Hydrocarbon-Degrading Bacteria and Potential Symbiosis with Deep-Sea Organisms: Using Ctenophores as a Model

Hydrocarbon-Degrading Bacteria and Potential Symbiosis with Deep-Sea Organisms: Using Ctenophores as a Model

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Abstract

Microbiomes and the functions of microbes as they relate to their host have been of interest in many areas of research. Microbes have been of great interest in the deep sea as they serve as potential symbionts for the organisms that reside there where they may participate in macro ecosystem nutrient cycling in an extreme habitat that is limited by lack of light, colder temperatures, and high hydrostatic pressure. Some organisms are known for being able to perform functions such as alkane degradation at the same efficiency at depth as they do at surface level and tend to be more abundant at depth. Such organisms include alkane-degrading bacteria such as *Alcanivorax* and various other microbes capable of hydrocarbon degradation. Bacteria capable of this function have also been detected as part of the microbiomes of ctenophores. It was hypothesized that ctenophores living in the photic zone of the ocean would have significantly different microbiomes than those living in the aphotic zone. Secondly, it was hypothesized that ctenophores living in the aphotic zone will have a higher relative abundance of hydrocarbon-degrading bacteria than photic-dwelling ctenophores. Lastly, it was hypothesized that genus-level diversity of hydrocarbon-degrading bacteria would increase with depth. Our results indicate that the microbiomes of ctenophores did in fact differ when comparisons between bacterial communities of photic samples were compared to aphotic samples, but neither the abundance nor diversity of hydrocarbon-degrading bacteria differed significantly between photic and aphotic ctenophores

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Chapter I.

Introduction

The ecological functions of microbes have been well studied in terrestrial environments; particularly the roles that microbes play in soils and nutrient cycling (Fierer, 2017). In terrestrial environments, microbes have been known to be involved in primary production using carbon fixation, nitrogen fixation, methanogenesis, and various other nutrient cycles (Fierer, 2017). The microbial communities in soils change drastically depending on environmental conditions (Fierer, 2017). Microbial ecological functions have also been studied in marine environments, and just like in soil, marine microbial communities change with environmental conditions (Amano, et al., 2022; Pavés & González, 2008). Such conditions in the ocean may be temperature, light availability, hydrostatic pressure, etc.

The notion that microbial functions are inhibited in the deep sea arose after the *Alvin* submersible sank to 1540 m in 1968 with a sandwich on board, which once recovered after 10 months, showed little to no decomposition (Jannasch et al., Science 1971). Prokaryotes in the deep sea are exposed to high hydrostatic pressure conditions which may have an impact on their biological processes; depending on whether they are piezophilic, piezotolerant, or piezosensitive (Amano, et al., 2022). Since prokaryotic organisms are affected by hydrostatic pressure, the community composition and physiological function of bacteria living symbiotically on deep-sea animals should be likewise affected.

Overall, a greater part of heterotrophic activity of prokaryotic communities is inhibited in the presence of high hydrostatic pressure (Amano, et al., 2022). As depth increases, heterotrophic microbial activity is thus thought to decrease. This has a major impact on oceanic carbon cycling, which in turn affects higher trophic levels in the deep sea that depend on prokaryotic biological cycles such as carbon fixation or methanogenesis etc. Microbial communities are well-known for their symbiotic functions across the tree of life from deep-sea corals to humans, whales, plants, and even insects and parasites (Amano, et al., 2022; Fredensborg, et al., 2020; Kellogg, 2019; Sanders, et al., 2015; Trivedi, et al., 2022). From soils to seas, microbiomes are clearly ecologically crucial. The functions of microbes in the deep sea include both general biogeochemical cycling, and specialized symbiotic relationships with individuals at higher trophic levels (Lan, et al., 2021; Pavés & González, 2008).

Estimates of prokaryotic carbon demand (PCD) of deep-sea microbes in the mesopelagic and bathypelagic layers of the Atlantic suggest that PCD is about one order of magnitude higher than the supply of sinking particulate organic carbon (POC). This implies a mismatch between previous understandings of carbon flux, i.e., the availability of carbon, and the probable carbon demand of prokaryotes in the deep sea where photosynthetic life is lacking (Amano, et al., 2022). Recalcitrant carbon that makes its way to the euphotic zone often comes from sinking POC from metabolic activity of microbes, and other organisms, and can persist in the water column for thousands of years (Jiao, et al., 2010). Sources of POC include detritus, fecal matter, living microbial cells, and other aggregated materials (Kharbush, et al., 2020). The downwelling recalcitrant carbon is likely more bioavailable than previously thought. This suggests that

there is an ecological niche for microbes that can metabolize carbon under high hydrostatic pressure using carbon sources that are available in the deep sea.

Much recalcitrant carbon takes the form of hydrocarbons such as alkanes and aromatics (Blumer, 1976; Li, et al., 2019). Alkanes are saturated hydrocarbons that can be linear (*n*-alkanes), cyclical (*cyclo*-alkanes), or branched (*iso*-alkanes). Alkanes are a relatively unreactive class of organic compounds found in crude oils and fuels. They are insoluble in water and pose an ecological threat to the environment when released in large quantities by anthropogenic sources. Despite the difficulty of degrading alkanes, there are microbes that have evolved effective strategies for harnessing them as a carbon source using specialized enzymes and metabolic pathways (Wentzel, et al., 2007). Other hydrocarbons include alkenes, alkynes, and aromatic hydrocarbons. These hydrocarbons in the marine environment originate from natural processes and anthropogenic sources (Kleindienst & Joye, 2019).

Anthropogenic sources of hydrocarbons include oil spills, which are considered point sources of pollution. Such spills may be from tanker ships, pipelines, or industrial waste drains (Kleindienst & Joye, 2019). Non-point sources of anthropogenic hydrocarbons involve contaminants that get into vectors such as ground water, which spread great distances from the initial source. Many non-point sources are land-based underground aquifers; therefore, they tend to disproportionately affect nearshore marine ecosystems. Non-point sources are more likely to affect near-shore ecosystems where floating hydrocarbons can accumulate in sediments (Kleindienst & Joye, 2019). Natural sources of hydrocarbons include cold seeps. Cold seeps are areas on the ocean floor where hydrocarbon-rich fluid seeps into the water column. Cold seeps are often found

along continental margins, and communities of organisms that live near cold seeps take advantage of the available hydrocarbon as an energy source (Kleindienst & Joye, 2019).

If microbes are utilizing carbon from alkanes and other recalcitrant hydrocarbons whose contribution to deep-sea organic carbon supply is poorly constrained, this may account for some of the discrepancy between estimated POC influx and PCD. If recalcitrant carbon is more bioavailable than previously thought, a large niche may exist in the deep sea for microbes able to metabolize it. The deepest parts of the ocean are recognized to have the highest known relative abundance of HCD bacteria (Liu, et al., 2019). The reason behind the higher proportion of HCD microbes in the deep as compared to coastal areas is not well understood, but likely has to do with the overall presence of hydrocarbons. According to researchers Liu, et al. (2019), the abundance of both alkane-degrading microbes and characterized alkane degradation genes, such as *alkB* and *almA,* increases at near bottom depths. They also found that the abundance of *Oceanospirillales,* a bacterial order in which hydrocarbon degradation is common, significantly increased in two near-bottom water samples, suggesting a bacterial niche boundary. Overall, this suggests a potential for niche partitioning of the water column by HCD microbes that can perform their metabolic functions at depth and those that cannot.

In addition to research examining the prevalence of free-living HCD bacteria in the deep sea, work has also been done on the symbiotic relationship between HCD bacteria in genus *Cycloclasticus* and their animal hosts, such as bathymodioline mussels. Evidence suggests that *Cycloclasticus* uses short-chain alkanes as a source of energy and carbon that is then shared with its host (Rubin-Blum, et al., 2017). Corals have been

known to harbor alkane-degrading bacteria (Ansari, et al., 2021) as well. The free-living bacterium *Alcanivorax borkumensis SK2* has been found to be closely related to several marine bacteria that are known to be symbionts, including the gill symbiont of the methane-utilizing coastal clam *Codakia costata* (Yakimov, et al., 1998). Additionally, phytoplankton have been shown to associate with HCD microbes. For example, the polycyclic aromatic hydrocarbon (PAH)-degrading bacterium *Porticoccus hydrocarbonoclasticus* was isolated from a marine dinoflagellate (Gutierrez, et al., 2012).

Though HCD microbes are ubiquitous in the marine environment, they tend to be low in abundance in the absence of hydrocarbons, whereas microbes capable of alkane degradation are likely to be found where alkanes are more prevalent (Kleindienst & Joye, 2019). Consider for example the case of *P. hydrocarbonoclasticus,* which is likely living in the photic zone of the ocean where phytoplankton produce PAHs that the bacteria can use for energy as an alternative to sugar. There are clearly diverse and distinct ecological roles that HCD bacteria play, both in the environment and within microbiomes of their hosts.

Despite the relatively recent development of interest in HCD bacteria, there is an assortment of proposed ecological functions and effects of hydrocarbon degraders. The drivers of these functions may be stimulated by both biotic and abiotic factors. Under some conditions, sheens and oil slicks stimulate phytoplankton production in surface waters, likely because of the interactions between phytoplankton and HCD microorganisms that proliferate in the presence of hydrocarbons (Kleindienst & Joye, 2019). In the water column, HCD microbes are associated with ascending gas bubbles, oil droplets, and dissolved hydrocarbons (Kleindienst & Joye, 2019). An accumulation of

hydrocarbons exists at the sea floor due to HCD microbes in the water column releasing large amounts of extracellular polymeric substances, which forms marine oil snow. The marine oil snow can sink and be deposited on the seafloor, along with crude oil components (Kleindienst & Joye, 2019). The accumulation of HCD microbes at greater depths may also be due to the release of hydrocarbons from cold seeps and other areas of tectonic activity that may release alkanes such as methane (Kleindienst & Joye, 2019; Li, et al., 2019).

This project focused on the Ctenophora, a phylum of exclusively marine invertebrates whose members have been very sparsely studied as bacterial hosts. The bacterial community of ctenophores has been known to harbor obligate alkane degraders such as species in the genus *Alcanivorax* (Wang & Shao, 2012). Specifically, ctenophores *Mnemiopsis leidyi* and *Beroe ovata,* have been known to possess Alphaproteobacteria In the genera *Thalassospira* and *Alcanivorax*, both of which are known to be capable of hydrocarbon degradation (Tinta, et al., 2019). The potential to degrade alkanes and other hydrocarbons is not limited to the genera *Alcanivorax* and *Thalassospira* found on these ctenophores. This ability has been characterized in many other microbes that can metabolize long-chain *n*-alkanes which may remain as an undiscovered part of the ctenophore microbiome (Wentzel, et al., 2007). Although ctenophores such as *M. leidyi* and *B. ovata* have some similarities within their microbiome, such as both harboring alkane-degrading microbes, ctenophores such as *M. leidyi*, *Beroe* sp., *Bolinopsis infundibulum*, and *Pleurobrachia pileus* have been known to have significantly dissimilar microbial communities overall with differences in abundance of potential alkane degraders (Hao, et al., 2015).

Even when collected in a small area, *M. leidyi* individuals were found to have distinctly different microbiomes from each other, although some similarities existed between individuals sampled closer to the same time frame (Jaspers, et al., 2019). This is consistent with the possibility that the immediate environment has a strong effect on the bacterial communities of ctenophores. It is possible that microbes living on individual ctenophores may be accommodated by their host in exchange for specific symbiotic functions, and that such symbioses depend in turn on the environment that the host inhabits at any given time. This idea is also supported by previous findings of temporal variation in *M. leidyi* bacterial communities, where they showed a shift in bacterial community composition throughout the season of sampling (Hao, et al., 2015). Researchers Daniels and Breitbart (2012) were also able to show that ctenophores have distinctly different microbiomes amongst different taxa, as well as the water column they are found in, but with some similarities between individuals of the same species sampled closer to the same time frame.

Daniels and Breitbart (2012) also show that ctenophores can carry bacteria similar to *Alcanivorax* and *Thalassospira*. Ctenophores release a large amount of dissolved organic matter (DOM) through excretion (Condon, et al., 2011; Nemazie, et al., 1993; Pitt, et al., 2009; Schnieder & Behrends, 1998). This release may stimulate growth in the local bacterial community (Condon, et al., 2011). Interestingly, bacterial communities in the surrounding water column of ctenophores can be shifted from predominantly *Alphaproteobacteria* to *Gammaproteobacteria* in response to the addition of ctenophore-derived DOM (Hao, et al., 2015). In a short laboratory incubation, when environmental conditions are fit for the ctenophore to add DOM into the local environment, there are

more *Gammaproteobacteria* than *Alphaproteobacteria,* directly affecting the free-living bacterial communities. Although ctenophores have been shown to have their metabolic function altered by decompression, it is plausible that if hydrostatic pressure imposes an environmental nutrient limitation on some ctenophores that their microbiomes might shift towards the *Alphaproteobacteria* due to a dearth of ctenophore-derived DOM (Bailey, et al., 1994). This would enhance the relative fitness of bacteria able to degrade carbon that is available in that environment, e.g., in the form of hydrocarbons. Alkane-degrading bacteria such as *Alcanivorax* are known to be able to still degrade alkanes even under high hydrostatic pressure (Liu, et al., 2019).

The scope of this investigation is two-fold. First, we tested whether the bacterial community on aphotic-dwelling ctenophores is dissimilar to that of photic-dwelling ctenophores. Second, we attempted to determine whether ctenophores found in aphotic depths have a higher percentage of alkane-degrading bacteria within their microbiomes than photic-dwelling species. The importance of answering these questions is also multifaceted. One layer of possibility is that bacteria capable of hydrocarbon degradation make recalcitrant carbon available in environments where carbon metabolism is limited by hydrostatic pressure, temperature, and light availability. Additionally, alkane-degrading bacteria have also been known to create biosurfactants and biofilms. There may be a symbiotic relationship between microbes that create biofilms, and their hosts (Klein, et al., 2010). Biofilms have been shown to form protective layers for many marine organisms (Armstrong, et al., 2001). Overall, understanding the answers to these questions help us understand practical applications to the potential ecological niche of HCD bacteria, such as bioremediation, biosurfactants production, and to understanding

carbon cycling in environments such as those on other planets regarding the search for life outside of our own planet.

Definition of Terms

Alkane Degradation: The metabolic degradation of complex hydrocarbons in which the carbon atoms are held together by single bonds. These hydrocarbons are often found in crude oil and petroleum.

Alkane-Degrading Bacteria: Bacteria that can metabolize hydrocarbons such as those found in crude oil.

Alkene: A non-aromatic hydrocarbon containing a carbon-carbon double bond.

Bathypelagic: The bathypelagic zone is part of the open ocean between 1,000 m and 4,000 m below the ocean's surface. Bathypelagic organisms are organisms that inhabit this zone of the ocean.

Carbon Fixation: The process by which inorganic carbon is converted to organic compounds by living organisms via photosynthesis or chemosynthesis.

Crude oils: Unrefined petroleum; a mixture of hydrocarbons in a liquid phase. Often found in underground reservoirs.

Classical Food Web: A collection of all the food chains in a single ecosystem that represent energy transfer from primary producers to consumers as that energy moves through the trophic levels.

Ctenophores: Invertebrates of the phylum Ctenophora. Ctenophora is an exclusively marine phylum whose name translates to "comb-bearing." Members of the phylum are also known as comb jellies, named after comb-like ciliary plates that they use for locomotion in the water.

Cyclo-alkanes: An alkane hydrocarbon in which the carbons of the molecule are arranged in the form of a ring.

Dissolved Organic Matter (DOM): A heterogeneous mixture derived mainly from decomposition of organic materials, often plant material, bacteria, and algae. It is found in all bodies of water, both marine and freshwater (Nebbioso & Piccolo (2013).

Holobiont: A host organism and the microorganisms that reside in or on it.

Hydrocarbon: Organic compound consisting of all carbon and hydrogen atoms.

Iso-alkanes: A branched-chain alkane that has a single methyl group on the next to last carbon in the chain.

k-mer: In bioinformatics, a k-mer is a substring of length k contained within a sequence (DNA, RNA, protein, etc.).

Mesopelagic: The mesopelagic zone in the ocean is often referred to as the "Twilight Zone," and is defined as 200 m to 1,000 m below the surface. It is the last zone that receives light from above. Mesopelagic organisms are organisms that reside in the open ocean within this zone.

Metagenomic NGS (mNGS): Metagenomic NGS refers to sequencing all DNA in a sample, which may contain mixed populations of microorganisms' and their host's DNA, and assigning these genomes to a reference genome to determine the taxonomy of the organisms present. See Next Generation Sequence (NGS).

Microbiome: The collection of all microbes, bacteria, fungi, and viruses that naturally live inside or on an organism.

Methanogenesis: An anaerobic metabolic process that generates methane as a waste product.

n-Alkane: Linear hydrocarbons that consist of hydrogen and carbon bonded by only single bonds.

Next generation sequencing (NGS): Also known as massive parallel sequencing, a method that processes multiple DNA sequences in parallel. Individual sequences are aligned to a standard genome to determine exactly what part of the genome is represented in the sample. See Metagenomic Next Generation Sequence (mNGS).

Nitrogen Fixation: When molecular nitrogen is converted back into ammonia, nitrates, or nitrites.

Particulate organic carbon (POC): The mass of carbon in a particular organic material that typically can be collected on a filter. It is also combustible, non-carbonate carbon.

Piezophilic: An organism that can undergo growth faster at >1 atm than at 1 atm.

Piezosensitive: Growth inhibited by applied hydrostatic pressure.

Piezotolerant: An organism that can tolerate high hydrostatic pressure, but whose growth is limited by it.

Potential HCD bacteria genera: Bacteria belonging to a genus that has had documented genes in some species within that genus known to degrade hydrocarbons.

Primary Producers: Organisms that can produce their own food via photosynthesis or chemosynthesis.

Prokaryotic carbon demand (PCD): Heterotrophic prokaryotic carbon demand is the measurement of heterotrophic biomass divided by respiration.

Unique fraction metric (UniFrac): A tool used to measure the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree

that leads to descendants from both environments versus just one environment (Lozupone, et al., 2011)

The Research Problem

The natural niche of microbes in the marine environment that are capable of hydrocarbon degradation is not well understood outside of the presence of petroleum (Chernikova, et al 2020). Previous research has shown that certain microbes, such as the species in the genus *Alcanivorax*, are able to degrade alkanes and other hydrocarbons (Schneiker, et al., 2006; Wang & Shao, 2012). HCD prokaryotes such as *Alcanivorax* have been known to exist in the marine water column and are also known to occur in the microbiomes of water-column animals, such as ctenophores *Mnemiopsis leidyi* and *Beroe ovata* (Tinta, et al., 2019). Although ctenophores have some similarities in their bacterial communities, overall, they have been found to have dissimilar microbiomes (Hao, et al., 2015). Even individuals of the same species are known to have distinctly different microbiomes from each other when sampled both at different locations and in proximity to each other during the summer of 2012 (Daniels & Breitbart, 2012; Jaspers, et al., 2019). More research is needed to understand these differences and why they occur.

Dissimilarities in the bacterial communities of ctenophores may be due to environmental conditions. Depending on the demands of a habitat, bacterial communities with different metabolic capacities may be present to meet those demands. Since HCD bacteria have been found on more than one species of ctenophore, the possibility exists that HCD bacteria may differ in amount on each individual based on the environment the ctenophore is in.

It is known that deep-sea habitats harbor a higher relative abundance of hydrocarbon degrading (HCD) bacteria than any other environment on Earth, and

bacterial strains isolated from 10,400 m have been shown to efficiently degrade hydrocarbons in conditions that simulated the deep sea (Liu, et al., 2019). Alkane-degrading bacteria appear in the water column itself in higher quantities in the deep sea than in the shallows (Liu, et al., 2019). Whether alkane or other HCD bacteria are more prevalent within the bacterial community on animals that inhabit deeper depths in the ocean, as compared to shallow-dwelling species, is not yet known. The first hypothesis is that ctenophores living below 200 m (aphotic) will have different bacterial communities than ctenophores living above 200 m (photic). The second hypothesis states that aphotic-dwelling ctenophores will have a higher relative abundance (based on metagenomic k-mer analysis) of bacteria with known hydrocarbon degradation capacity than photic-dwelling ctenophores.

To test this hypothesis, metagenomic data from three ctenophores captured below 200 m was assessed for bacterial community composition, and then compared to the metagenomic data from three ctenophores captured above 200 m. After community comparison, the diversity of HCD bacteria on each specimen was assessed using a set of gene sequences associated with hydrocarbon degradation. If the aphotic-dwelling ctenophores host a greater diversity of HCD bacteria than the photic-dwelling species, this would be a steppingstone on the path to understanding the ecological niche of HCD bacteria and how they may be associated with individual organisms.

Chapter II.

Materials and Methods

Metadata

This study investigated the microbiomes of ctenophores (three found in the photic zone, and three in the aphotic zone), and whether aphotic ctenophores hosted more recalcitrant-carbon-metabolizing bacteria than ctenophores from photic habitats. The metagenomic data for this study was provided by collaborators from the Monterey Bay Aquarium Research Institute located in Moss Landing, California. Samples collected include one individual (obtained at the noted depth and temperature) of each of the following species: *Bolinopsis microptera* (captive-reared; 12°C), *Hormiphora californensis* (10 m; 13°C), *Mertensiidae sp. T* (276 m; 8.59°C), *Euplokamis dunlapae* (1 m; 10°C), *Bathyctena chuni* (2161 m; 1.90°C), and *Platyctenida sp. T* (3958 m 1.49°C). Data was processed in the Girguis Laboratory for Ecophysiology, Biochemistry, and Engineering under the supervision of Jacob Winnikoff, NASA Postdoctoral Program Fellow, and Peter Girguis, Professor of Organismic and Evolutionary Biology at Harvard. This research did not involve any human or vertebrate subjects.

Metagenomic Next Generation Sequencing

Next generation sequencing (NGS) is a blanket term for several high-throughput sequencing methods. Such methods involve taking billions of nucleic acid fragments and sequencing them simultaneously and independently. The sequence “reads” generated can

be hundreds to tens of thousands of bases long, depending on the sequencing technique. Computers are then able to assemble those reads into one, or more genomes in the case of a holobiont. Metagenomic NGS (mNGS) additionally involves sequencing and analyzing DNA from the host organism's microbes. This study used mNGS to interrogate ctenophore microbiomes. To determine microbial community composition, we used Kraken2, a k-mer based analysis of raw reads. To assess the functional diversity of the microbes present in our samples, we assembled the reads into microbial genomes, which were then BLAST-queried using recalcitrant carbon metabolism genes gathered from online databases (Supplementary 1).

Community Composition

Once the metagenomic reads from the ctenophore samples were taxonomically identified using Kraken2 and compared to the Kraken2 database built from NCBI data, bacterial communities on each sample were compared using the unique fraction metric, or UniFrac. UniFrac is used to measure the phylogenetic distances between sets of taxa in a phylogenetic tree, as the fraction of the branch length of the tree that leads only to taxa from one of the two samples being compared. In simpler terms, it is used as a comparison tool for comparing communities in two samples where the total amount of evolution unique to each sample is measured. This tool was used to ascertain whether our samples have dissimilar microbiomes. A t-test was performed to determine the significance in differences between our photic samples and aphotic samples.

Percent of Potentially Hydrocarbon Degrading Genera

Next, the relative abundance of potentially HCD genera in the aphotic ctenophore samples were compared to that in the photic ctenophore samples by summing the percentages of DNA from genera, and some families that are known to feature HCD potential. Those percentages were then compared by comparing the total percentages for both photic samples and aphotic samples using a T-test. A linear regression was also run between samples determining whether OTU percentage of each sample to depth as a predictor as well as between each genus of bacteria found on each sample compared to sample depth. The Benjamini & Hochberg multiple testing correction was used to limit false positive occurrences while still allowing for discovery of statistically significant findings.

Gene Hits

After determining the dissimilarities of the ctenophore's microbiomes, the diversity of HCD bacterial genes was analyzed for each sample. This was accomplished by using the Basic Local Alignment Search Tool (BLAST) to align known recalcitrant carbon metabolism genes with sequences in the samples. The BLAST search that was performed was optimized for specificity. The specific search used a set of metabolic genes manually curated from the literature to query our metagenomes (Supplementary 2). The total number of distinct hydrocarbon degradation genes in each sample was counted. After finding unique hits, sequence matches, we obtained a bit score for the BLAST results to determine a median hit quality for each metagenome. We normalized the amount of hits by size of each assembled metagenome, yielding hits per million base pairs (Mbp). A t-test was used to compare the photic sample's hits per Mbp to the aphotic

sample's hits per Mbp. A linear regression was then fitted to test the relationship between hits per Mbp in each metagenome and depth of capture.

Diversity

Last, species diversity of HCD bacteria by sample was calculated for each sample using Simpson's Diversity Index (D). This was done by using the following formula: $D = \sum(n/N)^2$ where *n* is the percentage of microbes of a particular genus or family in a sample, and N is the total percentage of all genera in a sample. Once the diversity of each species was calculated, the diversity values of the photic samples were compared to the diversity values of the aphotic samples using a t-test. A linear regression was completed to relate each sample's diversity value to depth.

Chapter III.

Results

Metadata

Six individuals, one from each of the following species (obtained at the noted depth and temperature) had their DNA, as well as the DNA of their bacterial communities present on them, sequenced and assembled into one metagenome per sample through PacBio CCS long-read sequencing (mNGS): *Bolinopsis microptera* (captive-reared; 12°C), *Hormiphora californensis* (10 m; 13.3°C), *Mertensiidae sp. T* (276 m; 8.59°C), *Euplokamis dunlapae* (1 m; 10°C), *Bathyctena chuni* (1915 m; 2.09°C), and *Platyctenida sp. T* (3958 m 1.49°C) (Table 1).

Kraken2

The results of the mNGS assembled genomes for each individual ctenophore and its associated bacterial community were used to create six individual krona plots that show the taxa and percentage of each taxa present in the sample (Supplementary Figure 3). Each plot shows a percentage of eukaryotic, “Homo Sapien," DNA, which is likely the DNA of the ctenophore host as Kraken2’s database does not contain ctenophore k-mers. Each sample had a root that is divided up into eukaryotic DNA, prokaryotic DNA, a percentage of DNA that had no hits, and some viral hits. Each plot also shows the count of annotated reads for each group (Table 2).

Unique Fraction Metric

Unifrac was used to estimate the phylogenetic distances between sets of taxa within the phylogenetic tree of the bacterial communities within our samples, represented as a fraction of the branch length that leads to the descendants of mutually exclusive environments. The Unifrac scores among the photic samples were compared to those among the aphotic samples using a T-test ($t = 3.3575$, $df = 9.4708$, p-value = 0.0078). It was determined that the comparisons between bacterial communities of the photic samples were significantly different from the comparisons between bacterial communities on the aphotic samples, suggesting a difference in the variation among bacterial communities on the two groups of hosts. The photic samples are more similar to each other than to the aphotic samples (Figure 1.).

Percentages of Hydrocarbon Degrading Bacteria

Using the krona plots created from Kraken2 analysis, the percentages of DNA in each sample from bacteria that have been previously mentioned in the literature to have hydrocarbon degradation potential was identified (potentially HCD genera). The focus was on the genus level, quantifying congeners of potential HCD bacteria, but included some percentages on the familial level when Kraken2 was unable to annotate more specifically. Once individual percentages of potential HCD genera were recorded, the total percentages of potentially HCD genera on each host was calculated (Table 2). Total percentages of photic samples were then compared to the total percentages of aphotic samples using a t-test ($t = -0.46738$, $df = 3.2913$, p-value = 0.6695), which failed to reject the null hypothesis that there would be a difference in relative abundance of potential HCD genera when comparing photic samples to aphotic samples (Figure 2).

A linear regression was used to test whether depth at which each sample was captured predicted the relative abundance of potentially HCD genera (Figure 3). With a p-value: 0.09039, it was found that depth did not significantly predict the total percentage of potentially HCD genera on our samples. A multiple linear regression was then conducted to compare each genus or family of potentially HCD genera found on each sample to depth. A Benjamini & Hochberg multiple corrections test was applied to correct for false positive occurrences. After performing a series of linear regressions and applying the multiple corrections test, it did not appear that any specific genera responded to depth as a predictor. Several genera did have an intercept that was < 0.05 indicating they were found in some amount on each sample (Figure 4).

Hits per Million Base Pairs

Using a manually curated list of known hydrocarbon degradation genes, BLAST analysis revealed the number of hits for each sample that had actual matches to that curated list. First, bitscores were generated to give the mean hit quality for each metagenome (Figure 5). Each pair of matching query and target sequences was considered a unique hit, numbers of hits per million base pair (Mbp) was then calculated and compared between photic and aphotic depth groups (Figure 6). A t-test was performed that failed to reject the null hypothesis that there would be a difference in hits per a Mbp of assembled genome when comparing photic vs aphotic ($t = -1.3992$, $df = 2.9656$, p-value = 0.2572).

A linear regression was completed to determine if depth predicted number of gene hits per Mbp (Figure 7). The linear regression yielded a multiple R-squared: 0.07889, adjusted R-squared: -0.1514, and p-value: 0.5898. It does not appear that there is a

significant relationship between number of gene hits per Mbp and depth for these samples.

Diversity

Diversity of potentially HCD genera on each sample was calculated using the percentage of each genus or family of these bacteria found in each sample, as well as total of these percentages from each sample, using the formula $D = \sum(n/N)2$. The value D is the Simpson's Diversity Index. The smaller the value, the higher the diversity. A value of zero means infinite diversity, while a D-value of one would indicate no diversity. First a t-test compared the D-values of the photic samples to the D-values of the aphotic samples (p-value = 0.2412) (Figure 8). We failed to reject the null hypothesis that there would be a difference in diversity of HCD bacteria in photic samples versus aphotic samples.

We then ran a linear regression to compare D-values for each sample to depth to determine whether depth predicted a higher or lower D-value (multiple R-squared: 0.2139, adjusted R-squared: 0.01734, and p-value: 0.3558). Overall, the results of the linear regression do not suggest that there is a relationship between diversity of HCD bacteria and depth (Figure 9). Although there is no clear relationship, the aphotic samples seemed to remain very similar regardless of depth and do have a lower D-value than most of the photic samples, while the photic samples had much greater variation. Aphotic samples didn't have a D-value that decreased with depth like a gradient as hypothesized but were all overall lower than photic samples.

Tables

Table 1. Metadata.

Species	Depth	Temp	Remarks
Hormiphora californensis	10	13.30	Wild-caught in Tucker trawl
Bolinopsis microptera	1	12.00	F3 captive-bred specimen from Monterey Bay Aquarium
Mertensiidae sp. T	276	8.59	ROV Ventana
Euplokamis dunlapae	1	10.00	Collected at Friday Harbor Labs by Wyatt Patry
Bathyctena chuni	1915	2.09	ROV Doc Ricketts
Platyctenida sp. T	3958	1.49	ROV Doc Ricketts

Ctenophore species, depth, temperature, and remarks on location of capture.

Table 2. Kraken2 Results.

Host Species	Eukaryotic	Bacteria	No Hits	Other Root	Archaea	Viruses	Total Annotated Reads	Bacterial Annotated Reads
Hormiphora californensis	68%	5%	25 %	2%	0.1%	0.05%	17737	14646
Bolinopsis microptera	34%	28%	33 %	4%	0.5%	0.3%	8227	7369
Mertensiidae sp T	74%	16%	6%	4%	0.3%	0.2%	11345	9975
Euplokamis dunlapae	34%	39%	10 %	5%	0.4%	0.3%	12005	10944
Bathyctena chuni	67%	23%	7%	3%	0.4%	0.1%	14283	12487
Platyctenida sp. T	28%	14%	56 %	2%	0.2%	0.1%	5887	5336

Kraken2 results for all six samples. Results are visualized in krona plots (see supplementary figures 2a-2f) Includes total annotated reads and specifically bacterial annotated reads. Also includes ctenophore host reads (annotated as human due to database used) species name for each sample, the percent DNA annotated as each of the following categories: eukaryotic, prokaryotic, (bacteria and archaea), viruses, no hits, other root. Color differs for each plot as color is dictated by abundance annotated reads present in each category. See Table 2 for summary statistics analysis.

Table 3. Total Percentages of Potential HCD Genera.

Ctenophore Species	Total Percent Bacteria
Hormiphora californensis	11.827 %
Bolinopsis microptera	13.43 %
Mertensiidae sp. T	17.5328 %
Euplokamis dunlapae	17.139 %
Bathyctena chuni	11.9884 %
Platyctenida. sp. T	8.614 %

Each sample is matched with the total number of potentially HCD bacteria found on that sample.

Figures

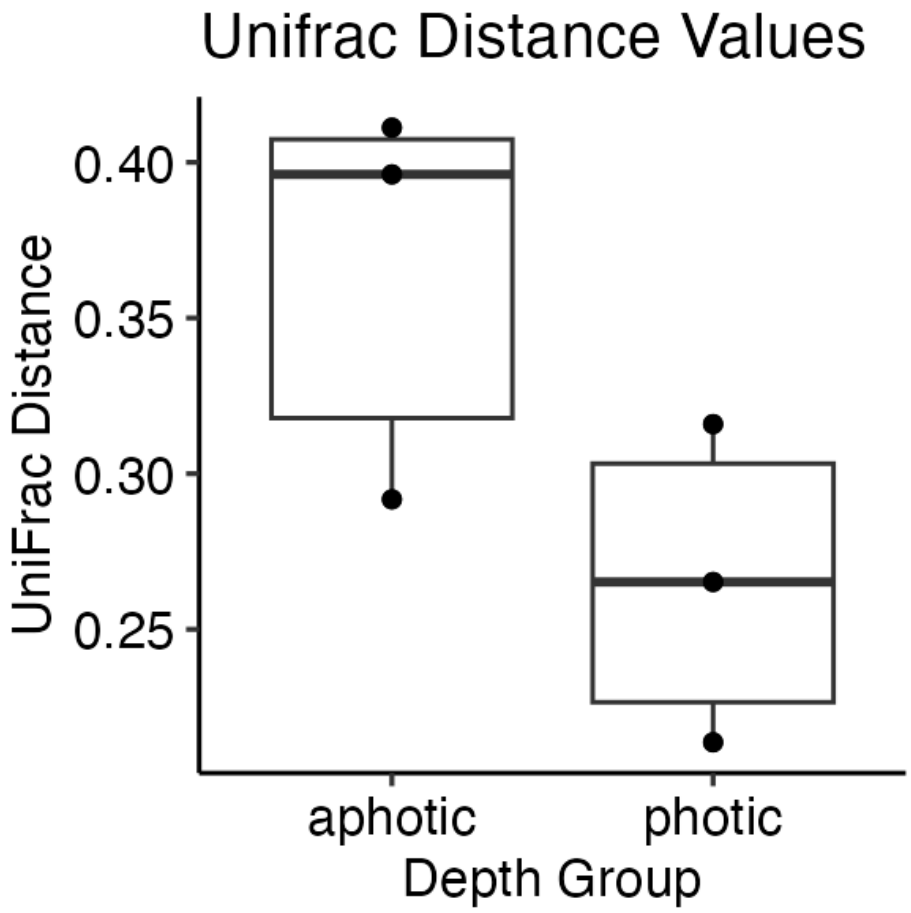


Figure 1. Unifrac Distance Values.

Unifrac distance measurements are compared between two samples, then comparisons are made between depth groups using a T-test. Comparison uses Unifrac Distance values to compare groups (p-value = 0.0078).

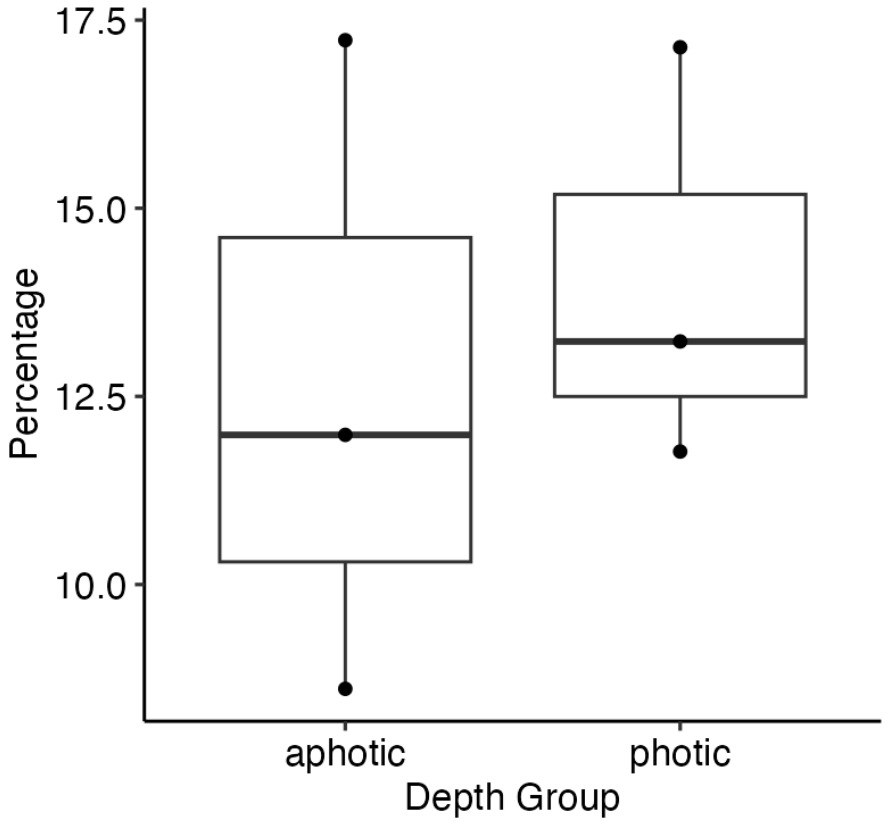


Figure 2. Total Percentages of Potentially Hydrocarbon Degrading Genera

Percentages of potentially HCD bacteria by genera or family were totaled for each sample. Photic samples were compared to aphotic samples (p-value = 0.6695).

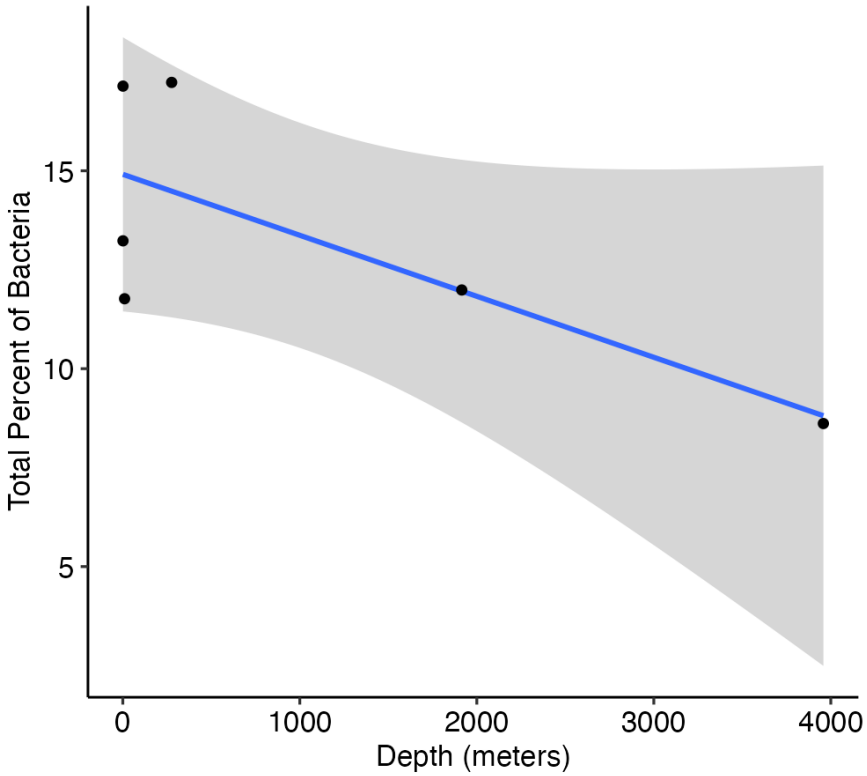


Figure 3. Total Percentages of Genera in Hydrocarbon Degrading Groups vs Depth.

The total percentage of potentially HCD bacteria on each sample as predicted by depth in meters at which the sample was captured.

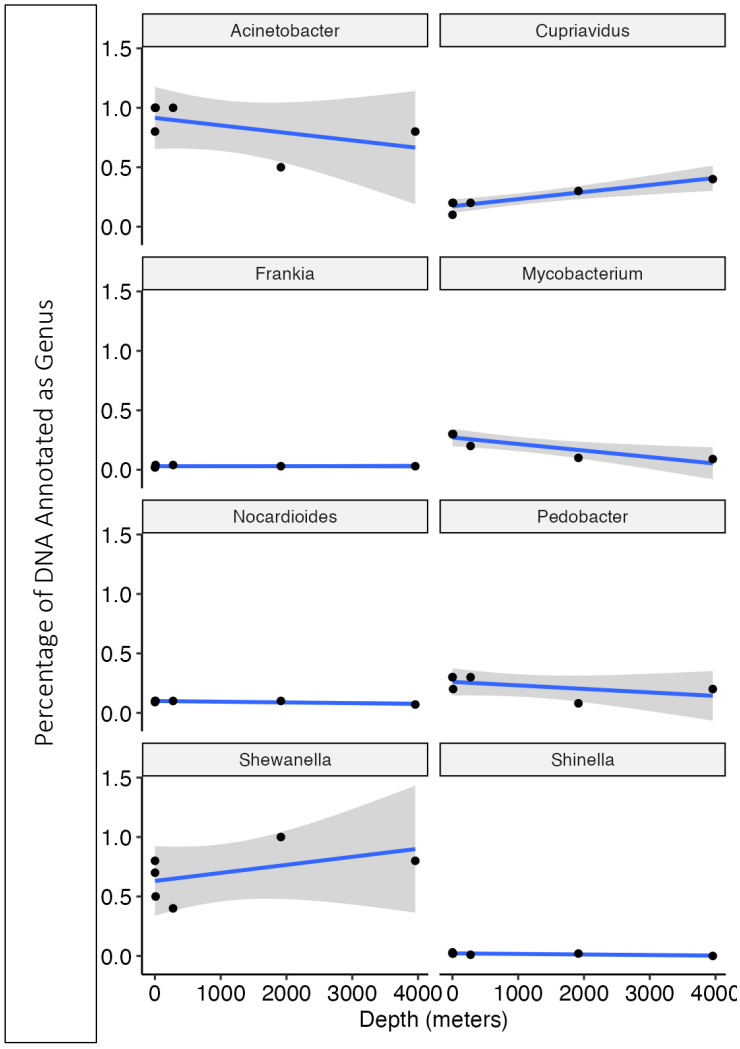


Figure 4. Depth as a Predictor of Potentially HCD Genera.

Linear regressions comparing depth (meters) to percentage of DNA annotated as genus resulting in 8 potentially HCD bacteria found to have an intercept p value of < 0.05.

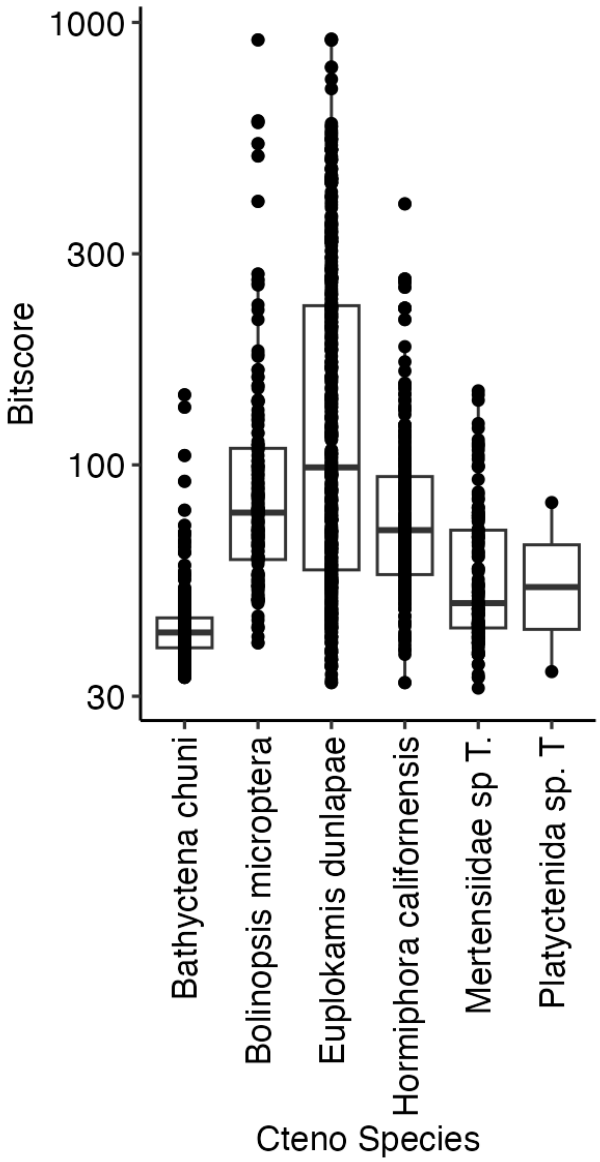


Figure 5. Bitscores of Unique Hits

Unique hits are counted when a known hydrocarbon degradation gene from the curated list of genes is matched with a genome from an organism in each sample. Box plots indicate median, first-, and third-quartile hit quality in each metagenome. Higher bitscores indicate higher quality alignments.

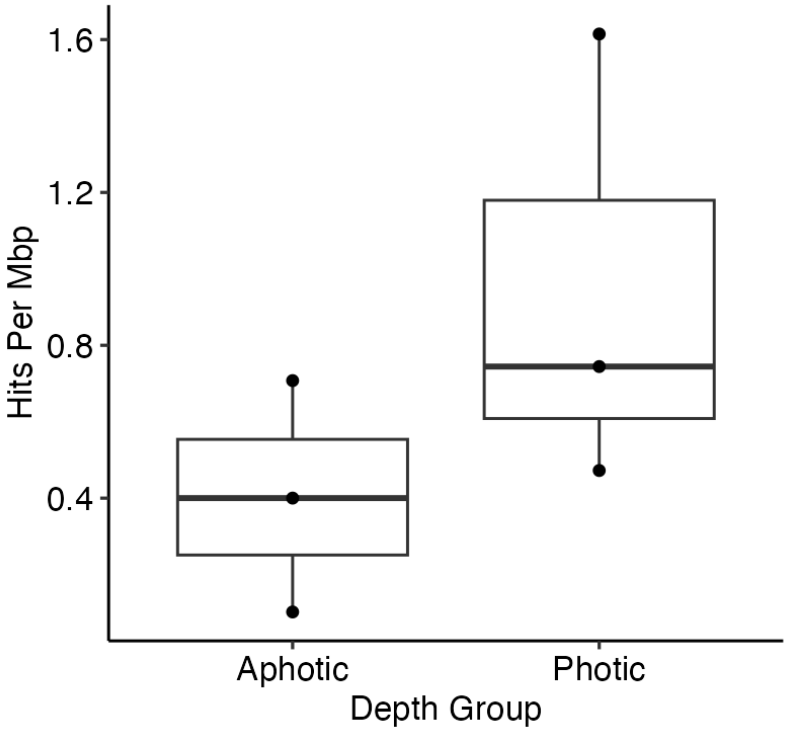


Figure 6. Number of Unique Hits per Mbp.

The number of unique hits per million base pairs (Mbp) analyzed from BLAST results that compared the metagenomes from each sample to a list of known HCD genes. Hits per Mbp are compared between depth groups (p-value = 0.2572).

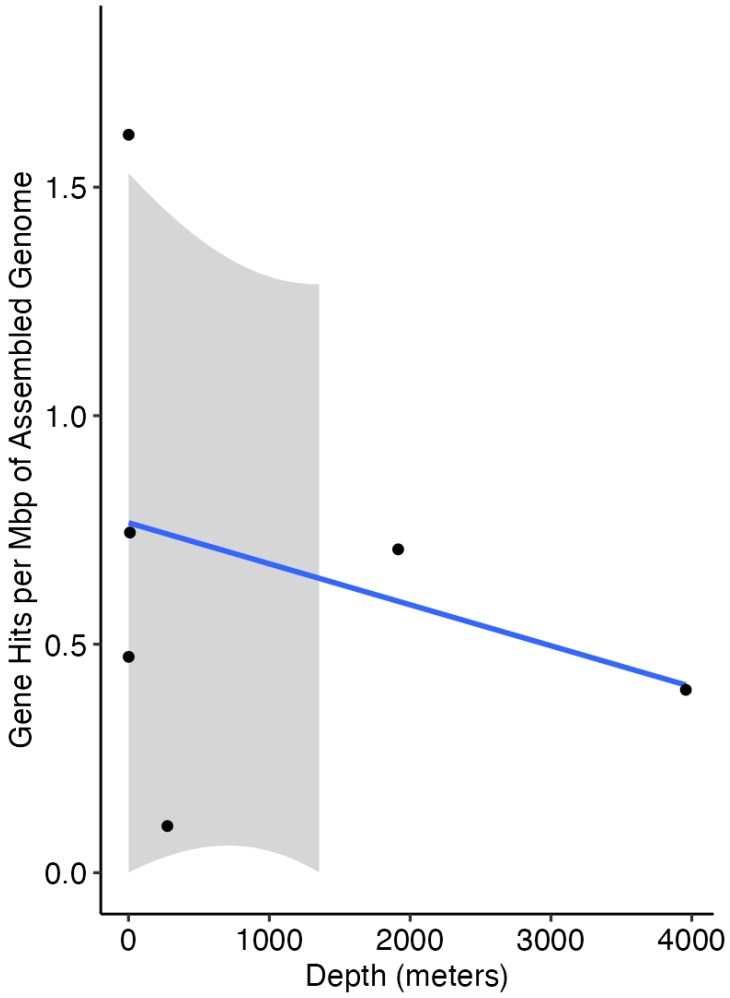


Figure 7. Number of Unique Hits per Mbp of Assembled Genomes vs Depth.

Number of gene hits per Mbp for each sample was compared to depth to determine if depth predicted a higher number of unique hits on that sample.

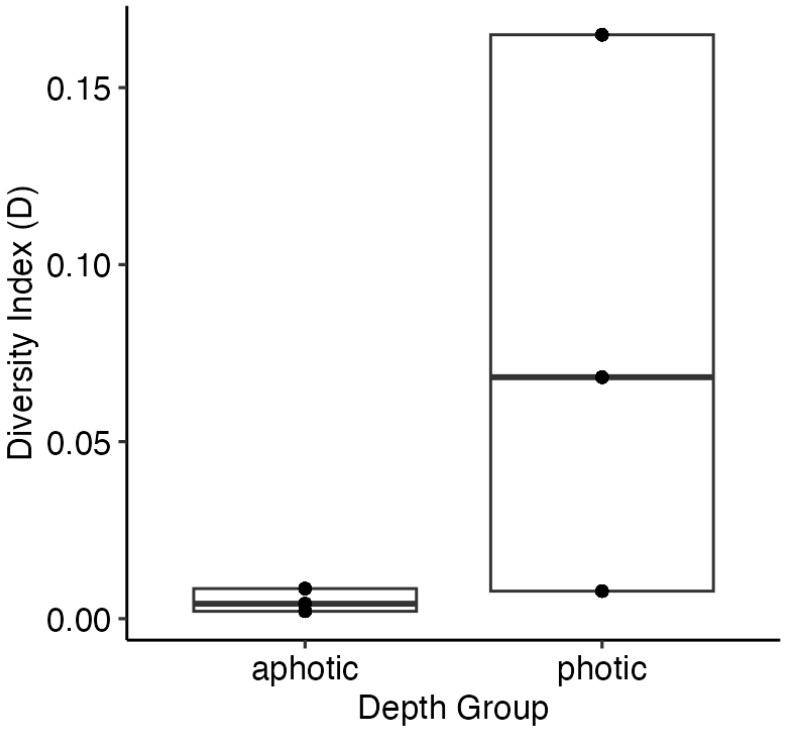


Figure 8. Simpson's Index Diversity Values (D) vs Depth Groups.

Aphotic samples are compared to photic samples. Comparison is between D-Values to indicate difference in species diversity between groups (p-value = 0.2412).

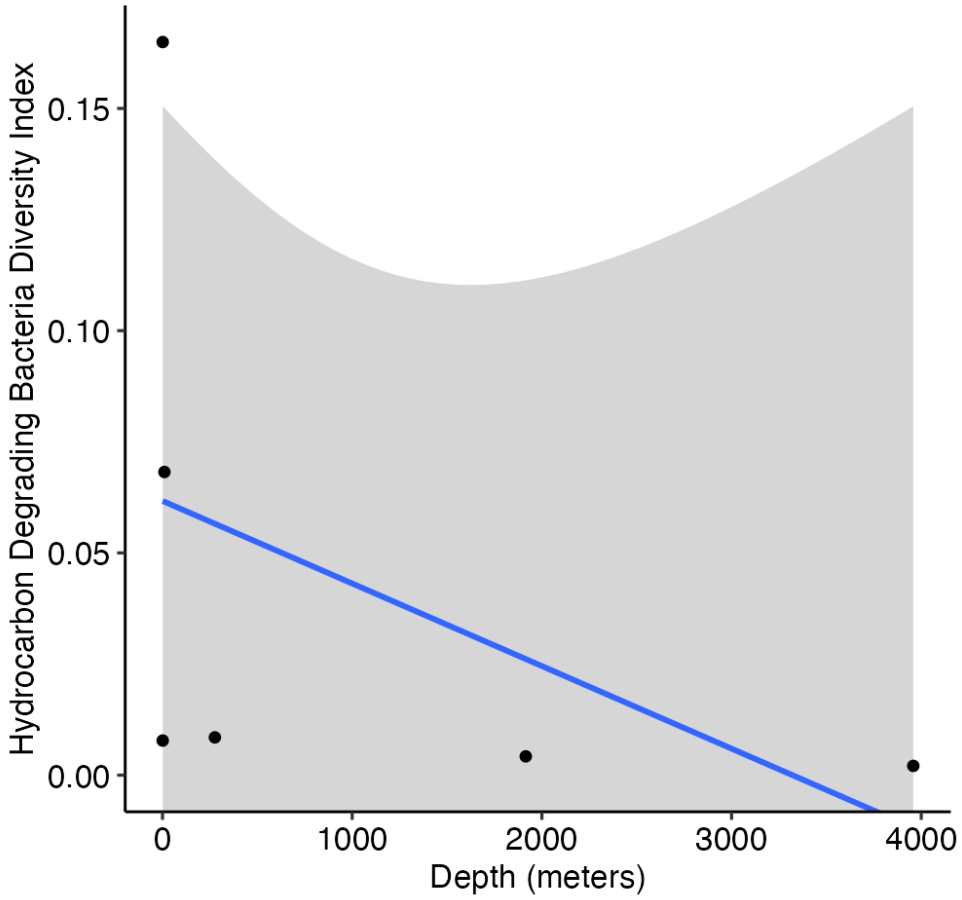


Figure 9. Depth as a Predictor of Potentially HCD Genera.

Aphotic The depth in meters is plotted against the Simpon's Diversity Index Value (D) for each sample. Values that are closer to zero have higher diversity than values that are closer to one.

Chapter IV.

Discussion

Research on deep-sea organisms is often limited due to constraints on sampling. It is very costly and time-consuming to go out to sea and obtain deep samples. The idea for this thesis was derived by knowing there was access to metagenomic data of only a few samples that were already available and provided by our collaborators at the Monterey Bay Aquarium Research Institute. It is unlikely to obtain more samples of ctenophores to compare bacterial communities at this time, which means our sample sizes are small. Although the sample size is small (one per species), sequence data are PacBio CCS reads, which improves assembly quality. For example, some samples such as *Euplokamis,* are deeply sequenced, which allows us to detect low-abundance members of the microbial community. The data are readily available from another project involving ctenophore genome assembly making these benefits possible. It was possible to cover a large range of depth with a small dataset by using only one species of each sample. It was advantageous to obtain all sequence data from the same individual from highly contiguous genomes, thus justifying one individual per species.

Additional limitations include incomplete characterization of alkane-degrading bacteria and the genes responsible for hydrocarbon metabolism. There certainly is some research in this area, as mentioned previously, but overall prior knowledge of these pathways, their ecological niche, etc. is limited. Although our dataset and sample size is small, it will likely contribute meaningfully to our knowledge in this area of research, which is currently underrepresented in the literature.

Overall, the topic of ctenophore microbiomes has been slowly growing in popularity the past few years and researchers have been gaining more insight on bacterial communities found on ctenophores. Since the Hao et al. study in 2015, more researchers have been looking into bacterial associations with ctenophores such as the Jaspers et al. 2019 paper that investigated the microbiota differences between invasive and native populations of *Mnemiopsis leidyi,* which found that all sub-populations of invasive *M. leidyi* were significantly different than native *M. leidyi.* Despite more research being done on the topic, there has not been any investigation into specifically HCD bacteria and potential symbiosis with ctenophores, or how HCD bacteria within ctenophore microbiomes relate to depth. So far, this study is the only known study focusing on the relationship between HCD bacteria and ctenophores across depth.

In sum, three hypotheses have been tested to investigate the relationship between HCD bacteria, ctenophores, and depth at which the host ctenophore is located. First, it was tested whether ctenophores in the photic zone would have significantly different bacterial communities than ctenophores living in the aphotic zone. Second, it was tested whether the aphotic zone ctenophore samples had a higher percentage of potentially HCD genera with known capacity for hydrocarbon degradation within their microbiome than photic-dwelling samples. Lastly, it was tested whether aphotic-dwelling species had a greater diversity of HCD genes than photic-dwelling ctenophores, as well as determining genus diversity among potential HCD bacteria genera. Determining the diversity of genes shows how many different potential HCD pathways may be present in a sample, but not how many different species of bacteria capable of these pathways are present. Additionally, determining the diversity of HCD bacteria shows how many genera of

bacteria are potentially able to perform such functions, but does not determine if they truly have the genes available to complete the function.

We predicted that sampled ctenophores from the photic zone would have broadly different bacterial communities than ctenophores from the aphotic zone. This prediction was not entirely supported by our data. A comparison of Unifrac distances within versus among depth groups did not reflect statistically significant clustering. There was less variation among photic samples than in aphotic samples. However, the mean distance between samples was different in each depth group, with aphotic microbiomes showing more variation ($P = 0.0078$). This is similar to the finding from the Hao, et al., 2015 study that compared four different species of ctenophores. Even with a larger sample size than this study, Hao, et al. also found that bacterial community composition was significantly different between species. This finding is also like that of the Jaspers et al. 2019 paper in which bacterial communities differed greatly even between hosts of the same species. Additionally, the Jaspers et al. 2019 paper discovered that bacterial communities also differed between microbiotic communities on the ctenophore and that of the water column surrounding the ctenophore. This suggests that the difference in bacterial communities is not necessarily linked to the species of bacteria found in the surrounding environment. This knowledge was the basis for the second and third hypotheses tested in this study.

If the immediate environment and the bacteria available in that environment are not determining factors for the bacteria found on ctenophore hosts, then possible explanations would be other environmental factors such as light availability, hydrostatic pressure, temperature, or a combination of environmental factors. Our second hypothesis

looked specifically for a difference in the percentage of potentially HCD genera between photic zone- and aphotic zone-dwelling ctenophores, essentially quantifying congeners of bacteria with HCD potential and comparing them between depth groups. Hydrocarbon degradation gene hits per Mbp were determined to not be significantly different between photic and aphotic groups and therefore this information did not support the hypothesis that there would be potentially more diversity of HCD bacteria within the aphotic group. Therefore, it does not appear that light availability or abundance of potentially HCD bacteria in low light to no light conditions to aid in carbon cycling explain the difference between bacterial community compositions on these six samples. The results of the linear regression comparing hits per Mbp to depth also does not support the idea that hydrostatic pressure constraints are predictors for an increase in potential HCD bacteria presence.

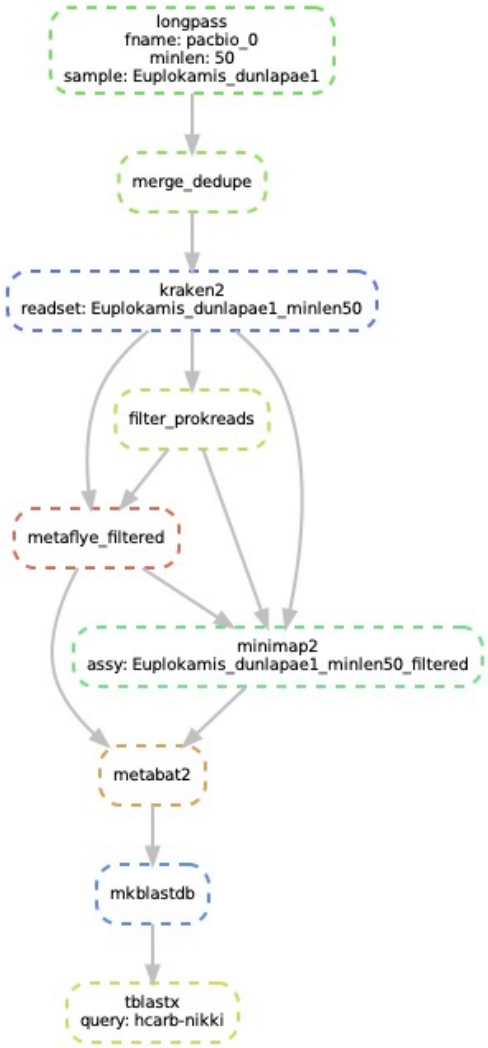
It was also predicted that photic-dwelling ctenophores would have a lower OTU diversity, higher Simpson's index (D), regarding bacteria that had HCD potential due to organic carbon being more available in the photic zone where photosynthesis is possible. Aphotic-dwelling species of ctenophores were predicted to have a higher diversity, lower Simpson's index (D), than photic-dwelling species due to the availability of recalcitrant carbon in areas where photosynthesis is not an option, and HCD bacteria are known to be more abundant particularly in the benthic zones.

Our quantitative data do not support either of these predictions as diversity values (D) showed that no true relationship seemed to exist between the D-values of the photic-dwelling species versus the aphotic dwelling species, as well as no relationship to depth when comparing each species D-value to its known depth at capture. However,

qualitatively, all aphotic ctenophores had high HCD genus diversity and two of the photic ctenophores had relatively low diversity. This suggests that work on this topic is worth extending with more samples. HCD genus diversity for the aphotic samples are similar in value when compared to each other, while photic samples have a significantly higher range in values. Although it needs to be taken into consideration that at least one of the photic samples was captive bred and raised in a planktonkreisel tank (1 meter depth), while the other two were not captive bred and found at 10 meters. The captive bred sample was interestingly the sample with the lowest diversity (D-value closer to 1) as compared to all other wild caught samples.

These results indicate that, as found in other studies, the microbiomes of ctenophores seem to differ across different taxa. They also seem to differ over temporal periods, and across habitats. Our results did not give any sort of overall determination as to why the microbiota of ctenophores differs, or what factors predict the organisms involved in each individual's microbiome. It does seem that there is potential for this data to paint a slightly different picture if more samples were involved. It may be a possibility that with more samples we could see higher levels of variation among the photic samples regarding hydrocarbon degrading bacterial diversity, while in aphotic samples we see increased diversity according to D-values, but less variation. Overall, the conclusion made is that the microbiomes of ctenophores appear to be very different across the board, however, new questions arise about what the differences mean in terms of what functions are these microbes providing for the ctenophore, if any?

Supplementary Tables and Figures



Supplementary Figure 1. Data Flow Chart.

The long pass reads for each sample with read length at which 50% of base pairs are longer or at least equal to this value are merged and then run through Kraken2. All prokaryotic reads are filtered. Metalflye k-mer based raw reads are filtered. Minimap2 aligned DNA sequences from metaflye, prokaryotic, and Kraken2 reads. Metabat2 is used to reconstruct genomes from minimap2 and metaflye results into FASTA files using mkblastdb. FASTA files are queried against the manually curated hydrocarbon gene list.

Bathyctena chuni Krona Plot

Supplementary Figure 2a. *Bathyctena chuni* Krona Plot

Krona Plot of Kraken2 results for Bathyctena chuni*. Includes total annotated reads and specifically bacterial annotated reads. Also includes ctenophore host reads (annotated as human due to database used) species name for each sample, the percent DNA annotated as each of the following categories: eukaryotic, prokaryotic, (bacteria and archaea), viruses, no hits, other root. Color differs for each plot as color is dictated by abundance annotated reads present in each category. See Table 2 for summary statistics analysis.*

Bolinopsis microptera Krona Plot

Supplementary Figure 2b. *Bolinopsis microptera* Krona Plot.

Krona plot for the Bolinopsis microptera*. Information provided is the same as supplementary figure 2a with color mapping being the exception as it changes according to abundance in each plot.*

Euplokamis dunlapae Krona Plot

Supplmentary Figure 2c. *Euplokamis dunlapae* Krona Plot.

Krona plot for the Euplokamis dunlapae*. Information provided is the same as supplementary figure 2a with color mapping being the exception as it changes according to abundance in each plot.*

Hormiphora californesis Krona Plot

Supplemnentary Figure 2d. *Hormiphora californensis* Krona Plot

Krona plot for the Hormiphora californensis. *Information provided is the same as supplementary figure 2a with color mapping being the exception as it changes according to abundance in each plot*

Mertensiidae sp. T Krona Plot

Supplementary Figure 2e. Mertensiidae sp. T Krona Plot

Krona plot for the Mertensiidae sp. T. Information provided is the same as supplementary figure 2a with color mapping being the exception as it changes according to abundance in each plot

Platyctenida sp. T Krona Plot

Supplementary Figure 2f. Platyctenida sp. T Krona Plot

Krona plot for the Platyctenida sp. T. Information provided is the same as supplementary figure 2a with color mapping being the exception as it changes according to abundance in each plot

Supplementary Table 1. Manually curated list of known hydrocarbon degradation genes.

Hydrocarbon Degradation Genes List

List includes gene name, species that gene can be found within, accession number, and sources.

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