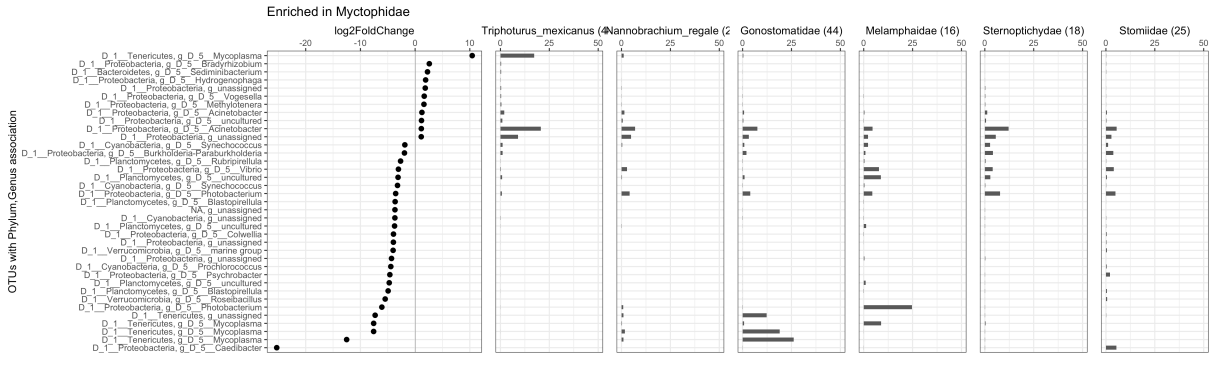


Figure 17. Relative abundance, OTU level in Myctophidae.

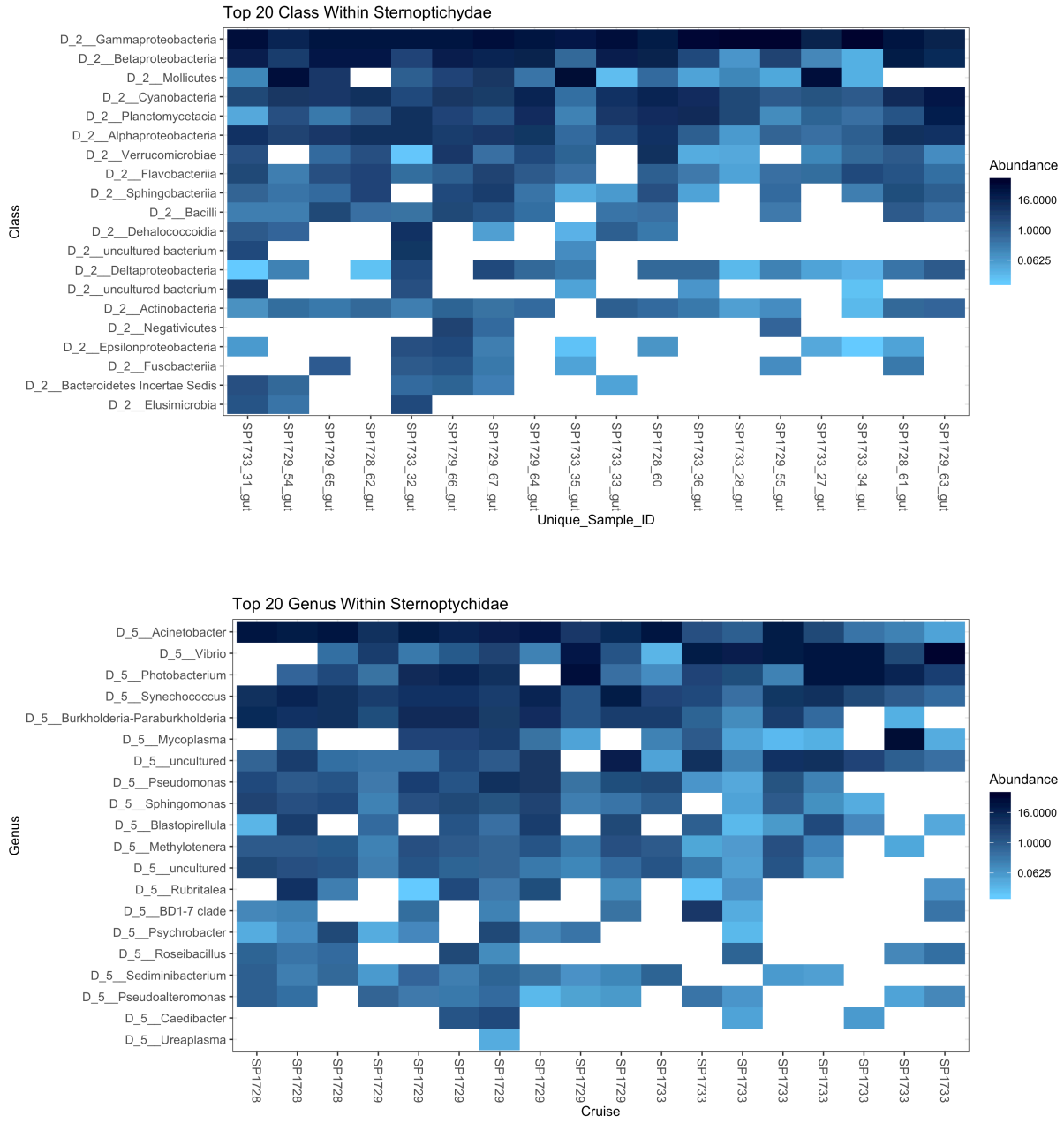


Figure 18. A. Microbial abundance within Sternoptychidae, top 20 classes. Most abundant class is Gammaproteobacteria, followed by Betaproteobacteria. B. Top genera include *Acinetobacter, Vibrio, Photobacterium,* and *Synechococcus.*

Table 3. Fish count and measurements.

Family	Count	Species	Size range (mm)	Mean length (mm)	Mean Weight (g)
Gonostomatidae	44	2	24-51	38	0.3131
Melamphaidae	16	2-3	32-94	53	4.6417
Myctophidae	44	2	37-65	51	1.2875
Sternoptychidae	18	5-6	8-46	24	0.7452
Stomiidae	25	2	50-280	166	12.1850

Table 4. Fish species numbers.

Family	Species	Count
Gonostomatidae	*Cyclothone signata*	3
	Cyclothone pacifica	41
Myctophidae	*Triphoturus mexicanus*	42
	Nannobrachium regale	2
Melamphaidae	*Scopelogadus bispinosus*	13
	Poromitra crassiceps or *Melamphaes lugribis*	1
	Unknown Melamphaidae	2
Sternoptychidae	*Argyropelecus affinis*	4
	Argyropelecus aculeatus	8
	Argyropelecus sladeni	1
	Argyropelecus sp.	1
	Sternoptyx diaphana or *Sternoptyx pseudobscura*	1
	Sternoptyx obscura	1
Stomiidae	*Idiacanthus antrostomus*	2
	Stomias atriventer	23

Table 5. Cruise data.

Cruise	Date	Time in	latitude/longitude in	Time out	latitude/longitude out	Depth (m)
SP1728	10/14/17	14:35	N32º 44.85 W117º 34.035	~17:35	32º39.79'N 117º31.33'W **	550-570 dipped to 618
SP1729	10/15/17	14:00	32º45.5'N 117º35.0'W*	17:07**	32º39.393'N 117º30.754'W**	590-620
SP1733	10/29/17	11:51	N32º 40.221 W117º 31.806	14:45	N32º 48.997 W117º 36.331	822
SP1608 (seawater)	5/7/16					50m 340m
SP1527 (seawater)	11/14/15		32 47.293 N 117 30.333W			832m

Figure 19. Assortment of Sternoptychidae (*Argyropelicus* spp.) and Melamphaidae (*Scopelogadus bispinosus*) before and during dissection.

Appendix

Diet study on mesopelagic fish, CO1 primer design

Work was also done on a pilot group of fish, attempting to ascertain diet from gut samples that were PCR amplified using cytochrome oxidase one gene (CO1) primers. This gene is used to 'barcode' animals, essentially matching a known sequence in databases with a sequence extracted from animal tissue[38]. Because of the high quantity of host DNA in each extracted sample, it was quickly realized that host specific blocking primers would be needed to minimize amplification of the host while, ideally, maximizing amplification of prey tissues. Host tissue was sent for Sanger sequencing, then host specific sequences were isolated using a computer program, Geneious (Geneious v8 (https://www.geneious.com)). Sequences were designed to overlap the same bases on the CO1 gene as the amplification primers designed by Mathieu Leray did[39]. Ideally, the exact sequence match would anneal to host DNA and block amplification primers from being able to anneal. Final sequences were ordered as primers with a 3-carbon addition on the 3' end, this addition would literally stop amplification during the PCR reaction. Before ordering, primers were tested *in silico* against a group of marine species CO1 sequences obtained from the BOLD database in order to ascertain whether the primers were likely to block non-target sequences. In all, four primers were designed and ordered. It was determined through extensive testing that primers needed to be at least genus specific (Figure 20). However, even primers that were tested on the host they were designed for were only able to block a limited amount of host DNA. It was also determined that the High Fidelity Q5 polymerase that was generally used in the Allen lab for MySeq Illumina sequencing runs was incapable of amplifying the shortened CO1 region. Instead it was necessary to use NEB 2X Taq polymerase. After sequencing, reads were processed and sequenced samples of blocked host intestine were compared to sequenced unblocked samples. Results showed less than a 10% gain of non-host species sequences (Figures 21, 22). Also, the issue arose that the database did not well represent the

full array of possible diet items in the California Current. As such, there might be limited matches even if the primers were extremely effective. For these reasons, this pilot study did not go further. Should it be re-visited, it is the opinion of those who worked on it that stomach samples be extracted rather than gut samples. This would ideally allow a greater proportion of non-host DNA to be present.

Stable isotope analysis on mesopelagic fish to compare trophic levels and microbiome

In order to pair microbial data with trophic positions using stable isotope data, two pilot studies were done with mesopelagic fish. The first was a simple test to determine whether washing a tissue sample with ethanol (EToH) would significantly change the carbon to nitrogen ratio. Ten paired samples (washed and unwashed tissue) across five fish families (Myctophidae, Stomiidae, Sternoptychidae, Bathylagidae, and Gonostomatidae) were submitted for isotope testing to UC Santa Cruz. Results showed that in all but one sample the washing did not significantly change the results (Figure 23). An unexpected takeaway from the pilot study was that mesopelagic fish are incredibly oily and need to be lipid extracted before they can be sent for isotope testing. A second group of 31 samples was chosen. Tissues were taken from samples where the microbial intestinal community had already been sequenced. Samples were lipid extracted, 5 samples were lost in the extraction process, the remaining 26 samples were placed in tin cubes and sent to UCSC for testing (Figure 24). Results showed very similar patterns on a PCoA to microbial analysis of the intestinal communities from the paired fish (Figure 25). These results were interesting enough to suggest a more inclusive study with fish from a variety of pelagic zones be done.

Tissue preparation for isotope analysis

Samples were taken from fish tissue above the lateral line, or when not enough tissue was available, all the lower portion of the fish was taken. While care was taken to remove skin and avoid bones, it must be noted that due to the small size of the fish and the lack of muscle tissue quantity,

much skin remained on many samples and all most certainly contain bones. These samples were washed during dissection with only MilliQ water and were stored in eppendorf tubes at -80C until use. When defrosted, samples were washed with 500ul of MilliQ water and checked again for skin that was, if possible, removed. As noted above, bones were not visible and it was not possible to remove them with any certainty. A pilot study of ten paired samples, two samples from each of five fish families was done to determine whether washing tissue with ethanol changed the carbon signature. The paired washed and unwashed tissues were defrosted and again rinsed with MilliQ H2O. Tissue was lyophilized over a twenty-four hour period at -50C. After tissue was dried it was homogenized to powder using a metal spatula. A quantity of between 50-100ug was weighed into a foil cup, rolled and folded into a small cube, and placed in a 96 well plate. This plate was sent to the Stable Isotope Lab at University of California, Santa Cruz for isotopic analysis. Samples were analyzed for delta 13 Carbon and delta 15 Nitrogen.

Lipid Extractions

(From a protocol used in the Kurle lab)

After analyzing the paired data from the washed and unwashed samples, it was observed that the fatty tissues of the mesopelagic fish resulted in high carbon to nitrogen ratios. It was suggested that to obtain more accurate ratios lipid extractions should be done. Of the samples washed with only MilliQ H2O during dissection, thirty-one were chosen to undergo lipid extractions. Choices were made in order to maximize species within fish families, and to have an end result of at least five fish per family for analysis. These samples were defrosted, washed with 2 mL MilliQ and where possible, skin was removed. Bones were again not seen but were assumed to be present. Holes were poked in the tops of labeled eppendorf tubes and tissues were placed inside for lyophilization over a twenty four hour period. After the samples had dried they were placed inside a folded weighing paper and homogenized by crushing with a curved metal spatula. (*Stomias, N. regale, T. mex* very oily fish). The

following lipid extraction was done entirely in a fume hood. A water bath was preheated in a sonicator to 60C. The ground samples were placed in clean, labeled 15mL glass centrifuge tubes and moved to the fume hood. To each tube, 10mL of petroleum ether was added and then tubes were covered with cap that had pre-drilled holes. Capped tubes were placed in a rack within the sonicator and sonicated for 10 minutes at 40khz. After sonification, tubes were centrifuged at 12,000xg for five minutes. Remaining petroleum ether was poured off and remaining drops were pipetted away. Samples and tubes were rinsed down with ultra pure H2O and excess H2O was poured off. An amount of 10mL of ultra pure H2O was added to each sample and sonicated for 10 minutes at 40khz in 60C bath. The samples were then centrifuged for 10 minutes at 12,000xg, water was poured off and last amounts pipetted off. Some samples lost tissue during this step. Tissue was then returned to clean eppendorf tubes and lyopholized for another 24 hours. Due to the hole in the top of the eppendorf tubes, some samples lost tissue during this process. After lyophilization was finished, samples were weighed and prepped in the tin cups as per the above isotope analysis preparation protocol. A total of five samples lost too much tissue to be tested, only twenty-six samples were sent to UCSC.

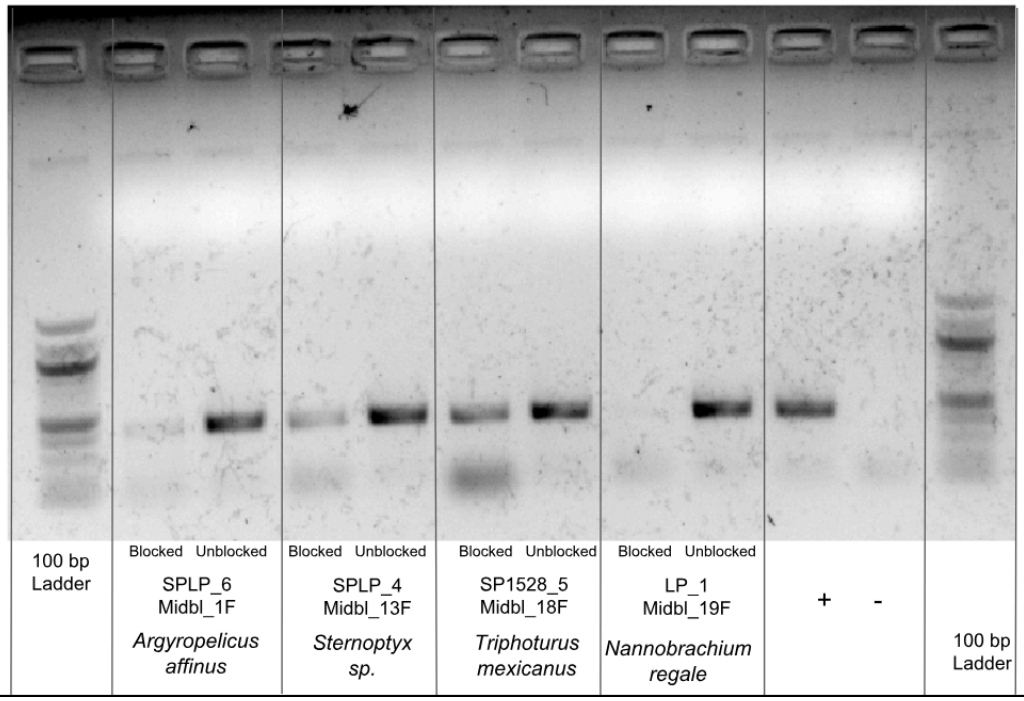


Figure 20. Gel electrophoresis image demonstrates blocking primers tested on host voucher tissue. Primers Midbl_1F and Midbl_19F appeared to be more effective at blocking host tissue than Midbl_13F and Midbl_18F.

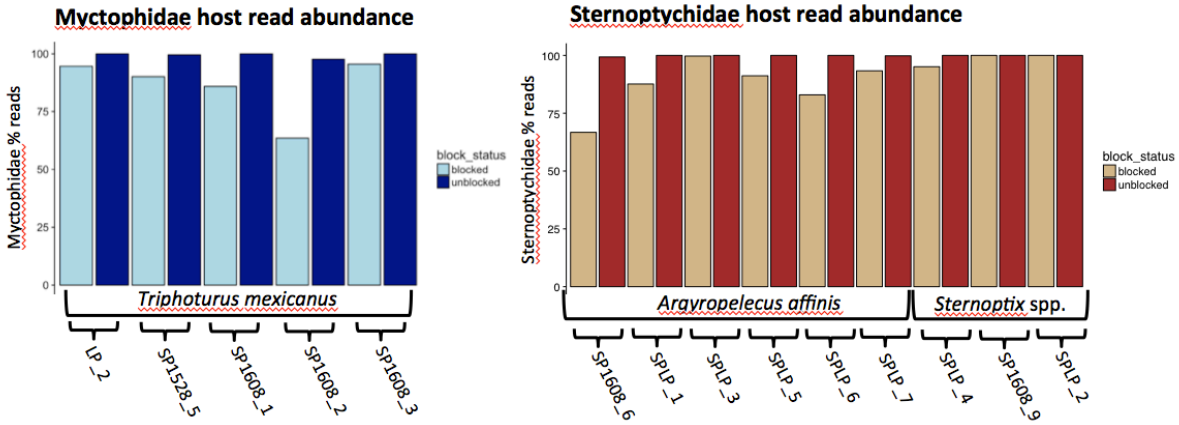


Figure 21. Host read abundance of blocked and unblocked intestinal tissue shows host specific blocking primers do block some host DNA but the majority of amplification is still host. (Image courtesy of Jessica Blanton).

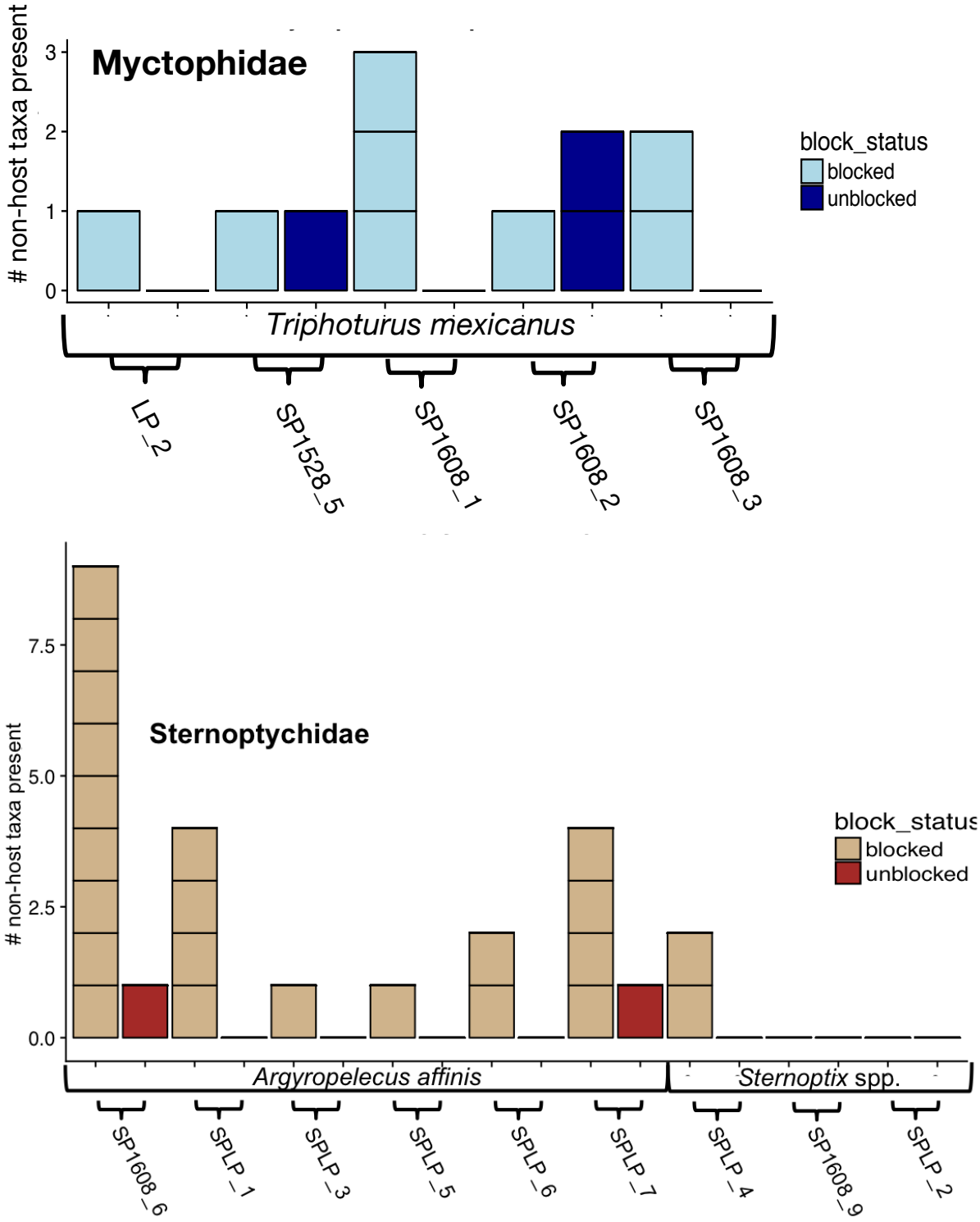


Figure 22. Number of DNA non-host sequences that amplified in sequenced blocked and unblocked intestinal tissue. Sequenced blocked samples reveal that even with blocking primers, the majority of sequences are still host (All images in Fig. 22 courtesy of Jessica Blanton).

Table 6. Primers used for amplification, primers designed and used for blocking host DNA

Primer name	Purpose	Primer sequence
ill_515f	16S V4, forward amplification	5’ - TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG YCA GCM GCC GCG GTA A - 3’
ill_806RB	16S V4, reverse amplification	5’ - GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACN VGG GTW TCT AAT - 3’
C3myc_806rb	18S blocking (Jess)	5’ - GGT TTC TAA TCG TCT TCG AAC CTC CGA C/3SpC3/ - 3’
CO1V1R	CO1 amplification, full length, reverse	5’ - TAG ACT TCT GGG TGG CCA AAG AAT CA - 3’
CO1V1F	CO1 amplification, full length, forward	5’ TTC TCA ACC AAC CAC AAA GAC ATT GG -3’
ill_jgHCO2198_R	CO1 amplification, reverse	5’ - GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCT TAI ACY TCI GGR TGI CCR AAR AAY CA - 3’
ill_mlC01intF	CO1 amplification, forward	5’ - TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGG GWA CWG GWT GAA CWG TWT AYC CYC C - 3’

Table 6. (Continued)

Midbl_1F	*Argyropelicus affinis* blocking primer	5’ - TTA TCC CCC TCT TTC CAG CAA / 3SpC3/ -3’
Midbl_2F (not used in study)	*Nannobrachium regale* blocking primer	5’ - ATC CCC CTC TAG CAG GCA A/3SpC3/ -3’
Midbl_18F	*Triphoturus mexicanus* blocking primer	5’ - CCC ACT TGC GGG GAA TCT CGC C/3SpC3/ -3’
Midbl_13F	*Sternoptyx* spp. blocking primer	5’ - TGT TTA CCC TCC TCT TGC TGG AAA CT/3SpC3/ -3’
Midbl_19F	*Nannobrachium regale* blocking primer	5’ TTT ATC CCC CTC TAG CAG GCA ATC T/3SpC3/ -3’

Table 7. Mastermix for 16S rRNA gene amplification

Master mix (16S rRNA V4)	1X
H2O	10.5 uL
ill515F	0.5 uL
ill806rb	0.5 uL
Hot Start 2X NEB Taq (MO496S)	12.5 uL
Template	1 uL

Table 8. Amplification cycle for 16S rRNA gene

Cycle (16S rRNA V4)	Temp (ºC)	Time	Repeated
Initial denature	94º C	1 minute	1 X
Denature	94º C	30 seconds	35 X
Anneal	50º C	30 seconds	35 X
Extension	68º C	1 minute	35 X
Final extension	68º C	5 minutes	1 X
Hold	4º C	infinite	

Table 9. Mastermix for CO1 gene amplification

Master mix (CO1V1F/R, illmlCO1intF/ illjgHCO2198R)	1X
H2O	10.5 uL
CO1V1F or illmlCO1intF	0.5 uL
CO1V1R or illjgHCO2198R	0.5 uL
Hot Start 2X NEB Taq (MO496S)	12.5 uL
Template	1 uL

Table 10. Mastermix for CO1 gene amplification with host-specific blocking primers

Master mix (CO1V1F/R, illmlCO1intF/ illjgHCO2198R)	1X
H2O	9.5 uL
CO1V1F or illmlCO1intF	0.5 uL
CO1V1R or illjgHCO2198R	0.5 uL
Hot Start 2X NEB Taq (MO496S)	12.5 uL
Template	1 uL
Host-specific blocking primer (5x)	1 uL

Table 11. Amplification cycle for CO1 gene

Cycle (CO1V1F/R, illmlCO1intF/ illjgHCO2198R)	Temp (ºC)	Time	Repeated
Initial denature	94º C	1 minute	1 X
Denature	94º C	30 seconds	35 X
Anneal	48º C	30 seconds	35 X
Extension	68º C	1 minute	35 X
Final extension	68º C	5 minutes	1 X
Hold	4º C	infinite	

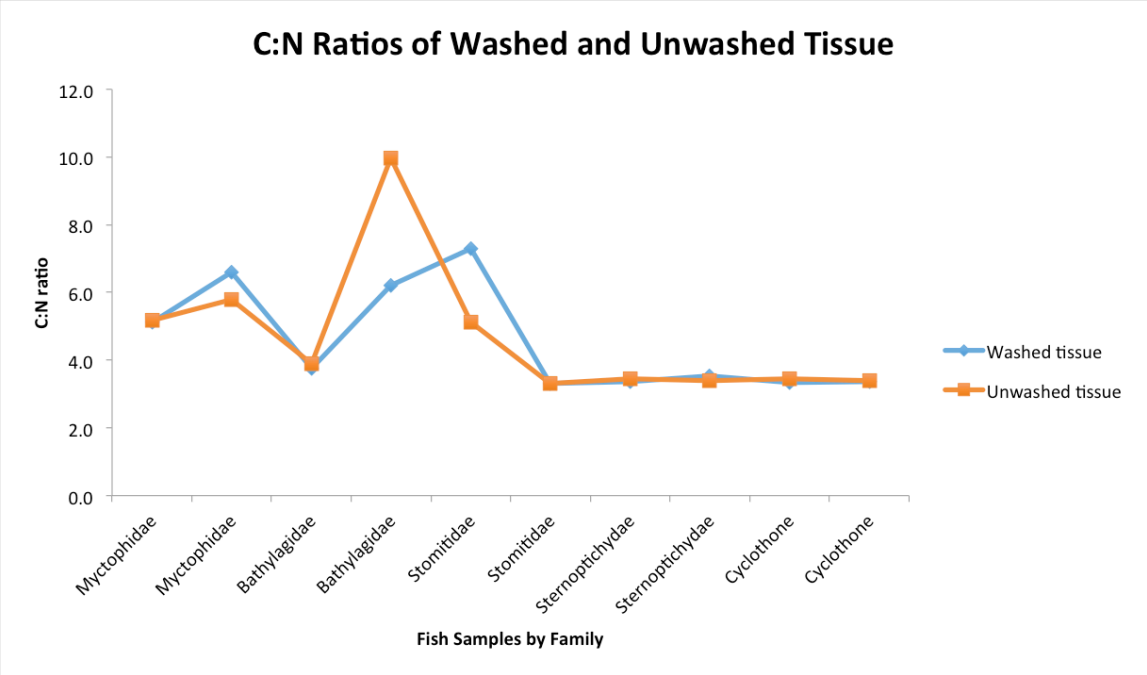


Figure 23. C:N ratios of washed and unwashed tissue. Pilot study to determine whether to wash tissue with EtOH.

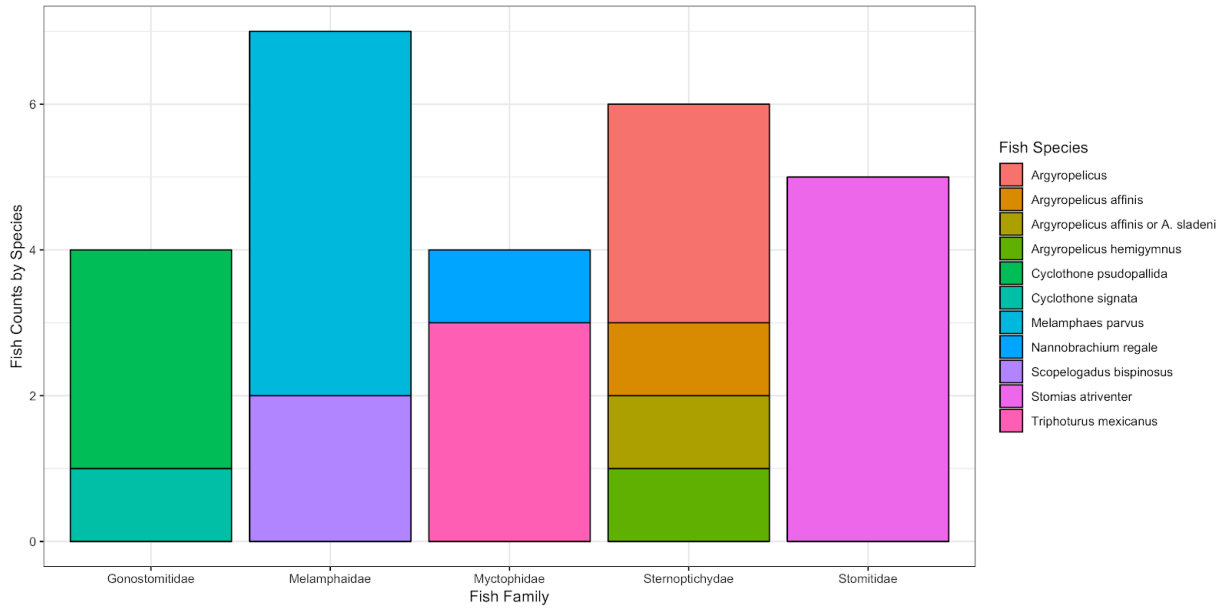


Figure 24. Fish counts colored by species, as sent for isotope analysis after lipid extraction.

Table 12. Single factor Anova tests for Delta 13 Carbon and Delta 15 Nitrogen.

Single Factor Anova, Delta Carbon 13

SUMMARY

Groups	Count	Sum	Average	Variance
Gonostomatidae	4	-78.6	-19.65	0.1212
Melamphaidae	7	-142.33	-20.33285714	0.84082381
Myctophidae	4	-80.04	-20.01	0.5946
Sternoptichydae	6	-118	-19.66666667	0.551266667
Stomiidae	5	-97.48	-19.496	0.06738

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.717992271	4	0.679498068	1.396475381	0.269428892	2.840099807
Within Groups	10.21819619	21	0.486580771			
Total	12.93618846	25				

Single Factor Anova, Delta Nitrogen 15

SUMMARY

Groups	Count	Sum	Average	Variance
Gonostomatidae	4	58.76	14.69	5.863
Melamphaidae	7	103.17	14.73857143	1.585447619
Myctophidae	4	58.65	14.6625	1.468825
Sternoptichydae	6	86.62	14.43666667	0.599386667
Stomiidae	5	80.83	16.166	0.38333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9.871020568	4	2.467755142	1.437989416	0.256429837	2.840099807
Within Groups	36.03841405	21	1.716114955			
Total	45.90943462	25				

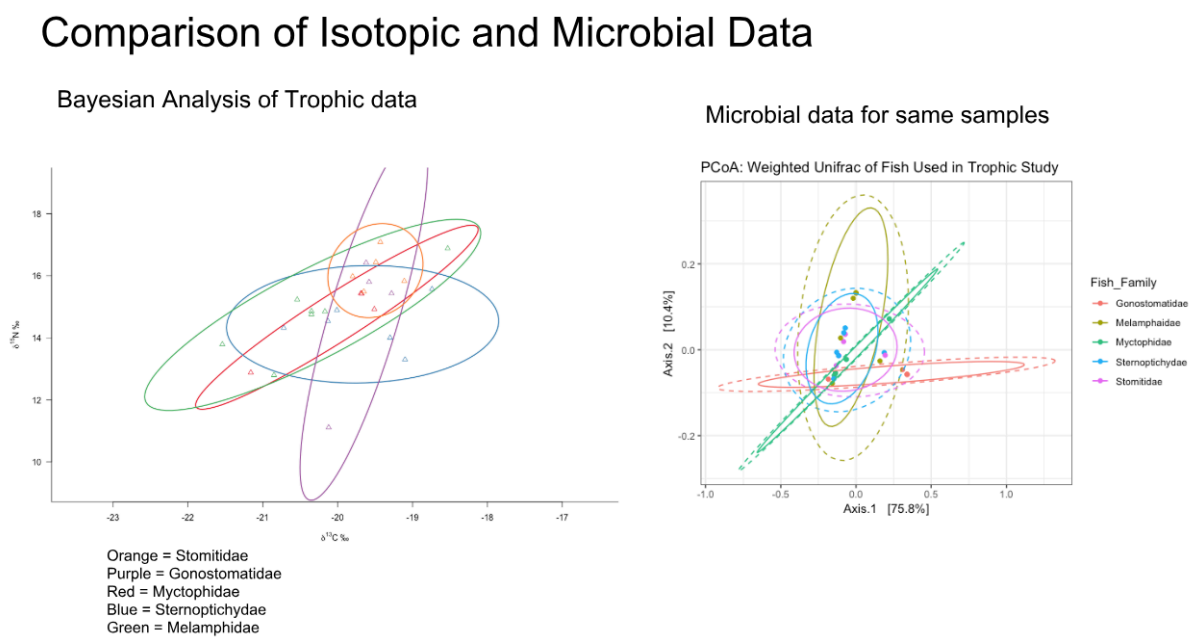


Figure 25. Visual comparisons of trophic niche and microbial data for paired samples suggests correlation.

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