Determining the spatial and seasonal influences of microbial community composition and structure from the Hawaiian anchialine ecosystem

by

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Abstract

Characterized as coastal bodies of water lacking surface connections to the ocean but with subterranean connections to the ocean and groundwater, habitats belonging to the anchialine ecosystem occur worldwide in primarily tropical latitudes. Such habitats contain tidally fluctuating complex physical and chemical clines and great species richness and endemism. The Hawaiian Archipelago hosts the greatest concentration of anchialine habitats globally, and while the endemic atyid shrimp and keystone grazer *Halocaridina rubra* has been studied, little work has been conducted on the microbial communities forming the basis of this ecosystem's food web. Thus, this dissertation seeks to fill the knowledge gap regarding the endemic microbial communities in the Hawaiian anchialine ecosystem, particularly regarding spatial and seasonal influences on community diversity, composition, and structure. Briefly, Chapter 1 introduces the anchialine ecosystem and specific aims of this dissertation. In Chapter 2, environmental factors driving diversity and spatial variation among Hawaiian anchialine microbial communities are explored. Specifically, each sampled habitat was influenced by a unique combination of environmental factors that correlated with correspondingly unique microbial communities. Notably, salinity was the one water chemistry factor with strong explanatory power and influence in driving microbial community structure. Chapter 3 examines seasonality in these Hawaiian anchialine microbial communities across an 18-month period. Although there was evidence that microbial community structure varied across the wet and dry seasons, these changes were minimal overall and the greatest shifts were in relative abundance of oxygenic and anoxygenic phototrophs, with oxygenic phototrophs more abundant during wet seasons and anoxygenic phototrophs during dry seasons. The specific microbial consortia found in the four distinct layers composing the unique orange laminated cyanobacterial-bacterial crusts from select Hawaiian anchialine habitats are discussed in Chapter 4. As with laminated

microbial mats from other ecosystems, greater taxonomic richness within the community occurred deeper within the crust structure, with these crusts apparently, and unusually, oxygenated at both their top and bottom surfaces. Therefore, oxygenic phototrophs were most abundant in the top and bottom of the crusts, with anaerobic metabolisms largely confined to the middle two layers. Finally, Chapter 5 discusses the conclusions of the preceding chapters and future research directions.

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Chapter 1. Dissertation Introduction

1.1 Introduction to the anchialine ecosystem

Anchialine habitats are coastal features with fluctuating salinities that lack surface connections to the ocean [1–4]. In 1963, Holthuis described shrimp collected from landlocked pools of water that fluctuated due to assumed connections to the sea [5]. Ten years later, Holthuis referred to these pools as 'anchialine,' from the Greek "anchialos" for "near the sea," and defined the habitat as pools of salt or brackish water that fluctuate with the tide despite lacking open connections with the sea [1]. This definition was subsequently revised to reduce its ambiguity by specifying the requirement of a significant terrestrial a well as marine influence and generalizing the required salinity to incorporate a wider range of salt concentrations [3]. Habitats fitting this ecosystem definition can take the form of open pools, flooded caves, and submerged cave passages [4]. While rare, instances of anchialine habitats have been identified across the globe in primarily tropical latitudes, including Bermuda [6], islands across the South Pacific, the Ryukyu Islands, the Philippines [2], the Sinai Peninsula [1], the Caribbean [7], Australia [8], Indonesia [9], Europe [4], and Hawaii [5].

The Hawaiian Archipelago is home to the only naturally-occurring anchialine habitats in the US as well as the greatest concentration of them in the world [10]. Of the eight high Hawaiian Islands, anchialine habitats naturally occur on three; ponds on Maui and the island of Hawaii occur in basalt basins while those on Oahu are found in fossilized coral (*i.e.*, calcium carbonate) basins. Other instances of habitats fitting the anchialine definition have also been recorded in the Hawaiian Archipelago. For instance, a single anchialine habitat, known as Sailor's Hat crater, was formed on the island of Kaho'olawe by the testing of military explosives in 1965 [11], but since appears to have lost its subterranean connections to the open ocean (T. Iwai personal communication). Similarly, Lake Kauhakō on Molokai was classified as an anchialine habitat by Stone [12], but the subterranean connections to the ocean appear to have been blocked and Kauhakō is now considered a meromictic lake and not an anchialine habitat [13]. Historically, many anchialine habitats on Maui and Hawaii were used for potable water, bathing, and aquaculture by native Hawaiians [12, 14, 15]. Notably, particular anchialine habitats

in the Cape Kinau region of Maui and Kona region of Hawaii both exhibit a bright orange laminated cyanobacterial-bacterial crust found nowhere else in the world [16, 17]. Of particular interest is that though identical in appearance and lamination, these crusts assembled rapidly and repeatedly due to occurring in relatively young (*i.e.*, <200 yrs old) lava fields that would have been rendered sterile during their formation, as well as assembling independently on each island, since Maui and Hawaii have never been geologically connected.

The anchialine ecosystem is known for having high species diversity, with numerous endemic species both worldwide [18] and in Hawaii [1, 5, 11]. Specifically, studies of Hawaiian anchialine habitats continue to document new species [19] and undiscovered genetic diversity [20] among those species already described. While some endemic anchialine organisms, like the atyid shrimp, *Halocaridina rubra*, have been fairly well studied [2, 5, 17, 21], the unique microbial communities growing as a distinctive orange laminated crust in certain regions of Maui and Hawaii have been poorly characterized. What is known about anchialine microbial communities in Hawaii is largely based on morphological identification of taxa comprising these crusts [16, 17, 22]. Additionally, Donachie et al. [13] examined the taxonomic diversity of water column microbiota from an anchialine pool on Southeast Island in the Pearl and Hermes Reef by partial prokaryotic small subunit ribosomal DNA (16s rDNA) genes amplified by the polymerase chain reaction (PCR) and sequenced from the community. However, a wide scale examination and documentation of microbial taxonomic diversity, community composition, and structure of the Hawaiian anchialine ecosystem, including those habitats with the unique orange crust, has not been done to date.

Historically, examination of anchialine microbial diversity was based on microscopy. For instance, the initial work by Wong examined 17 anchialine habitats from Cape Kinau, Maui and described the algal and microbiotic community through light microscopy [16]. Four types of pond benthos were described based on community appearance and dominant taxa: matted cyanobacterial crust, marine algae and cyanobacteria, *Ruppia maritima*, and marine macrophytic chlorophytes and rhodophytes. In all cases, these communities were species rich, with the following examples. Twenty species of chlorophytes

belonged to the genera *Caulerpa, Chaetophora, Chlorella, Cladophora, Cladophoropsis, Dictyosphaeria, Enteromorpha, Microdictyon, Microspora, Stigeoclonium, Struvea, Ulothrix,* and *Valonia*. Forty-one species of Cyanobacteria belonged to the genera *Calothrix, Chroococcus, Dermocarpa, Gomphosphaeria, Lyngbya, Microcoleus, Oscillatoria, Pleurocapsa, Schizothrix, Scytonema,* and *Spirulina*. Five species of Rhodophyta belonged to the genera *Ahnfeltia, Amphiroa, Hildenbrandtia, Lithophyllum,* and *Porolithon.* Seventy-three species of diatoms were observed, as well as two species of Chrysophyta and three species of dinoflagellate [16]. Along with this, Maciolek examined 35 anchialine habitats from Cape Kinau, including those studied by Wong, and reported 71 diatom species, 20 Chlorophyta species, 2 Chrysophyta species, 41 Cyanobacteria species, 3 dinoflagellate species, and 5 Rhodophyta species [22]. All of the species reported by Wong [16] were also observed by Maciolek [22]. This was followed by work from Bailey-Brock and Brock, which imaged specimens of the orange microbial crust as the food source for *H. rubra* from Hawaii using scanning electron microscopy (SEM) and described the structure as primarily composed of diatoms and filamentous chlorophytes growing over cyanobacteria in successive layers similar to those observed in stromatolites or algal mats [17]. The carbonate crystals found between cell layers, representing previous crust surfaces, were produced by Cyanobacteria [17]. In addition to species identified by Wong [16] and Maciolek [22], *Rhizosolenia* sp. and two species of folliculinids were reported [17].

Accurate identification of microbial taxa, populations, and communities using microscopy is often impossible because of the lack of diagnostic morphological characters [23, 24]. One means to circumvent this limitation is the use of DNA sequence data, which allows for more accurate membership identification from diverse microbial communities [23]. For example, surface water samples collected from an anchialine pool on Southeast Island in the Pearl and Hermes Reef by Donachie et al. resulted in 16S rDNA sequences belonging to the Alphaproteobacteria, Gammaproteobacteria, and Firmicutes [13]. Pure cultures were also grown and identified as belonging to three alphaproteobacterial lineages, one cyanobacterial lineage, 11 gammaproteobacterial lineages, and three Firmicutes lineages [13]. Thus, application of a sequence-based approach to the complex microbial communities present in Hawaiian

anchialine habitats would likely provide the ability to identify a greater diversity of community members than studies based on microscopy.

Currently, Hawaiian anchialine habitats are at an extreme risk of degradation and destruction, primarily due to urbanization, development, and invasive species introductions [11, 17]. For example, development along the coastline of Hawaii has resulted in the destruction of many known habitats, with a single project in 1985 destroying over 130 habitats [25], and many remaining anchialine habitats near development being impacted by nutrient run-off [25–27]. Furthermore, the introduction of poeciliids (*Poecilia* spp. and *Gambusia affinis*) and tilapia (*Oreochromis* spp.) results in a modified diel migration of *H. rubra* [28–32] and subsequently alters microbial community biomass, productivity, and nutrient content [2, 66]. Indeed, previous studies regarding *H. rubra* suggest that it may act as a keystone grazer that is responsible for the maintenance of the laminated crust over an algal monoculture [17, 30, 33–35]. Unfortunately, projected increases in sea level due to climate change are expected to exacerbate the situation by providing fish access to a greater proportion of Hawaiian anchialine habitats than are currently impacted [36]. *Halocaridina rubra* also faces predation by the invasive shrimp *Macrobrachium lar*, which also results in altered *H. rubra* behavior and abundance and thus impacts the microbial community [37].

Alterations to microbial communities of aquatic environments can have dramatic impacts on the entire ecosystem since their members are responsible for the majority of aquatic primary production as well as most carbon and nitrogen cycling [38, 39]. The anchialine ecosystem appears to be no different, in that microbial communities are the primary producers supporting higher trophic levels [7, 40]. For example, anchialine cave habitats in the Yucatan Peninsula contained chemoautotrophic nitrifying bacteria that may provide significant levels of primary production [7], and Bahamian anchialine blue holes were dominated by anoxygenic phototrophic bacterial mats [41]. Dalton et al. [30] demonstrated that the microbial community formed the base of food webs in anchialine habitats on the island of Hawaii and can exert bottom-up influences on higher trophic levels. Clearly, understanding the microbial

community that supports Hawaiian anchialine habitats is vital to understanding the functioning of the entire ecosystem before it is irreversibly degraded and/or destroyed.

In order to further scientific understanding of the anchialine ecosystem in the Hawaiian Islands, gathering greater knowledge of the microbial community at the base of the food web is essential. My dissertation research sought to address this need by examining the impact of spatial and seasonal factors on the microbial community and by attempting to elucidate distinctions in the four colored layers present in the unique orange laminated crust community via the application of high-throughput next-generation sequencing of environmental DNA. Below, I provide a brief description of the rationale and hypotheses of each chapter.

1.2 Environmental factors driving spatial variation and diversity among *Bacteria* and micro-*Eukarya* communities of the Hawaiian anchialine ecosystem

Spatial distribution is a prominent theme in ecological work, since identifying the range across which organisms can be found provides clues about the environmental conditions and factors required for their survival. Furthermore, investigating environmental conditions required for an organism's growth can then be scaled to the community level to learn more about both individual species' niches and the conditions required to sustain a specific community. While variation in microbial populations across spatial scales is a relatively new concept [42], spatial variation in microbial communities should be influenced by distance and habitat size in the same way as macroorganisms [43]. For example, Anderson-Glenna et al. [44] evaluated microbial biofilm communities along a stretch of pristine river and found that variability in biofilm community composition across downstream sites was correlated with increased pH, temperature, calcium, magnesium, and sodium. Additionally, Ramette and Tiedje [45] examined the relationships between abundance of the *Burkholderia cepacia* complex isolated from crop monocultures, environmental heterogeneity, and spatial distance across multiple scales and found that *B. cepacia* complex abundance exhibited spatial variation influenced by environmental factors, including soil variables and crop species. Despite identifying variables contributing to the distribution of the *B. cepacia*

complex, most variation was unexplained [45] and reinforces the need for further work examining how environmental variables drive the structuring of microbial communities.

The impact of environmental factors on microbial community structure has been little studied in the anchialine ecosystem. However, those studies that have been done suggest geographic proximity does not necessitate similarity in microbial communities among anchialine habitats. For example, adjacent (~20 km) Bahamian blue holes were found to have distinct microbial communities, despite their proximity [41]. Similarly, anchialine pools within 100 m of each other inside a single cave in Mallorca, Spain, were also found to contain distinct communities [46]. Indeed, the abiotic diversity of the ecosystem [3, 4, 8, 47, 48] allows for complex structuring of microbial communities within a single habitat. For instance, Bundera Sinkhole, Australia, exhibited distinct complex vertical stratification of temperature, salinity, dissolved oxygen, and hydrogen sulfide in the water column that influences the distribution of phytoplankton, aerobic heterotrophic bacteria, white sulfur-oxidizing bacteria, and nitrifying bacteria [49]. Specifically, most sulfur reducers in this case belonged to Deltaproteobacteria and Deinococci, chemolithoautotrophic ammonia-oxidizers belonged to Thaumarchaeota, halophilic anaerobes belonged to Bacteroidia, and hydrogen sulfide oxidizers belonged to Gammaproteobacteria [50]. In addition, Humphreys et al. [50] observed that the microbial ecosystem in Bundera Sinkhole was stratified by taxonomic class across depth.

The possible environmental factors involved in structuring the microbial community have not been previously identified for the Hawaiian anchialine ecosystem. Anchialine habitats in Hawaii are found across a range of environmental factors, particularly basin substrate (basalt vs. fossilized coral), temperature (17-30°C), and salinity (1-16) [51], and microbial community structure is expected to reflect this diversity in environmental factors. For example, the orange microbial crust has only been observed in anchialine habitats in particular regions of Maui and Hawaii [17, 22]; these regions sharing the unique orange microbial crust phenotype possess basalt basins, but the other environmental factors influencing crust presence have not been examined. Due to the great diversity in environmental factors across anchialine habitats, it was hypothesized that each habitat would have a unique microbial community

structure associated with its unique geographic location and environmental conditions, and that any similarities in microbial community structure between habitats would correlate with similarities in environmental conditions.

1.3 Seasonal stability in Hawaiian anchialine microbial communities across an 18-month period

Microbial communities can be influenced by seasonal variation in environmental factors, resulting in consistent and repeating temporal changes in community structure. For example, such temporal shifts in microbial communities have been documented in lakes [52, 53], rivers [44], aquifers [54], geothermal springs [55, 56], and the coastal ocean [57]. A specific instance is the study by Lymer et al. [53], which examined three lakes in central Sweden and identified temperature and dissolved organic carbon concentration as being most important in explaining bacterial community composition. Additionally, date and temperature were strongly correlated with bacterial community composition across seasons. Furthermore, seasonal shifts in the microbial consortia in Lake Kinneret, Israel, altered the nutrient availability for higher trophic levels [52]. Anderson-Glenna et al. [44] also found that seasonal fluctuations in temperature correlated most closely with microbial community variation in riverine biofilms. Temporal fluctuations in microbial communities from shallow portions of the Doñana aquifer in Spain also exhibited correlations with changing seasonal temperature, although communities in deeper regions of the aquifer did not exhibit such temperature-correlated fluctuations [54]. Temperature and phosphate concentration were also correlated with temporal fluctuations in microbial mat communities from geothermal springs in the Philippines, likely through the addition of rainfall-related runoff during the wet season [55]. Similarly, microbial communities found in the hot springs of Tengchong, China, exhibited differences in richness and diversity due to seasonal changes in water nutrient levels and temperature [56]. Nelson et al. [57] identified distinct temporal variation in coastal ocean microbial communities, but were unable to correlate them to routine oceanographic chemical measurements and thus suggested that biological and/or ecological processes may have been responsible for the observed variation.

Currently, only two studies have examined seasonal or temporal variation in microbial communities from the anchialine ecosystem. Specifically, for anchialine caves on Mljet Island in the Adriatic Sea, total bacterial abundance, relative abundance of high- and low-nucleic acid bacteria, and the relative influence of bottom-up vs. top-down control all varied across the sampling period [58]. Furthermore, anchialine habitats in Quintana Roo, Mexico, were found to exhibit seasonal fluctuations in bacterioplankton density hypothesized to be linked to seasonal variation in freshwater and organic matter inputs [59]. Specifically, Alcocer et al. found bacterial cell density to be greater during the rainy than the dry season in five anchialine caves, but could not correlate this with any specific water quality variables. Given this, seasonal variation was assumed to be driven by addition of exogenous microbes introduced by rainwater influx relying on organic carbon sources also introduced by the same route [59].

Documentation of temporal changes in microbial communities can lead to a better understanding of the ecological functions for specific taxa whose abundance, and potential ecological significance, fluctuate seasonally. For example, Brown et al. [60] examined coastal marine microbial communities and found heterotrophic taxa to be most abundant during spring when nutrients and prey sources were abundant. In contrast, phytoplankton taxa that had been less abundant during spring dominated the summer community along with other taxa assumed to either rely on the phytoplankton or occupy the same niche [60]. Gilbert et al. [61] examined microbial communities in the Western English Channel and found seasonal fluctuation in expression of photosynthetic genes with the exception of proteorhodopsin, which exhibited a constant rate across the year. In this case, bacterial and archaeal diversity was greatest during winter, corresponding with an increased rate of photosynthetic gene expression [61]. In the Mediterranean Sea, *Roseobacter*, Gammaproteobacteria, Bacteroidetes, and the SAR11 group of Alphaproteobacteria exhibited differences in seasonal patterns of activity, measured as uptake of glucose, amino acids, and ATP [62]. In this case, differential seasonal activity implies that different taxa contribute to marine nutrient cycling differentially depending on season [62].

Although temporal or seasonal fluctuations have been observed in numerous microbial communities, stability in the face of fluctuating environmental conditions has also been documented for

hypersaline microbial mats [63–65], cyanobacterial desert soil crusts [66], hot spring microbial mats [67], and phototrophic microbial/cyanobacterial mats found in a meromictic hypersaline lake [68, 69]. The laminated nature of many cyanobacterial-bacterial mats and crusts allows for niche formation that in turn allows greater taxonomic diversity, and metabolic activity is largely due to the cyanobacterial component. Specifically, the metabolic diversity displayed by Cyanobacteria enables them to survive in extreme environments and facilitate mat or crust formation by driving productivity, separating oxygenated and anoxygenated niches, and secreting the extracellular polymeric substances (EPS) that further create cohesion [70]. As a result, laminated cyanobacterial-bacterial mats or crusts allow for greater community diversity and functional redundancy, characteristics Yannarell et al. [65] posited allowed Bahamian hypersaline microbial mats to have little to no compositional change in the cyanobacteria component due to seasonal hurricane activity [64]. Thus, it is possible that the laminated nature of the orange cyanobacterial-bacterial crusts found in the Cape Kinau region of Maui and the Kona region of Hawaii may increase the resistance of these communities to factors associated with seasonal fluctuations.

While it remains to be determined whether Hawaiian anchialine microbial communities exhibit temporal variation, the presence of seasonal changes in environmental variables suggests the potential to do so. Specifically, Hawaii experiences seasonal fluctuations in factors such as rainfall that correspond with fluctuations in dissolved nutrients [71]. These varying nutrient levels in groundwater could influence anchialine microbial community composition due to greater nutrient levels being found in groundwater than adjacent sea water [72]. For most regions of the state, January exhibits the greatest monthly rainfall and June the minimum [73], so nutrient levels in Hawaiian anchialine habitats are expected to peak during the wet winter season (*i.e.*, November-April). Therefore, seasonal variation in Hawaii's groundwater nutrient levels is hypothesized to result in corresponding variation in microbial community taxonomic diversity and relative abundances; alternatively, the laminated cyanobacterial-bacterial crust community is resistant to seasonal variation.

1.4 Comparison of microbial consortia composition in the layers of laminated cyanobacterial-bacterial mats found in select Hawaiian anchialine habitats

Microbial mats exhibiting lamination, with taxa having vertically stratified distributions in response to chemical or other gradients, have been valuable study subjects in advancing scientific techniques and knowledge. The discovery of *Taq* polymerase from *Thermus aquaticus* [1, 2], isolated from microbial mats from hot springs in Yellowstone National Park, Montana, USA [3] has enabled the rapid advancement of genetic lab techniques. Furthermore, bioremediation techniques have benefited from the study of laminated microbial mats, particularly in addressing contamination from sources such as aquaculture effluent [4, 5] and mine drainages [6, 7]. Study of laminated microbial mats has also advanced our understanding of the evolution of life and ecosystem function, as fossilized mats in the form of stromatolites have helped with estimating when life first evolved [8–11] and also contribute to understanding how it may potentially have arisen on other planets [12].

Although laminated crust communities have not been documented from other anchialine habitats, vertical stratification in water chemistry, particularly salinity and dissolved oxygen levels, inherent to this ecosystem can vertically structure microbial communities within the water column [8, 41, 46, 49, 58]. An example of this is Bundera Sinkhole in Australia, where aerobic heterotrophs and phytoplankton are concentrated near the surface, while chemolithoautotrophic Thaumarchaeota and sulfur-reducing Deltaproteobacteria were most abundant below the halocline [50]. Likewise, anchialine blue holes in the Bahamas were also found to have increased microbial density in the form of colored biofilms at, or just below, the halocline that were composed primarily of anoxygenic phototrophs [41]. Additionally, anchialine caves from both Mljet Island in the Adriatic Sea [58] and Mallorca, Spain [46], exhibited changes in microbial abundance through the water column that correlated with water chemistry.

Lamination in microbial mats or crusts is driven by physical and chemical gradients across their depth that are created and maintained by the organisms composing the structures themselves [74]. Of particular note are gradients of oxygen and light, which are both abundant at the surface, creating an oxygenated photic zone, below which an anoxygenated photic zone exists were oxygen becomes depleted

and light limiting [74]. Under the photic layers, anaerobic heterotrophic and chemotrophic organisms can be found [69, 75–79], where the lack of oxygen and light creates suitable unoccupied niches. Both the structure, and gradients within the mat or crust, allow for greater niche diversity [48] that in turn allows for greater species richness and diversity further into the mat [42, 44–46, 49]. Common in the oxygenated photic zone, Cyanobacteria are thought to be instrumental and major contributors to primary production as photoautotrophs, and are also vital to the mat's structural integrity as contributors of filamentous taxa and secretors of extracellular polymeric substances [10].

Despite their importance to mat and crust formation and structure, Cyanobacteria typically contribute only 10-20% of the total microbial population [10, 41]. Indeed, increases in species richness and diversity with greater depth, representing the anoxygenated photic and anoxygenated aphotic zones, of the structure have been observed in hypersaline environments [69, 78, 80, 81] and salt marshes [82]. Heterotrophic organisms commonly found in such situations include diatoms, Bacteroidetes, and Proteobacteria [69, 74, 77–79, 81–84] while common anoxygenic phototrophic organisms include members of Chromatiales, Rhodobacterales, and Rhodospirilalles as well as sulfate-reducing bacteria like Syntrophobacterales [10, 38–47]. Such organisms typically utilize the near-infrared wavelengths of visible light that pass through the oxygenated photic zone. In mat and crust structures with well-developed sulfur-cycling consortia, a black layer of iron sulfide precipitate due to sulfate reduction can be found at the very bottom [79, 85].

Given the lack of other laminated communities being described from anchialine habitats outside the Hawaiian Archipelago, the orange laminated cyanobacterial-bacterial crust communities found in the Hawaiian anchialine ecosystem share more in common with those from other ecosystems. Previous microscopy studies identified constituents such as Cyanobacteria, diatoms, and algae as composing the bulk of the crust community [16, 17], and specifically *Chrooccoccus* sp., Ulotrichales, and *Microcystis* sp. were identified as composing the bottom green layer of the crust [16]. Due to the laminations observed in these anchialine crust microbial communities, it was hypothesized that the consortia of the

four distinct layers would demonstrate similar stratifications in taxa and functional groups as found in other laminated microbial mats.

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Chapter 2. Environmental factors driving spatial variation and diversity among *Bacteria* and micro-*Eukarya* communities of the Hawaiian anchialine ecosystem

2.1 Abstract

The Hawaiian Archipelago is home to numerous anchialine habitats, defined as nearshore bodies of water with subsurface freshwater and seawater connections, and a number in the regions of Cape Kinau (Maui) and Kona (Hawaii), respectively, possess unique, laminated orange cyanobacterial-bacterial crusts that have independently assembled on these two islands in comparatively young basalt fields. Currently, little is known about the diversity and composition of microbial communities from anchialine habitats, including these orange crusts, or the potential environmental factors driving their community structure. Here, benthic and water column *Bacteria* and micro-*Eukarya* communities from nine anchialine habitats on Oahu, Maui, and Hawaii were surveyed using high-throughput amplicon sequencing of the V6 (prokaryotic-specific) and V9 (eukaryotic-biased) hypervariable regions of the 16S- and 18S-rDNA genes, respectively. While benthic communities from habitats with cyanobacterial-bacterial crusts were more similar to each other than to habitats lacking it on the same island, each habitat had distinct benthic and water column microbial communities. Significant environmental drivers for these patterns included annual rainfall, longitude, site, aquifer, watershed, ammonium, dissolved organic carbon, and salinity. Future conservation efforts to preserve Hawaiian anchialine ecosystems should take into account their habitat-specific uniqueness in *Bacteria* and micro-*Eukarya* diversity and community structure.

2.2 Introduction

Examining microbial communities from unusual habitats and ecosystems has proved invaluable in growing biological understanding of the world as well as in developing new technologies. For example, research on laminated microbial mats found in hotsprings of Yellowstone National Park, Montana, USA, led to the discovery of *Taq* polymerase [1, 2], which revolutionized genetic lab techniques while also providing insight into how life evolved on Earth [3, 4] and potentially other planets [5]. Furthermore, microbial mat communities with bioremediation potential have been identified from "extreme" environments created by aquaculture effluent [6, 7] and mine drainages [8, 9]. While increased knowledge of microbial ecology, evolution, and potential application can result from examining communities from unusual environments, it is surprising that some habitats and ecosystems remain understudied to the point that even basic information regarding the taxonomic diversity contained within them remains largely lacking. Microbial communities from anchialine ecosystems are one such example of this situation.

Having a specific definition, the anchialine ecosystem is characterized by near-shore bodies of water with both fluctuating volumes and salinities but lacking surface connections to the ocean [10–13]. Habitats fitting this definition are found worldwide across the tropics [10, 11, 13–18] and occur within a variety of basin substrates, including karst caves, blue holes, cenotes, natural wells and springs, fossilized coral reefs, and basalt (*i.e.* lava) fields of varying ages [10, 11, 13]. Due to their simultaneous connections to the ocean and groundwater aquifer, anchialine habitats can exhibit potentially complex clines in nutrient concentration and temperature in addition to widely varying salinities over the tidal cycle [12, 14, 16]. Although high levels of species richness and endemism have been well-documented among macroorganisms from anchialine ecosystems [10, 19–25], much less is known regarding the microbial communities that occur within them [26–30], thus hampering efforts towards understanding the potential roles microbes play in the functioning of these ecosystems.

In the few studies done to date, anchialine microbial communities were found to play a major role in nutrient cycling and primary production [26, 27], including being the basis of the food web [31].

Furthermore, microbial community compositions and distributions in anchialine habitats can be strongly affected by environmental factors such as water chemistry, resulting in variable community structures along clines. For instance, vertically-structured microbial communities correlating with environmental clines were reported from the Bundera Sinkhole [29, 30] and between adjacent (~ 20 km) Bahamian blue holes [28]. In the Hawaiian Archipelago (Fig. 1a), home to the world's highest concentration of anchialine habitats, environmental factors such as being an open pool vs. cave (Fig. 1b, c), basin substrate, temperature, and salinity vary greatly [32–34] and likely drive variation among constituent microbial communities. Of these, a number of anchialine habitats in the regions of Cape Kinau (Maui) and Kona (Hawaii) harbor distinctive, laminated orange cyanobacterial-bacterial crusts (Fig. 1b, d) that are found nowhere else in the world [35, 36]. While appearing phenotypically identical with similar lamination, these crust communities are notable in that they assembled 1) independently of each other since Maui and Hawaii have never shared a physical connection, and 2) relatively quickly and repetitively on each island due to occurring in comparatively young (*i.e.*, < 200 yrs old) lava fields rendered sterile by high temperatures during their formation. Although previous surveys of these crusts via light and scanning electron microscopy revealed numerous members from the Cyanobacteria, Bacillariophyta, and Ciliophora, they were limited to the identification of morphologically-distinct taxa and only briefly mentioned the presence of "various cocci and bacilli" prokaryotic cells [35, 36] with no assessment of their taxonomic affinities.

Here, the diversity, composition, and structure of benthic and water column microbial (*i.e.*, *Bacteria* and micro-*Eukarya*) communities from anchialine habitats on Oahu, Maui, and Hawaii (the three Hawaiian Islands where these habitats naturally occur) are reported. Given the breadth of factors occurring either singularly or in combination, such as being an open pond vs. cave, having differing basin substrates, and varying nutrient levels or salinity, it was hypothesized that each habitat across the Hawaiian anchialine ecosystem possesses a unique microbial community that reflects its particular geographic location and environmental factors. Further, it was hypothesized that if similarities in microbial community structure are identified between habitats, such instances will correlate to similarities

in their environmental factors. This study is the first to examine the diversity, composition, and structure of both *Bacteria* and micro-*Eukarya* communities across such a range of habitats belonging to the anchialine ecosystem.

2.3 Materials and methods

2.3.1 Sites and Sampling

Benthic and water column samples were collected during the summer of 2010 from nine anchialine habitats on the islands of Oahu, Maui, and Hawaii. These sites included both open ponds and caves and were each also characterized by categorical and continuous environmental factors including basin type (*e.g.*, categorical) and water chemistry (*e.g.*, continuous) (Fig. 1 and Table 1, see below). Additional environmental factors were drawn from the Hawaii Statewide GIS Program, such as aquifer designation, annual rainfall, and mean annual solar radiation data [37, 38].

Samples were collected over 12 days to minimize the potential for temporal bias. About 100 g of the orange cyanobacterial-bacterial crust or benthic material (when crusts were absent) were collected from three sampling locations at each site with disposable sterile spoons and preserved in RNALater (Thermo Fisher Scientific, MA, USA), 95% ethanol, and 3.7% formalin or flash frozen in 10% dimethyl sulfoxide (DMSO) and 10% glycerol with liquid N_2. Ethanol-, DMSO-, and glycerol-preserved samples were archived as part of the Hawaiian Anchialine Microbial Repository in conjunction with The Ocean Genome Legacy Center (http://www.northeastern.edu/ogl/) under accession numbers S23033-S23083. Water column microbial communities were sampled at two sampling locations at each site by filtering ~1 L of water collected ~5 cm below the surface through sterile 0.2 μm Sterivex (Millipore, MA, USA) filter units and preserved by flooding with cell lysis buffer (Qiagen, CA, USA). Additionally, ~0.25 L of filtered water from one sampling location at each site was collected for water chemistry analyses at the University of Hawaii at Hilo Analytical Laboratory to quantify dissolved organic carbon (DOC), ammonium (NH_4^+), nitrite (NO_2^-) + nitrate (NO_3^-), total dissolved nitrogen (TDN), orthophosphate (PO_4^{3-}), total dissolved phosphorus (TDP), silica (Si), and salinity.

2.3.2 Sequence Data Generation

Extraction of DNA from RNALater-preserved benthic materials or whole crust samples utilized MoBio PowerSoil DNA Isolation Kits (MOBIO, CA, USA) according to the manufacturer's instructions with the exception of utilizing bead-beating rather than vortexing. DNA from water column filters was isolated with Gentra Puregene Yeast/Bacteria Kits (Qiagen, CA, USA) according to Amaral-Zettler et al. [39] with the modification that DNA was pelleted by centrifugation at 13-16,000 g for 5 minutes, washed in 750 µL 70% ethanol, and centrifuged again at 13-16,000 g for 3 minutes. The supernatant was then discarded and the DNA pellet allowed to dry for 5-15 minutes before being resuspended in 50 µL of Gentra DNA rehydration buffer (Qiagen, CA, USA) warmed to 65° C. To examine whether taxa were heterogeneously distributed within a site, DNA was extracted in most cases from samples belonging to two of the sampling locations within a site as well as with two separate extractions of benthic whole crust samples from the QB and MAKA3 sites.

Extracted DNA was shipped to the HudsonAlpha Institute for Biotechnology, Inc. Genomic Services Laboratory (Huntsville, AL) for amplification via the polymerase chain reaction (PCR) in duplicate (Fig. 2), with each reaction using 20 ng of DNA template, except in cases of low DNA concentration where the template volume was divided equally between the two PCRs. Two ribosomal DNA (rDNA) regions (Fig. 2) were PCR amplified per sample: the V6 hypervariable region of the 16S-rDNA using the *Bacteria*-specific primers 967-985F and 1078-1061R from Gloor et al. [40] and the V9 hypervariable region of the 18S-rDNA using the *Eukarya*-biased primers 1389F and 1510R from Amaral-Zettler et al. [39]. Additionally, two sequencing runs were performed of the dual barcoded amplicons (Fig. 2) to obtain 100-bp paired-end (PE) reads on an Illumina HiSeq 2500, with each run done on independent flow cells to minimize the potential for sample handling errors.

2.3.3 Operational Taxonomic Unit (OTU) Clustering

Sequence reads were processed in PandaSeq v.2.5 [41] to align the paired-ends, trim off primer sequences, and filter out those with uncalled bases. The FASTQ Quality Filter, part of the FASTX-Toolkit v.13.2 [42], was then used to filter reads using a conservative quality score cut-off of 30 over at least 75% of the sequenced nucleotides. Potentially chimeric sequences were filtered using USEARCH61 [43] as distributed in the QIIME v.1.8 pipeline [44]. Within QIIME, sequences were clustered into operational taxonomic units (OTUs) at a conservative 95% sequence similarity and 0.005% abundance through the `pick_open_reference_otus.py` workflow using UCLUST [43] and the 99% clustered GreenGenes 13.8 [45] and the 99% clustered Silva 111 [46] databases as initial cluster references for the V6 and V9 hypervariable regions, respectively. Notably, the 0.005% OTU abundance filter was adopted as recommended by Bokulich et al. [47] for improvement of clustering results. For each OTU cluster, the most abundant sequence was selected to act as the reference for that cluster. Taxonomic identities were assigned to cluster references using megaBLAST v.2.2.26 [48] to the appropriate curated database mentioned above at a sequence identity of ≥90% and e-value of $1x10^{-6}$. OTUs were aligned against the appropriate curated database with PYNAST v.1.2.2 [49] under default parameters (*i.e.*, minimum length of 75% median input length, minimum identity 75%) and any OTUs failing to align were filtered from the final OTU tables (see below).

2.3.4 Analyses of Community Composition and Environmental Factors

Alpha diversity, measured as the number of observed OTUs, as well as Shannon [50] and Inverse Simpson [51] diversity indicies, was calculated on the final OTU abundance tables and plotted using PhyloSeq v.1.10.0 in the R v.3.1.3 statistical environment [52, 53]. Specifically, Shannon diversity quantifies the uncertainty in predicting what OTU/taxon the next sampled sequence would belong to, such that higher Shannon values reflect greater diversity [50]. On the other hand, Simpson's index quantifies the probability that two random sequences belong to the same OTU/taxa, with a lower index reflecting greater diversity. Due to the inverted relationship between Simpson's index and diversity, its inverse was used since it reports the richness of a perfectly even community that would have the same diversity as the

observed sample. Rarefaction curves were generated for the three diversity metrics in R using ten replicates of 1, 10, 100, 1000, 10,000, 20,000, and 30,000 randomly selected sequences per sample in order to gauge the effectiveness of the sampling depth at capturing the diversity and composition of the Hawaiian anchialine *Bacteria* and micro-*Eukarya* communities under examination.

Microbial community compositions were visualized as stacked bar plots of the proportion of each taxonomic group present at a sample site using the `summarize_taxa_through_plots.py` script from the QIIME v.1.8 pipeline [44]. Additionally, core OTUs (*i.e.*, those present in all samples) for benthic and water column communities from both orange cyanobacterial-bacterial crust and cave sites were identified using the script `compute_core_microbiome.py` [44] . To compare community composition and examine how categorical and continuous environmental factors contributed to microbial diversity and structure between sites, a non-metric multidimensional scaling (NMDS) ordination with 95% confidence ellipses was created using both a Jaccard dissimilarity coefficient matrix and a Bray-Curtis dissimilarity matrix transformed to even sampling depth, in the R package PhyloSeq v.1.10.0 [52, 53]. Specifically, the Jaccard dissimilarity coefficient returns the proportion of unshared taxa between samples and only considers their presence or absence [54] while the Bray-Curtis dissimilarity metric is based on the abundance of OTUs shared between communities [55]. The individual explanatory power of the continuous environmental factors on sample ordinations was investigated with the envfit function (999 permutations) in the R package vegan v.2.3.1 [56]. Fitted vectors generated from continuous environmental factors with significant explanatory power (α=0.05) were then scaled by their explanatory power (r) and overlaid on the NMDS ordinations. Using the bioenv function in the R package vegan [56], the combination of categorical and continuous environmental factors best predicting the observed OTU abundances was also examined, and was calculated as the subset for which Euclidean distance resemblance matrix had a maximum Spearman correlation with the final OTU table.

2.3.5 Data Accessibility

Raw Illumina sequence reads were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under BioProject ID Number PRJNA325159 and SRA Sample Accession Numbers SRS1524866, SRS1524868, SRS1524873, SRS1524883, SRS1524885, SRS1524927, SRS1524929, SRS1524931, SRS1524943, SRS1524948, SRS1524949, SRS1524950, SRS1524956, SRS1524967, SRS1524974, SRS1524975, SRS1524978, SRS1524979, SRS1524980, SRS1524982, SRS1525031, SRS1525034, SRS1525038, SRS1525042, SRS1525049, SRS1525050, SRS1525052. Furthermore, documentation of all R code, QIIME scripts, and additional commands utilized in these analyses can be downloaded from http://www.auburn.edu/santosr/sequencedatasets.htm.

2.4 Results

2.4.1 Sites and Sampling

A total of 30 biological samples from three non-crust and six orange cyanobacterial-bacterial crust sites were successfully PCR amplified and sequenced for the V6 and V9 rDNA hypervariable regions (Table 2). Of the 30, 19 and 11 were benthic and water column samples respectively. Water chemistry analyses from these anchialine habitats revealed that they ranged from fresh to saline and varied widely in their dissolved nutrient concentrations and other environmental factors (Table 1). Notably, sites sharing aquifers and/or watersheds did not necessarily have similar nutrient or environmental profiles. For example, KBI and MAKA3 occur in the same aquifer and watershed, yet were more dissimilar than SKIP and QB that only share a watershed (Table 1).

2.4.2 Sequence Data Generation and OTU Clustering

The V6 sequencing produced a total of 19,806,349 demultiplexed Illumina PE reads with an average ($\bar{x}$) of 83,925 reads/sequencing replicate sample, hereafter referred to as a sample. For V9, a total of 13,128,796 PE reads were generated ($\bar{x}$ = 56,589 reads/sample). Following alignment, quality filtering, chimera-checking, and abundance filtering, 1,881,683 V6 and 1,833,571 V9 sequences were retained,

representing 90% and 86% reductions, respectively, in each dataset. While significant proportions of the sequence data were eliminated via filtering, such stringent parameters were used due to the short length of the Illumina reads (see below) as well as to reduce the noise-to-signal ratio. Thus, the post-processing average numbers of sequences per sample for V6 and V9 were 15,946 and 15,806, respectively. Lengths of the V6 sequences ranged from 65-80 bp ($\bar{x}$ = 74 bp) while those of V9 ranged from 85-163 bp ($\bar{x}$ = 122 bp). No V6 OTUs were removed due to failure to align in PYNAST, resulting in 1,776 OTUs in the final dataset. In contrast, 18 V9 OTUs failed to align and were excluded from the final OTU table; removal of the 5,421 sequences associated with these excluded OTUs reduced the V9 total to 1,828,150 ($\bar{x}$ = 15,759 reads/sample) and 1,319 OTUs. The majority (*i.e.*, 88.6%) of V6 OTUs were assigned taxonomy using the GreenGenes 13.8 database [45], with the exception being 157 OTUs encompassing 213,912 sequences (*e.g.*, 11.4% of the total sequences in the final OTU table). Comparisons of sequences from these unassignable OTUs to NCBI's GenBank nr database [57, 58] using BLASTN v.2.3.0 [48] revealed affiliations with uncultured samples primarily belonging to the Alphaproteobacteria, Cytophaga/Flavobacteria/Bacteroidetes group, and Deltaproteobacteria, but also Acidobacteria, Actinobacteria, Chlorobi, Chloroflexi, Chlorophyte chloroplasts, Cyanobacteria, Deinococcus-Thermi, Enterobacteria, Firmicutes, Fusobacteria, Planctomycetes, and Verrucomicrobia, at low e-values (data not shown). Additionally, 15 V6 OTUs were identified as coming from eukaryotic chloroplasts, representing 1.01% of the total V6 sequences (*e.g.*, 0%-16% per sample, $\bar{x}$ = 1.03%) classified to this organelle. Ten V9 OTUs, composed of 7,328 sequences or 0.136% of the total number, failed to be assigned taxonomy using the Silva 111 database [46]. Comparisons of sequences from these unassigned V9 OTUs against NCBI's GenBank nr database with BLASTN identified matches with Alveolata, Amoebozoa, Apusozoa, Bacillariophyta, Chlorophyceae, Ciliophora, Cryptophyceae, Dinoflagellata, Mycetozoa, Oomycota, Rhizaria, Rhodophyta, and Stramenopiles, again at low e-values (data not shown).

2.4.3 Analyses of Community Composition and Environmental Factors

Samples from the same site and environment (*e.g.*, benthos or water column) were most similar to each other regardless of which biological sample, DNA extraction, PCR, or sequencing run data were generated from (data not shown). For this reason, all replicates for any given benthic or water column sample were combined per site for most downstream analyses. Rarefraction analyses suggested that OTU richness, as measured via alpha diversity, was not saturated at sampling depths of 30,000 sequences per sample for either the *Bacteria* V6 (Fig. 3a) or *Eukarya*-biased V9 datasets (Fig. 3b). In contrast, curves of both Shannon and Inverse Simpson diversity flattened and reached apparent saturation at sampling depths equal to or greater than 10,000 sequences per sample (Fig. 3a and 3b). Rarefying without replacement at 5,000 and 4,000 sequences for V6 and V9, respectively, estimating the same three diversity metrics 1,000 times, and testing the results with the Kruskal-Wallis Rank Sum test failed to identify significant differences between samples grouped by site or whether they were from the benthos or water column (data not shown). This implies sample diversity was not structured by site or whether they were from the benthos or water column.

At the phylum level, orange cyanobacterial-bacterial crust and cave anchialine microbial communities shared a majority of taxa, but at differing levels of abundance (V6: Fig. 4a; V9: Fig. 4b). Using the `compute_core_microbiome.py` script in QIIME, all crust site samples from both the benthos and water column had core V6 and V9 OTUs belonging to Actinobacteria, Alphaproteobacteria, Bacteroidetes, Gammaproteobacteria, Verrucomicrobia, and Heterokonta. Cyanobacteria were identified as core V6 OTUs in benthic crust samples from both Maui and Hawaii, but were only core OTUs in the water column samples of crust sites from Hawaii. On the other hand, all samples from the two cave sites had core V6 and V9 OTUs belonging to Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Fusobacteria, Gammaproteobacteria, Verrucomicrobia, Ciliophora, and Dinoflagellata. Fungal groups were identified as members of the core water column communities from orange cyanobacterial-bacterial crust sites on Hawaii, but not Maui, as well as from all cave site samples. Thaumarchaeota, the only recovered Archaeal phylum, was exclusive to the benthic community at one cave site (*i.e.*, PU) and found nowhere else. Furthermore, the two cave sites, occurring

on different islands, shared relatively high numbers of benthic *Bacteria* V6 OTUs (*i.e.*, 136) (Table 3). Conversely, the benthic and water column microbial communities of orange cyanobacterial-bacterial crust sites from Maui and Hawaii possessed relatively few mutual OTUs. For example, only 10 and 14 V6 and V9 OTUs, respectively, were shared across islands while benthic crust samples from either Maui or Hawaii had ~5-7X more V6 and V9 OTUs in common among sites (Table 3). A similar pattern of low OTU sharing was also identified among water column microbial communities from orange cyanobacterial-bacterial crust sites on the two islands (Table 3). The specific V6 and V9 OTUs identified as core community constituents from benthic and water column samples of the Hawaiian anchialine ecosystem are provided in Appendix1.

In the NMDS plots, samples grouped by both site and whether they were from the benthos or water column, forming tight and distinct clusters with limited overlap between them in nearly all cases (Fig. 5a and 5b). Differences between the Jaccard or Bray-Curtis NMDS ordinations and fitted envfit vectors were minimal (data not shown), thus only the Bray-Curtis ordinations for the V6 (Fig. 5a) and V9 (Fig. 5b) OTUs are presented. While the benthic orange cyanobacterial-bacterial crust communities on Maui and Hawaii were more similar to each other than to their water column communities, they clustered with minimal overlap and correlate with the unique environmental factors and water chemistry at each site (Table 1).

Consideration of individual environmental factors with the envfit analysis revealed all categorical factors as being significant at $P < 0.001$, with environment (*i.e.*, benthic or water column) accounting for the least variation (*i.e.*, V6 r^2=0.146, V9 r^2=0.189) and site for the most variation (*i.e.*, V6 r^2=0.857, V9 r^2=0.858). Furthermore, the bioenv function tested 33,554,431 possible combinations between 1) site, sample ID, and whether a sample originated from the benthos or water column; 2) the ten categorical factors (Table 1), and; 3) the twelve continuous environmental factors (Table 1) for both datasets. These analyses identified presence or absence of the cyanobacterial-bacterial crust community, whether the sample originated from the benthos or water column, and salinity as the best model (r=0.755) for the *Bacteria* V6 OTUs. On the other hand, presence or absence of the cyanobacterial-bacterial crust

community, whether the sample originated from the benthos or water column, whether the site was an open pond or cave, and longitude was identified as the best model (r=0.854) for the *Eukarya*-biased V9 OTUs. While all of the continuous environmental factors were significant predictors of the *Bacteria* V6 sample ordination, only nine of the twelve were significant for the *Eukarya*-biased V9 (Fig. 5). Specifically, nitrite (NO_2^-) + nitrate (NO_3^-), silica (Si), and total dissolved nitrogen (TDN) were only predictive for the V6 data, while salinity was the single continuous environmental factor with the strongest explanatory power for both the *Bacteria* and micro-*Eukarya* communities from this sampling of Hawaiian anchialine habitats (Appendix 2).

2.5 Discussion

2.5.1 Microbial Diversity of the Hawaiian Anchialine Ecosystem

This study represents both the first detailed genetic survey of *Bacteria* and micro-*Eukarya* diversity, as well as the first attempt to identify potential environmental factors driving spatial variation in microbial community composition and structure, across Hawaii's anchialine ecosystem. While every member of the microbial community was not sampled, flattened rarefaction curves of Shannon and Inverse Simpson diversities (Fig. 3a and 3b) suggest recovery of the major taxonomic players in the surveyed communities. In support of this, comparison of taxa from this study with prior light [35] and scanning electron [36] microscopy surveys of orange cyanobacterial-bacterial crust materials from Maui and Hawaii identified appreciable overlap with specific taxa in the cyanobacteria, diatoms, dinoflagellates and green algae (Appendix 1), including members of the historic cyanobacterial subsections I-IV as well as 8 of 32 diatom, 2 of 3 dinoflagellate and 2 of 12 green algae genera [34]. Potential reasons this study did not recover all of the previously identified taxa include the fact that the exact same sites were not sampled or that regional environmental conditions on each island may have shifted in the 17+ years since these previous surveys, with subsequent changes in community composition of these orange cyanobacterial-bacterial crusts. Additionally, revisions in species names and taxonomic ranks since 1975

and 1993, the years in which these other surveys were published [34, 35], may also contribute to the failure to recover the exact same taxa that were previously identified.

Of the few studies examining the microbial diversity of Hawaii's soils or freshwaters, phyla identified as occurring in young volcanic soils and forests on the island of Hawaii included Acidobacteria, Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Chloroflexi, Clostridia, Cyanobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Fibrobacteres, Firmicutes, and Planctomycetes [59–62], which were also recovered in this survey of the Hawaiian anchialine ecosystem. Furthermore, most taxa identified in a survey of five Hawaiian lakes [63] were also found in this study, with the exception of Euryarchaeota and the candidate divisions. While the distinctive orange cyanobacterial-bacterial crust communities found in the regions of Cape Kinau (Maui) and Kona (Hawaii) are unique to the Hawaiian anchialine ecosystem, there was also overlap with taxa from other anchialine habitats. For example, an anchialine pool on Pearl and Hermes Atoll in the Northwest Hawaiian Islands possessed members of the Cyanobacteria, Firmicutes, and Gammaproteobacteria [63], with a subsequently cultured Gammaproteobacterium appearing to belong to a novel genus [64]. Along with this, a survey of the microbial communities of two anchialine caves on Mljet Island in the Adriatic Sea identified Deltaproteobacteria, Epsilonproteobacteria, and Gammaproteobacteria as the major resident bacterial lineages, with abundances of the latter and former being concentrated at the surface and below 30m, respectively, and Epsilonproteobacteria distributed ubiquitously [65]. While Epsilonproteobacteria and Deltaproteobacteria might be considered relatively minor contributors to the Hawaiian anchialine microbial communities examined here, Gammaproteobacteria accounted for 14.79% of the total V6 sequences generated in this study. Specifically, most of the Gammaproteobacteria sequences belonged to members of the Alteromonadales, Oceanospirillales, Thiotrichales, and Vibrionales. Likewise, all of the bacterial taxa previously identified in two anchialine Bahamian blue holes were also recovered from the Hawaiian anchialine ecosystem, including Chlorobi, Deltaproteobacteria, Gammaproteobacteria, Planctomycetes, Verrucomicrobia, Firmicutes, Lentisphaerae, Bacteroidetes, Spirochaetes, and Nitrospirae [28].

While the two cave sites on different islands shared relatively high numbers of benthic *Bacteria* OTUs, identification of core *Bacteria* and micro-*Eukarya* OTUs among the orange cyanobacterial-bacterial crust communities of Maui and Hawaii revealed limited overlap between islands, with both the benthos and water columns on the same island having higher numbers of shared core OTUs and more similar communities than with the other island (Table 3). Additionally, higher numbers of *Bacteria* and micro-*Eukarya* OTUs were identified as core community members on Hawaii than Maui, despite orange cyanobacterial-bacterial crust sites on Hawaii having greater geographic separation, and the potential for isolation by distance, than those on Maui. One possibility for the greater core community diversity on Hawaii may be due in part to the lower salinity of these habitats compared to orange cyanobacterial-bacterial crust habitats on Maui (Table 1), which allows for the persistence of a wider range of taxa. Such a situation, where diversity and structure in aquatic microbial communities negatively correlated with increasing salinities (also see below), has been reported from numerous high-altitude Tibetan lakes [66, 67], which share a number of abiotic factors (*e.g.*, strong UV radiation, oligotrophy, low terrestrial input of organic carbon resources) with many Hawaiian anchialine habitats possessing these distinctive orange cyanobacterial-bacterial crust communities (Table 1).

2.5.2 Environmental Drivers of Microbial Diversity in the Hawaiian Anchialine Ecosystem

While this study did not explicitly test whether anchialine habitats in Hawaii possess the vertical stratification observed in water column microbial community of other anchialine habitats due to various physical and chemical clines [16, 29, 30], spatial variation, even in geographically adjacent habitats, was identified. Unique microbial communities despite geographic proximity have also been reported from anchialine blue holes in the Bahamas [28] as well as anchialine pools in a single cave in Mallorca, Spain, where distinct communities occurred within 100 m of each other [18]. Given that close proximity does not necessitate similarity in environmental factors, as seen in the Bahamian blue hole study [28], the Spanish cave study [18], and sites in this study that shared aquifers and watersheds, reiterates the abiotic complexity of anchialine habitats and this ecosystem in general [12–14, 16, 68].

Of the environmental factors examined here, salinity had some of the highest explanatory power for the observed community variation, suggesting it may be the dominant water chemistry parameter as similarly determined for high-altitude Tibetan lakes [66, 67]. Concerning the *Eukarya*-biased V9, longitude was also identified as influential and corresponds with island and the presence of the unique orange cyanobacterial-bacterial crust, since such communities occur across short longitudinal gradients on Maui and Hawaii (but a relatively long latitudinal gradient on Hawaii, Fig. 1). Examination of other individual environmental variables also identified the categorical factors of site, aquifer, and watershed as well as the continuous factors of ammonium and DOC as also having high explanatory power. Given this, the presence of unique benthic and water column communities at each site results in the significance of site, aquifer, and watershed, with salinity, ammonium, and DOC being major drivers of *Bacteria* and micro-*Eukarya* community diversity and structure in the Hawaiian anchialine ecosystem at the level of individual habitats. For example, and as mentioned earlier, salinity of the anchialine habitats examined here correlated with island, such that sites on Hawaii had the lowest and those on Maui had the highest salinity (Table 1), which likely drives differences in overall community diversity. These findings correspond with those of Wong [35], who identified salinity as important in dictating the dominant algal or cyanobacterial taxa in anchialine pools at Cape Kinau, Maui. In riverine bacterioplankton communities, increased salinity results in changes to both community composition and metabolism, leading to less consumption of DOC [69]. Coincidentally, the anchialine habitats examined here with greater salinity also had higher levels of DOC (Table 1), suggesting an analogous situation where *Bacteria* and micro-*Eukarya* communities at higher salinity sites are consuming less DOC. In support of this, benthic epilithon communities from crust-containing anchialine habitats on Hawaii responded differentially, with subsequent shifts in community composition to nitrogen (N) and phosphorus (P) enrichment depending on salinity [70], implying the influences of salinity on member composition ultimately impact nutrient utilization by, and function of, these microbial communities.

2.5.3 Considerations for Conservation Efforts of Hawaiian Anchialine Habitats

Given that the abiotic and biotic uniqueness of individual Hawaiian anchialine habitats is only now being appreciated [71], it is unfortunate that they have been vulnerable both historically and currently to destruction and degradation, primarily due to coastal development and invasive species introductions [25, 36, 68]. For example, a single development project in 1985 destroyed over 130 habitats [72]. Furthermore, the introduction of poeciliids (*Poecilia* spp. and *Gambusia affinis*) and tilapia (*Oreochromis* spp.) induces diel migratory behavior in the endemic atyid shrimp and keystone grazer *Halocaridina rubra* [31, 33, 73–75] that leads to alterations in microbial community biomass and productivity as well as increased nutrient load of impacted habitats [2, 66]. Unfortunately, the threat of invasive fishes to habitats and biota of the Hawaiian anchialine ecosystem is expected to intensify, because predicted sea level rise due to global climate change will allow them to access uninvaded habitats [76]. While a number of shrimp species found in Hawaii's anchialine ecosystem are listed as State of Hawaii and Federal candidates for protection (http://dlnr.hawaii.gov/wildlife/files/2013/09/Fact-Sheet-anchialine-shrimps.pdf), there are currently no conservation efforts seeking to protect anchialine habitats in the islands with the goal of preserving their *Bacteria* and micro-*Eukarya* diversity. Indeed, the need for specific attention towards preserving global microbial diversity has been highlighted by the scientific community [77–79]. Given this, future conservation efforts should take into account that the phenotypic similarity between the unique orange cyanobacterial-bacterial crust communities found on Maui and Hawaii masks their distinctiveness and that unique microbial diversity and communities apparently occupy each habitat across the islands.

While the technological advancement of high-throughput amplicon sequencing allowed us to elucidate the diverse *Bacteria* and micro-*Eukarya* communities present in a range of habitats belonging to the Hawaiian anchialine ecosystem, much remains to be investigated. For example, finer taxonomic assignment is difficult given the relatively short length of Illumina sequence reads and the conservative clustering parameters removed both OTUs failing to align with the reference databases and rare OTUs, which potentially represent novel taxa or the controversial 'rare biosphere' [80–82]. Additionally, the influence of temporal dynamics and changes on microbial community diversity and/or composition

spanning from habitat creation to senescence are unknown. In any case, the data and analyses presented here illustrate how geographic location and associated environmental factors significantly drive *Bacteria* and micro-*Eukarya* diversity, composition, and structure among benthic and water column microbial communities of the Hawaiian anchialine ecosystem.

2.5.4 Conclusions

In this study, high-throughput amplicon sequencing identified significant differences in *Bacteria* and micro-*Eukarya* diversity, composition, and structure across anchialine habitats on the Hawaiian Islands of Oahu, Maui and Hawaii. Each habitat proved to have a unique microbial community, and multiple categorial and continuous environmental factors, including site, watershed, salinity, and DOC, appear to be significant drivers for these patterns. While the distinctive orange cyanobacterial-bacterial crust communities from Maui and Hawaii were more similar to each other in community composition than to non-crust communities, they were distinguishable not only by island, but by site as well. Future efforts aiming to preserve the Hawaiian anchialine ecosystem should take into account the unique *Bacteria* and micro-*Eukarya* diversity within them as conservation plans are considered and developed.

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2.7 References

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Table 1. Sampled anchialine habitats from the islands of Hawaii, Maui and Oahu and their corresponding categorical and continuous environmental factors. Site abbreviations: OWAI – Waianae, Oahu; HM – Hanamanioa, Maui; QB – Queen's Bath, Maui; SKIP – Skippy's Pond, Maui; WC – Waianapanapa Cave, Maui; KBI – Keawaiki Bay, Hawaii; MAKA3 – Makalawena Beach, Hawaii; PB – Pohue Bay, Hawaii; PU – Puhi'Ula Cave, Hawaii.

Categorical Environmental Factors	**Sites**								
	OWAI	**HM**	**QB**	**SKIP**	**WC**	**KBI**	**MAKA3**	**PB**	**PU**
Island	Oahu	Maui	Maui	Maui	Maui	Hawaii	Hawaii	Hawaii	Hawaii
Habitat Type	Pond	Pond	Pond	Pond	Cave	Pond	Pond	Pond	Cave
Benthic Substrate	Calcium Carbonate	Basalt	Basalt	Basalt	Basalt	Basalt	Basalt	Basalt	Basalt
Orange Cyanobacterial-Bacterial Crust	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No
Fish	No Fish	No Fish	No Fish	No Fish	Poeciliids	Tilapia	Poeciliids & Marine	No Fish	No Fish
Goats	No	Yes	Yes	Yes	No	No	Yes	No	No
Open to Public	No	No	No	No	Yes	Yes	Yes	No	No
Aquifer [37]	Waianae	Kahikinui	Central	Kahikinui	Hana	Hualalai	Hualalai	SW Mauna Loa	SE Mauna Loa
Watershed [37]	Kaupuni	Kanaio	Ahihi Kinau	Ahihi Kinau	Honomaele	Kiholo	Kiholo	Kauna	South Point
Potential Warm Groundwater [37]	No	Yes	Yes	Yes	No	No	Yes	No	No
Continuous Environmental Factors									

Latitude	21.45	20.58	20.6	20.6	20.79	19.89	19.79	19.01	19.06
Longitude	158.2	156.41	156.43	156.42	156.01	155.9	156.03	155.8	155.55
Annual Rainfall (mm) [37, 38]	547.9	364.7	363.9	366.4	1925.4	242.9	320.4	611.5	819.8
Mean Annual Solar Radiation (Watts/m^2) [37]	208.5	216.6	193.5	196.2	208.9	226.5	224.8	180.7	193.4
Salinity (ppt)	20.5	15	26.5	24	5	5	4	6	4
Nitrite and Nitrate (NO_2+NO_3, µM)	56	48.1	24.6	23.9	28.5	79.8	46.3	23.7	41.1
[1] Orthophosphate (PO_4, µM)	1.47	1.65	ND	ND	2.46	1.24	7.24	0.75	2.37
Silica (Si, µM)	778	418	383	355	315	666	897	613	667
[2] Ammonium (NH_4, µM)	ND	1.41	ND	ND	1.47	1.41	1.16	2.33	1.15
Dissolved Organic Carbon (DOC, µM)	94.7	44.1	54.2	40.1	15.4	38.3	14.5	43.9	10.6
Total Dissolved Nitrogen (TDN, µM)	57	49.1	24.9	23.6	25.3	73.4	42.9	24.9	39.7
[3] Total Dissolved Phosphorus (TDP, µM)	1.7	1.53	ND	ND	2.44	1.25	7.5	0.7	2.41

1 Not detectable (ND) <0.10 µM

2 Not detectable (ND) <1.00 µM

3 Not detectable (ND) <0.50 µM

Table 2. Number of biological samples, technical samples (produced by duplicate PCR reactions of a single biological sample), and the total number of samples sequenced from two Illumina runs analyzed in this study from sampled anchialine habitats on the islands of Hawaii, Maui and Oahu.

			OWAI	HM	QB	SKIP	WC	KBI	MAKA3	PB	PU
V6	Benthos	Biological Samples	2	2	4	2	1	2	4	1	1
		Duplicated Samples	4	4	8	4	2	4	8	2	1
		Total Samples Sequenced	8	8	16	8	4	8	16	4	2
	Column	Biological Samples	2	1	0*	2	2	2	0*	2	0*
		Duplicated Samples	4	2	0*	4	4	4	0*	4	0*
		Total Samples Sequenced	8	4	0*	8	8	8	0*	8	0*
V9	Benthos	Biological Samples	2	2	4	2	0*	2	4	2	1
		Duplicated Samples	4	3	8	4	0*	4	8	4	1
		Total Samples Sequenced	8	6	16	8	0*	8	16	8	2
	Column	Biological Samples	2	1	0*	2	2	2	0*	2	0*
		Duplicated Samples	4	2	0*	4	4	4	0*	4	0*
		Total Samples Sequenced	8	4	0*	8	8	8	0*	8	0*

**Samples were collected and sent for sequencing but failed to amplify.*

Table 3. Number of *Bacteria* (V6) and micro-*Eukarya* (V9) OTUs shared by all samples in specified sample groups (*n*=number of samples sequenced) from sampled anchialine habitats on the islands of Hawaii and Maui.

Sample Groups	**# V6 Core OTUs**	**# V9 Core OTUs**
All Cave Benthos	136 (*n*=6)	N.D.*
All Cave Water Column	65 (*n*=8)	11 (*n*=8)
Maui Crust Benthos	56 (*n*=32)	62 (*n*=30)
Hawaii Crust Benthos	76 (*n*=28)	48 (*n*=32)
All Crust Benthos	10 (*n*=60)	14 (*n*=62)
Maui Crust Water Column	31 (*n*=12)	33 (*n*=12)
Hawaii Crust Water Column	168 (*n*=16)	86 (*n*=16)
All Crust Water Column	11 (*n*=28)	2 (*n*=28)

**Not determined due to V9 amplification failure of benthic samples from WC*

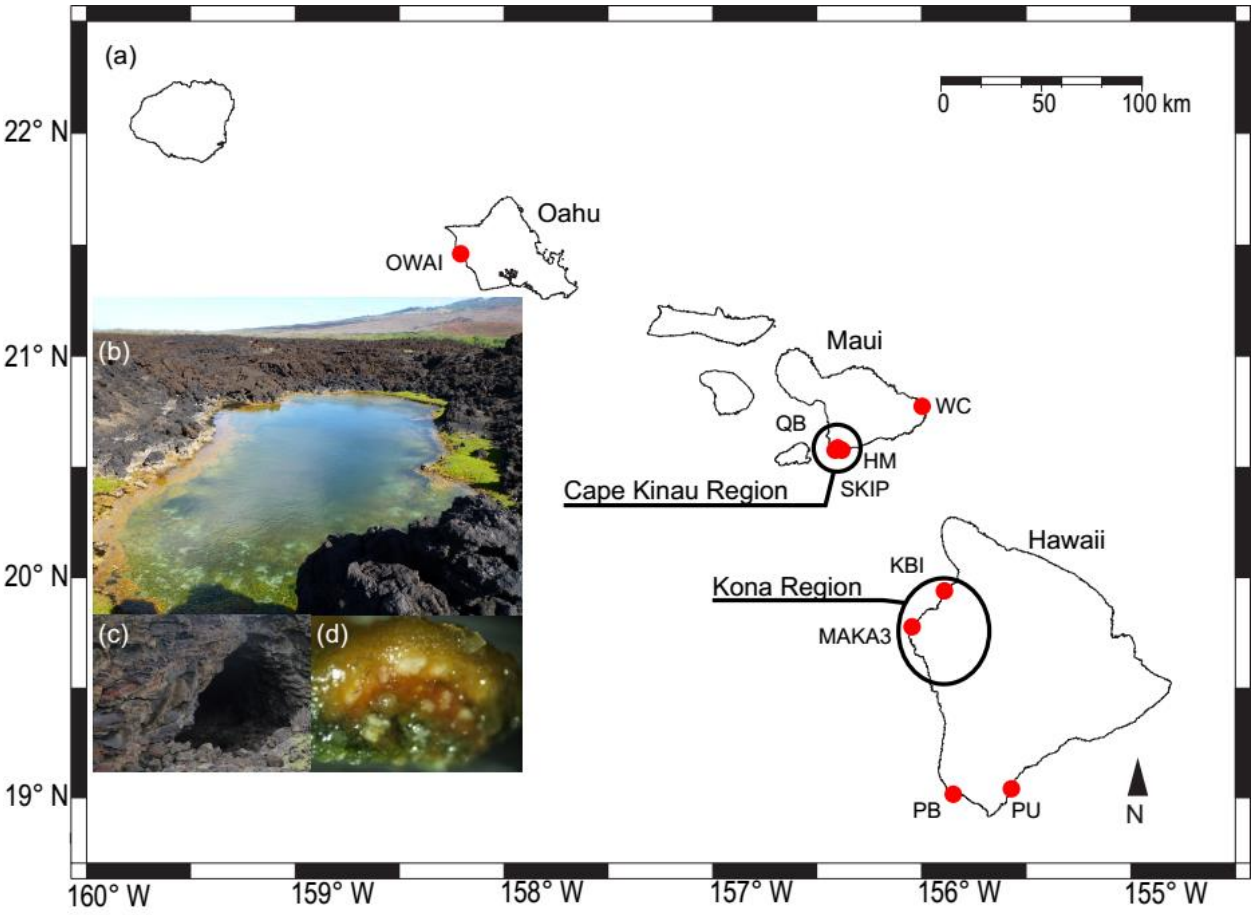


Fig. 1 a Map depicting sampling sites of anchialine habitats on the islands of Oahu, Maui, and Hawaii with the regions of Cape Kinau, Maui and Kona, Hawaii indicated (open circles) **b** Example of a Hawaiian anchialine open pool habitat (*i.e.*, site SKIP) with the orange cyanobacterial-bacterial crust found in Cape Kinau, Maui and Kona, Hawaii **c** Example of a Hawaiian anchialine cave habitat (*i.e.*, site PU) on Hawaii **d** Close-up of dissected laminated orange cyanobacterial-bacterial crust found exclusively in the Cape Kinau region of Maui and the Kona region of Hawaii

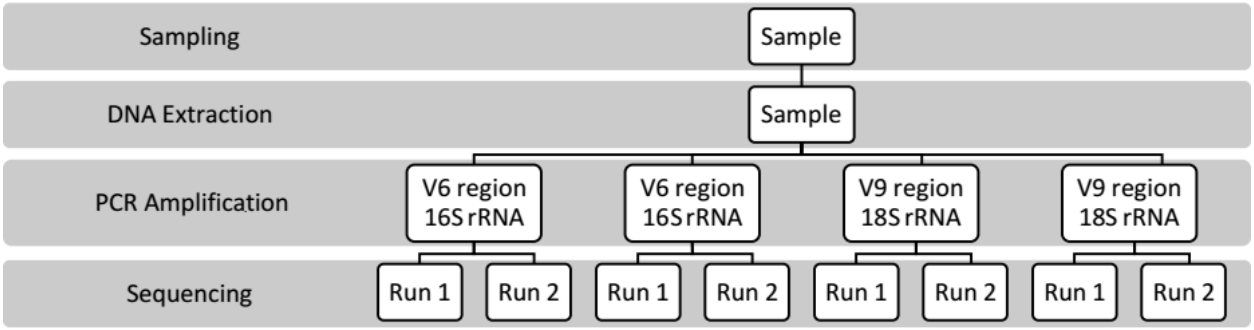


Fig. 2 Schematic of data generation showing processing of a Hawaiian anchialine microbial sample from DNA extraction through sequencing of the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene or *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene

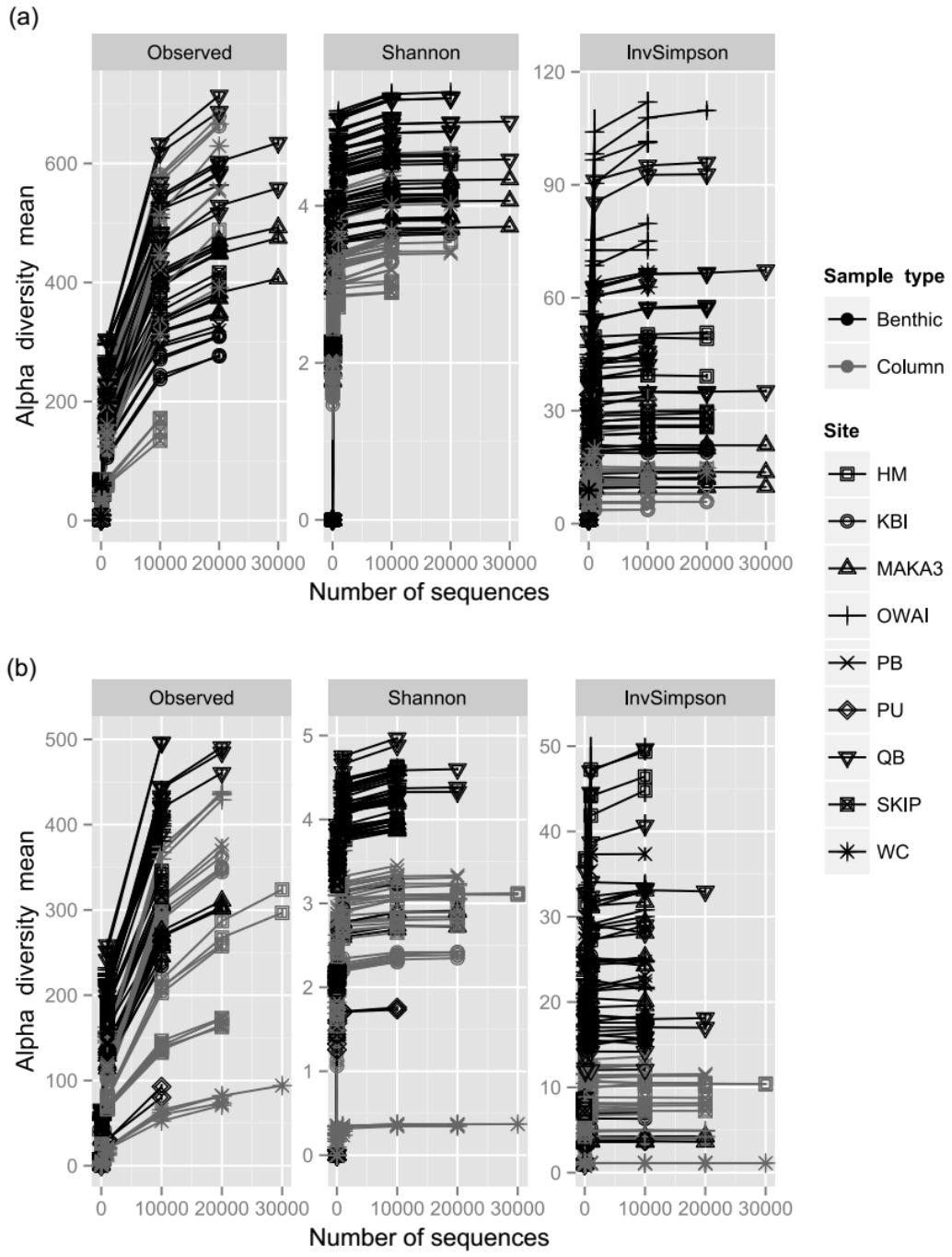
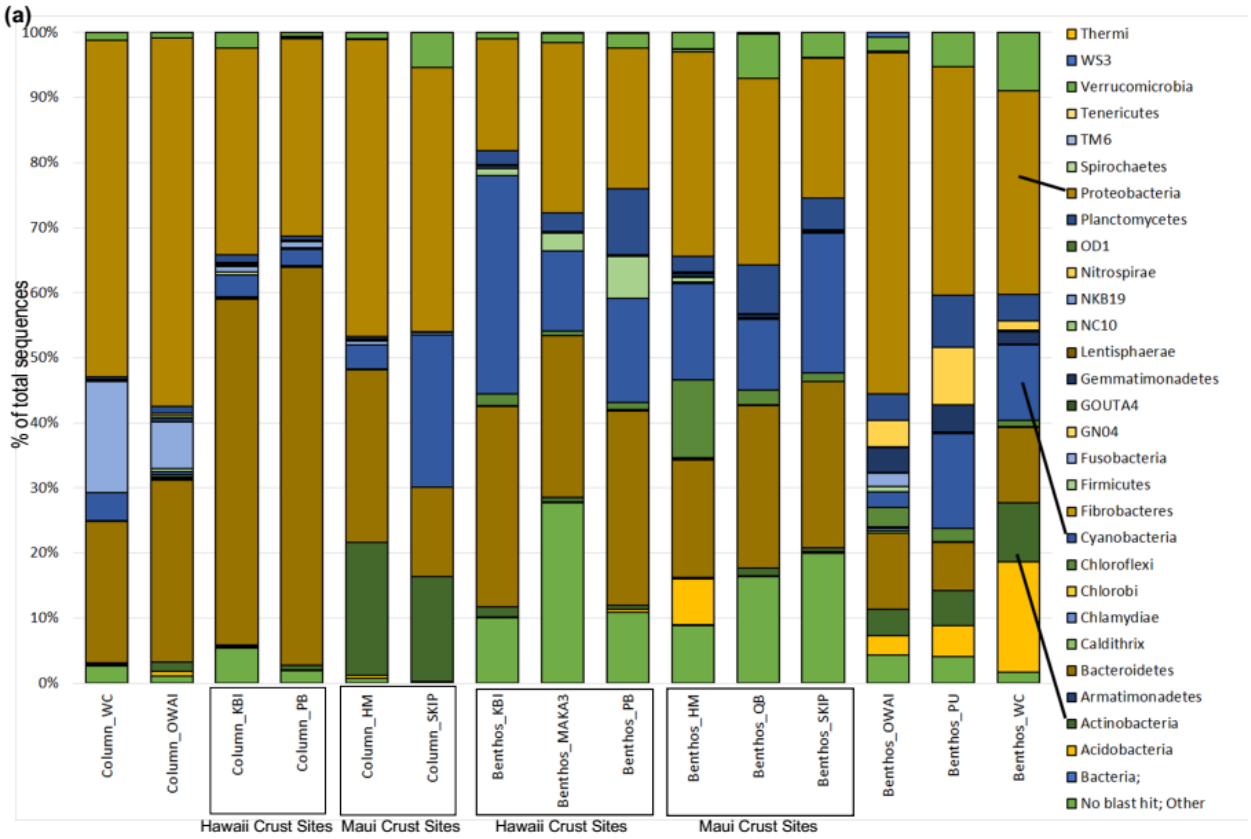


Fig. 3 Diversity estimates, specifically number of observed OTUs, Shanon diversity, and Inverse Simpson diversity, of the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene (**a)**, and the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene (**b**) Samples were grouped by benthos and water column communities within sites


(a)
% of total sequences
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Column_WC
Column_OWAI
Column_KBI
Column_PB
Column_HM
Column_SKIP
Benthos_KBI
Benthos_MAKA3
Benthos_PB
Benthos_HM
Benthos_QB
Benthos_SKIP
Benthos_OWAI
Benthos_PU
Benthos_WC
Hawaii Crust Sites
Maui Crust Sites
Hawaii Crust Sites
Maui Crust Sites
Thermi
WS3
Verrucomicrobia
Tenericutes
TM6
Spirochaetes
Proteobacteria
Planctomycetes
OD1
Nitrospirae
NKB19
NC10
Lentisphaerae
Gemmatimonadetes
GOUTA4
GN04
Fusobacteria
Firmicutes
Fibrobacteres
Cyanobacteria
Chloroflexi
Chlorobi
Chlamydiae
Caldithrix
Bacteroidetes
Armatimonadetes
Actinobacteria
Acidobacteria
Bacteria;
No blast hit; Other

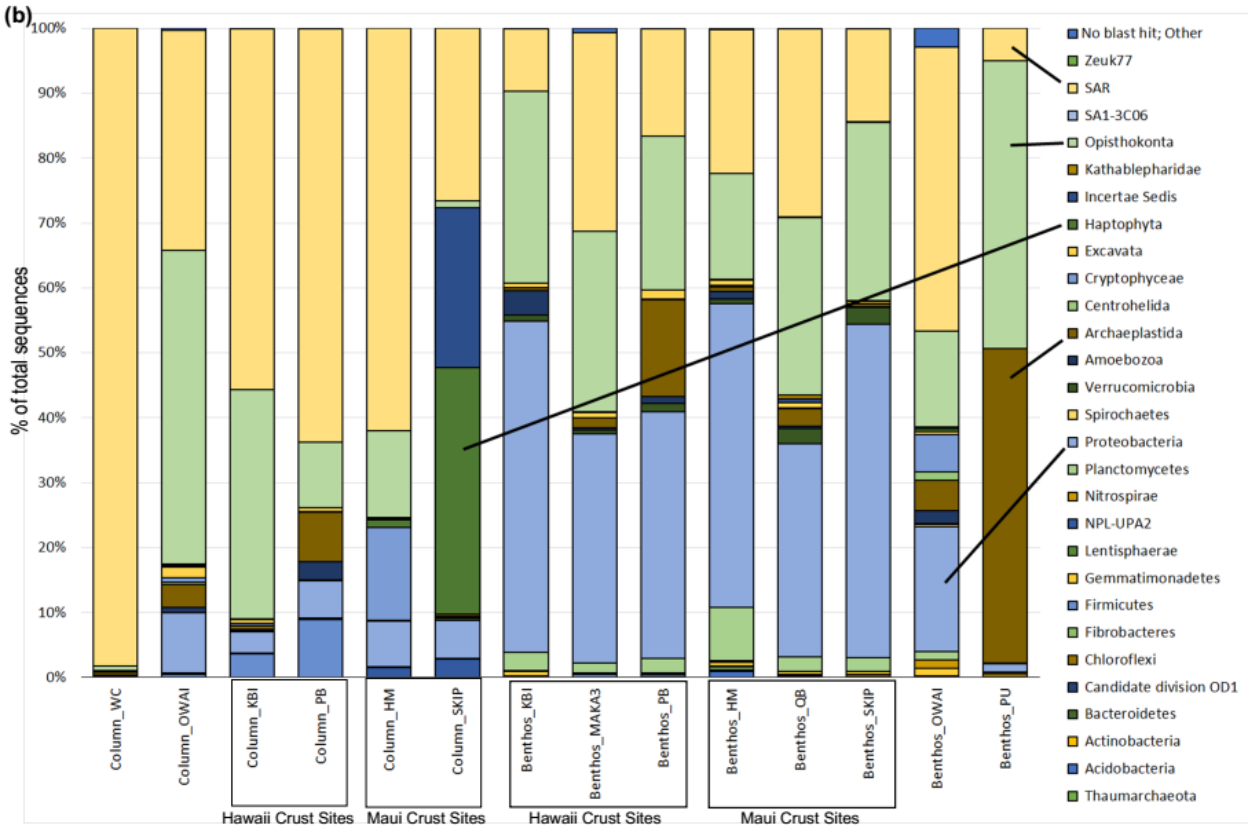

(b)
% of total sequences
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Column_WC
Column_OWAI
Column_KBI
Column_PB
Column_HM
Column_SKIP
Benthos_KBI
Benthos_MAKA3
Benthos_PB
Benthos_HM
Benthos_QB
Benthos_SKIP
Benthos_OWAI
Benthos_PU
Hawaii Crust Sites
Maui Crust Sites
Hawaii Crust Sites
Maui Crust Sites
No blast hit; Other
Zeuk77
SAR
SA1-3C06
Opisthokonta
Kathablepharidae
Incertae Sedis
Haptophyta
Excavata
Cryptophyceae
Centrohelida
Archaeplastida
Amoebozoa
Verrucomicrobia
Spirochaetes
Proteobacteria
Planctomycetes
Nitrospirae
NPL-UPA2
Lentisphaerae
Gemmatimonadetes
Firmicutes
Fibrobacteres
Chloroflexi
Candidate division OD1
Bacteroidetes
Actinobacteria
Acidobacteria
Thaumarchaeota

Fig. 4 Relative abundance of taxa identified in samples grouped by sample type and site **a** Bacterial phyla identified by the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene using the GreenGenes 13.8 database **b** Clades identified by the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene using the Silva 111 database

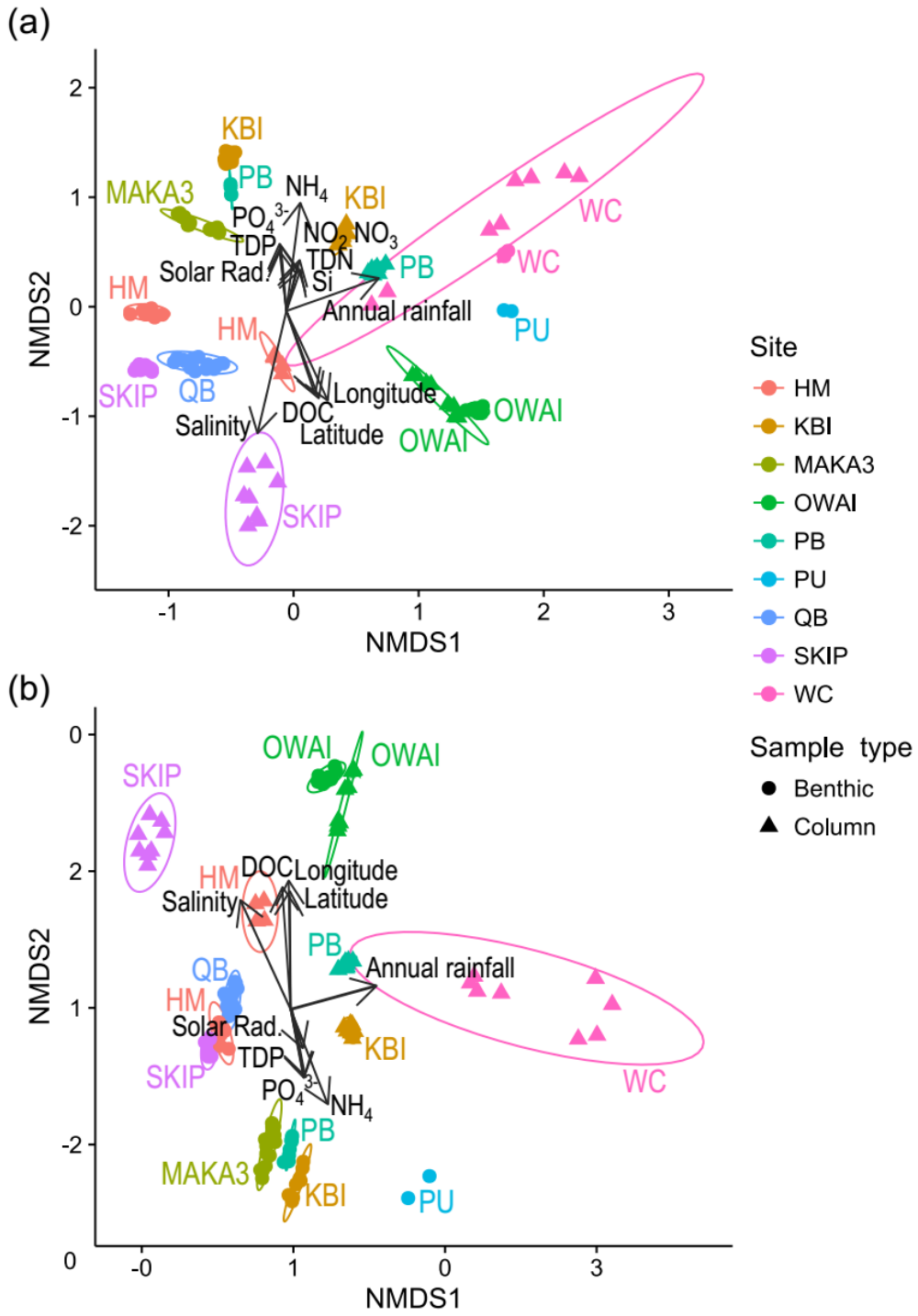


Fig. 5 Non-metric Multidimensional Scaling (NMDS) ordination using the Bray-Curtis Dissimilarity Index of samples grouped by benthic or water column microbial communities within anchialine sites that were surveyed. Environmental factors were fitted to and overlaid on the ordinations, and represent environmental gradients in ordination space **a** Samples generated using the *Bacteria*-specific V6

hypervariable region of the 16S-rDNA gene **b** Samples generated using the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene

Chapter 3. Seasonal stability in Hawaiian anchialine microbial communities across an 18-month period

3.1 Abstract

Environmental factors are known to influence the distribution and abundance of microbes, and understanding the impact of seasonal fluctuations in environmental factors provides further insight into microbial community function. The anchialine ecosystem, defined as tidally-influenced near-shore bodies of water with subsurface freshwater and seawater connections, has been relatively unstudied with regards to its microbial communities. Furthermore, anchialine habitats found in the Cape Kinau and Kona regions of Maui and Hawaii, respectively, exhibit distinctive, laminated orange cyanobacterial-bacterial crusts that are subject to seasonal fluctuations in water chemistry, but almost nothing is known about the degree to which these fluctuations might influence shifts in their community composition. To address this, benthic and water column microbial communities were surveyed from six habitats in these geographical regions during summer 2010 and spring, summer, and winter 2011 using high-throughput amplicon sequencing of the V6 (*Bacteria*-biased) and V9 (micro-*Eukarya*-biased) hypervariable regions of the SSU rRNA gene. While seasonal environmental variation was observed in habitat salinity, ammonium, dissolved organic carbon, total dissolved nitrogen, and nitrite and nitrate, spatial factors had a stronger influence on benthic and water column community composition than factors varying with season. In spite of this, approximately half of the third-level clades (i.e., approximately class level taxonomy) within the benthic and water column communities identified from this survey exhibited seasonal variation in relative abundance. Of these clades, changes in relative abundance for approximately three-quarters of them were correlated with at least one seasonally-varying factor. Overall, this study represents the first sequence-based survey of seasonal variation in Hawaiian anchialine microbial communities and explores

potential environmental and water chemistry factors which may mediate seasonal dynamics in these endangered habitats.

3.2 Introduction

While spatial factors are known to influence microbial communities in much the same way as macroorganisms [1], considerably less is known about how temporal or seasonal fluctuations impact the their compositions and distributions. Such information is important since microbes fulfill vital roles such as primary production [2] and facilitation of nutrient cycling [3], and thus any environmental factors influencing the microbial component of a community can potentially impact higher trophic levels. For example, phototrophic microbial mat communities in Yellowstone National Park, USA, were found to concentrate mercury from spring water and transfer it to grazing insect larvae and ultimately, the greater food web [4]. Seasonal environmental fluctuations have been found to compound such situations, with shifts in microbial consortia due to seasonal factors leading to altered nutrient availability for higher community trophic levels of Lake Kinneret, Israel [5].

The anchialine ecosystem, first defined in 1973 by Holthuis [6] as "pools with no surface connection with the sea, containing salt or brackish water, which fluctuates with the tides," includes any tidally-influence body of water that is characterized by physical and chemical stratification at the confluence of marine and groundwaters [7]. Such habitats are primarily localized to the tropics [6, 8–15], lack surface connections to the ocean [6, 8, 10, 16], and occur within a variety of basin substrates, including karst caves, cenotes, natural wells and springs, fossilized coral reefs, and coastal basalt (*i.e.*, lava) fields [6, 8, 10]. Given their simultaneous marine and groundwater connections, anchialine habitats often exhibit complex physical and chemical clines in addition to widely varying salinities across the tidal cycle [11, 12, 16]. Relatively little is known regarding the microbial communities that occupy anchialine habitats [13, 14, 17–22], despite studies documenting the great species richness and endemism of macroorganism communities from this ecosystem [6, 23–29].

Only two previous studies have examined seasonal variation in microbial communities from the anchialine ecosystem as well as the environmental and water chemistry factors potentially influencing such dynamics. Specifically, examination of bacterioplankton abundance in anchialine caves in the Yucatan Peninsula, Mexico, found greater density during the rainy season that appeared driven by

increased nutrient input in conjunction with transient surface bacteria being washed into the habitat [30]. Similarly, microbial communities from anchialine caves on Mljet Island in the Adriatic Sea were found to exhibit shifts in total bacterial abundance, relative abundance of high nucleic acid and low nucleic acid bacteria, and influence of bottom-up vs. top-down control across the 21 month sampling period [14]. However, no studies to date have explored the impact of seasonal environmental fluctuations on the microbial community of the anchialine ecosystem in the Hawaiian Archipelago (Fig 1a), that possesses the world's greatest concentration of anchialine habitats. Furthermore, habitats in the Hawaiian Islands occur across greatly varying environmental factors such as basin substrate, temperature, and salinity [22, 31–33] and their food web is based on their microbial communities [2, 34]. Interestingly, a distinctive, laminated orange cyanobacterial-bacterial crust (Fig. 1b, c) can be found in anchialine habitats within the Cape Kinau and Kona regions of the islands of Maui and Hawaii, respectively, and represents unique communities found nowhere else in the world [22, 34, 35]. In this context, laminated cyanobacterial-bacterial mat communities found in hot springs have been shown to exhibit greater richness and diversity during dry seasons since wet seasons physically disrupt mat structure while simultaneously increasing nutrient levels and lowering temperatures [36, 37]. In Hawaii, the year can be broken into two broad periods, dry (May through October) and wet (November through April) seasons [38], with groundwater nutrient levels tracking these differences and nutrient levels being greatest during the wet months [39]. Given this, seasonal changes in microbial community composition and distribution may potentially occur in the distinctive, laminated orange cyanobacterial-bacterial crusts of these particular Hawaiian anchialine habitats.

In this study, the diversity, composition, and distribution of benthic and water column (*i.e.*, *Bacteria* and micro-*Eukarya*) communities was examined across an 18-month period from anchialine habitats within the Cape Kinau and Kona regions of Maui and Hawaii, respectively. Due to the known seasonal variation in Hawaiian groundwater nutrient levels, it was hypothesized microbial community taxonomic diversity and relative abundance would exhibit dynamics correlating to particular environmental factors. Specifically, ammonium, dissolved organic carbon (DOC), and salinity were

expected to drive seasonal variation as these nutrients were identified in Chapter 2 as driving spatial variation in Hawaiian anchialine microbial communities. Alternatively, the laminated cyanobacterial-bacterial mat community may be resistant to seasonal variation.

3.3 Methods

3.3.1 Data Generation

Water column and benthic samples were collected from six anchialine habitats, three each on Maui and Hawaii, during the summer (July) of 2010 and spring (March), summer (July), and winter (December) of 2011. Habitats on Maui were located at Cape Hanamanioa (HM) and within the Ahihi-Kinau Natural Area Reserve at Skippy's Pond (SKIP) and Queen's Bath (QB). On Hawaii, habitats were located at Makalawena Beach (MAKA3), Kiawaiki Bay (KBI), and Pu'uhonua O Hōnaunau National Historical Park (PUHO3). Most sampled habitats contained the laminated cyanobacterial-bacterial crust with the exception of PUHO3, which was historically utilized for fish aquaculture and is now considered a degraded anchialine habitat. Due to the weather patterns of Hawaii, seasons can be subdivided into wet (November-April) and dry (May-October) [38], thus summer samples were considered as dry season and spring and winter samples considered as wet season. All habitats were open ponds, but varied in impact by fish, goats, and humans (Fig. 1 and Table 1). Additional environmental variables from the Hawaii Statewide GIS Program were included, including annual rainfall, rainfall during the month of sampling, rainfall 15 months prior to the month of sampling, and mean annual solar radiation [40, 41]. Rainfall 15 months prior to the month of sampling was included to account for rainwater input via gravitational movement of groundwater since previous work found that the average transit time of dye injected into inland wastewater reclamation injection wells traveling out to the ocean was this duration [42].

Each set of samples was collected within an 8-day span to minimize fine-scale temporal variation. Using disposable sterile sampling spoons, approximately 100 g of the benthos from each of three sampling locations per site was collected and preserved in RNALater (Thermo Fisher Scientific, MA, USA) for future processing. Additional samples were collected and preserved in 95% ethanol, formalin,

or flash frozen with liquid N_2 in dimethyl sulfoxide (DMSO) with 10% glycerol for archival in the Hawaiian Anchialine Microbial Repository in conjunction with The Ocean Genome Legacy (http://www.northeastern.edu/ogl/) under accession numbers S23033-S23083. For sampling water column communities, ~ 1 L of water at each of two sampling locations per site was filtered through sterile 0.2 μm Sterivex (Millipore, MA, USA) units and preserved by flooding with cell lysis buffer (Qiagen, CA, USA). Water chemistry analyses for each habitat were performed by the University of Hawaii Hilo Analytical Laboratory on ~ 0.25 L of filtered water to quantify dissolved organic carbon (DOC), ammonium (NH_4^+), nitrite (NO_2^-) + nitrate (NO_3^-), total dissolved nitrogen (TDN), orthophosphate (PO_4^{3-}), total dissolved phosphorus (TDP), silica (Si), and salinity.

3.3.2 Sequence Data Generation

DNA was extracted from benthos samples preserved in RNALater using MoBio PowerSoil DNA Isolation Kits (MOBIO, CA, USA) and from lysis-buffer preserved water column filters using Gentra Puregene Yeast/Bacteria Kits (Qiagen, CA, USA) according to the procedures in Chapter 2. To reduce the risk of failing to sample taxa distributed heterogeneously within a site, DNA was extracted from at least two of the sampling locations within a site and two separate extractions of most samples were performed (or three separate extractions in the case of MAKA3 benthos in summer 2010) in most cases. Amplification via the polymerase chain reaction (PCR) and sequencing of the extracted DNA samples were performed by the HudsonAlpha Institute for Biotechnology, Inc. Genomic Services Laboratory (Huntsville, AL, USA). Each amplification reaction utilized 20 ng DNA except in cases of low DNA concentration, where the template volume was divided equally between the two PCRs. Amplified samples were sequenced as dual-barcoded amplicons on an Illumina HiSeq 2500 platform to obtain paired-end 100 bp reads from one of two ribosomal DNA (rDNA) regions. Specifically, these regions were selected to target both prokaryotic and eukaryotic rDNA to maximize the proportion of diversity sampled. The V6 hypervariable region of the 16S rDNA was amplified using the *Bacteria*-specific primers 967-985F and 1078-1061R primers [43] and the V9 hypervariable region of the 18S-rDNA using

the *Eukarya*-biased primers 1389F and 1510R [44]. PCR reactions and sequencing runs were each performed in duplicate as described in Chapter 2. Raw high-throughput sequencing reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Experiment Accession Numbers SRX1877412, SRX1877424, SRX1888539-SRX1888787, SRX1902175, SRX1902315-SRX1902325, SRX1902328-SRX1902338, SRX1902341-SRX1902351, SRX1902354-SRX1902364, SRX1902367-SRX1902449, SRX1902452-SRX1902462, SRX1902465-SRX1902475, SRX1902478-SRX1902488, SRX1902490-SRX1902563, SRX1913131-SRX1913145, SRX1913151-SRX1913165, SRX1913171-SRX1913185, SRX1913192-SRX1913206, SRX1913214-SRX1913267, SRX1913269-SRX1913395, SRX1913401-SRX1913415, SRX1913421-SRX1913435, SRX1913442-SRX1913456, SRX1913465-SRX1913517, SRX1913519-SRX1913629SRP077079, BioProject ID Number PRJNA325159).

3.3.3 Operational Taxonomic Unit (OTU) Clustering

PandaSeq v.2.5 [45] was used to align forward and reverse sequencing reads, trim primer sequences, and filter any sequences with uncalled bases. Reads were further filtered using a conservative quality score cut-off of 30 over at least 75% of the read nucleotides using the FASTQ Quality Filter included in the FASTX-Toolkit v.13.2 [46]. USEARCH61 [47], as distributed in the QIIME v.1.8 pipeline [48], was then utilized to remove potentially chimeric sequences before utilizing UCLUST [47] in the `pick_open_reference_otus.py` workflow to cluster sequences into operational taxonomic units (OTUs). Sequences were clustered at 95% sequence similarity and 0.005% abundance using the 99% clustered Silva 111 database [49] as the initial cluster references for the V9 hypervariable region and the 99% clustered GreenGenes 13.8 database [50] for the V6 region. The 0.005% OTU abundance filter was applied as per recommendations by Bokulich et al. [51] for improvement of clustering results. Each OTU cluster, as represented by its most abundant sequence, was submitted for taxonomic identification using megaBLAST v.2.2.26 [52] (sequence identity ≥90%, e-value $1x10^{-6}$) and alignment using PYNAST v.1.2.2 [53] with the default parameters (*i.e.*, minimum length of 75% median input length, minimum

identity 75%) [53] to the appropriate curated databases discussed above. Those OTUs failing to align using PYNAST were removed from the final tables.

3.3.4 Analyses of Community Composition

Within the R v.3.1.13 statistical environment [54], the package PhyloSeq v.1.10.0 [55] was used to calculate three alpha diversity metrics on the final OTU abundance tables: the number of observed OTUs and Shannon [56] and inverse Simpson [57] diversities. Here, higher Shannon diversity values reflect greater community diversity by quantifying uncertainty in predicting to what OTU/taxon the next sampled sequence belongs. In contrast, Simpson's index of diversity measures the probability that two randomly selected sequences belong to the same OTU/taxon. For more intuitive interpretations, the inverse of Simpson's index are presented since greater index values correlate to greater diversity by specifically reporting the richness of a perfectly even community with the same diversity as the observed sample. Rarefaction curves were generated in R for the three alpha diversity metrics using ten replicates at sequencing depths of 1, 10, 100, 1000, 10,000, 20,000, and 30,000 sequences per sample in order to examine the effectiveness of the sampling depth at capturing community diversity. Differences in alpha diversity between samples grouped by whether they originated from the benthos or water column as well as season of sampling (*i.e.*, dry summers vs. wet winter/spring) were tested using one-way analyses of variance (ANOVAs) followed by Tukey's Honestly Significant Differences (HSD) post-hoc test in the R package agricolae v.1.2.3 [54, 58]. Differences with $p < 0.05$ were considered significant.

The `summarize_taxa_through_plots.py` script in the QIIME v.18 pipeline [48] was used to produce data tables corresponding to third-level clades (*i.e.*, class and approximately class in the GreenGenes and Silva databases, respectively) that were employed for later analysis. Whole sampled communities were visualized using non-metric multidimensional scaling (NMDS) ordination with 95% confidence ellipses in the R package PhyloSeq v.1.10.0 [54, 55]. Ordinations were made using the dissimilarity matrix resulting from applying the Bray-Curtis dissimilarity metric and the Jaccard dissimilarity coefficient on the final OTU tables after transformations to even sampling depth. The Bray-

Curtis dissimilarity metric is commonly used in ecological studies because it is based on the abundance of OTUs shared between communities [59]. In contrast, the Jaccard dissimilarity coefficient utilizes only the presence or absence of OTUs to return the proportion of unshared taxa between samples [60]. Environmental variables, including sample site, sample type, and season when sampled, with significant explanatory power (α=0.05) of the sample ordinations were identified using the envfit function (999 permutations) in the R package vegan v.2.3.1 [54, 61] and overlaid on the ordinations as vectors scaled by their explanatory power (r). Furthermore, the bioenv function in the R package vegan v.2.3.1 [61] was utilized to find the combination of continuous and categorical environmental variables whose Euclidean distance resemblance matrix maximized the Spearman correlation with the final OTU table. Variables identified by the bioenv function were considered the best predictors of the observed OTU abundances. The variation in environmental variables with sampling season was evaluated by one-way multivariate analysis of variance (MANOVA) followed by Wilk's Lambda post-hoc test. Univariate one-way analyses of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post hoc tests were then performed on water chemistry variables to identify which were specifically impacted by season type. One-way ANOVAs with Tukey's HSD post-hoc tests were also utilized to identify third-level clades whose relative abundance varied with season of sampling, and multiple regressions were then used to examine the relationship between clades whose relative abundance varied with sampling season and environmental variables that also varied with season type. All R code, QIIME scripts, and other commands employed in this study can be downloaded from http://www.auburn.edu/santosr/XXXXX.htm.

3.4 Results

3.4.1 Data Generation and OTU Clustering

Samples taken from 2010 (*i.e.*, summer) through 2011 (*i.e.*, spring, summer, and winter) from three anchialine habitats each on Maui and Hawaii were successfully sequenced for the V6 and V9 hypervariable regions of the 16S and 18S rRNAs, respectively. Of the 118 samples examined, 33 were from the water column and 85 were from the benthos (Table 2). Overall, sequencing effort produced a

total of 51,706,414 demultiplexed *Bacteria* V6 Illumina reads in each paired-end direction, with an average ($\bar{x}$) of 96,828 reads/sequencing replicate sample, hereafter referred to as a sample. For the *Eukarya*-biased V9 data, 33,046,764 demultiplexed reads in each paired-end direction were produced ($\bar{x}$ = 61,885 reads/sample).

Following alignment, quality-filtering, chimera-checking, and abundance filtering of the V6 data, 14,126,948 reads (a 72.68% reduction overall) were retained ($\bar{x}$ = 30,057 reads/sample). Similarly, 72.21% of the V9 reads were also removed during processing, resulting in 9,180,794 reads ($\bar{x}$ = 19,701 reads/sample). These stringent filtering parameters were employed to reduce noise-to-signal ratio and to reduce potential issues from the relatively short read lengths obtained from the Illumina platform (see below). Lengths of the V6 reads ranged from 63-80 bp ($\bar{x}$ = 74 bp), while V9 reads ranged from 65-163 bp ($\bar{x}$ = 125 bp). A single V6 OTU was removed due to failure to align in PYNAST, resulting in 1,656 OTUs, totaling 12,492,442 sequences ($\bar{x}$ = 26,579 sequences/sample), being retained. In contrast, 15 V9 OTUs failed to align and were removed from the final dataset, resulting in 8,450,946 sequences belonging to 1,211 OTUs ($\bar{x}$ =18,135 sequences/sample). Of the 1,656 and 1,211 OTUs in the final V6 and V9 datasets, 149 (213,912 sequences, 11.36% of total) V6 and 10 (13,799 sequences, 0.16% of total) V9 OTUs could not be assigned taxonomic identifications using either the GreenGenes 13.8 [50] or Silva 111 [49] databases. Similarity searches of the unassigned V6 OTUs to NCBI's GenBank repository [62, 63] using BLASTN v.2.3.0 [52] revealed affiliations primarily to members of the Acidobacteria, Actinobacteria, Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Chloroflexi, Cyanobacteria, Firmicutes, Verrucomicrobia, algal chloroplasts, and NC10 at low e-values (data not shown). For the unassigned V9 OTUs, searches to GenBank revealed associations with Stramenopiles, Alveolata, Rhizaria, Porifera, Anthozoa, Mycetozoa, Fungi, Amoebozoa, Angiosperms, Metamonada, Chlorophyta, and Rhodophyta. Thirty-seven V6 OTUs were identified as most likely originating from eukaryotic chloroplasts, which represented 3.31% of the total sequences (range of 0%-37.38%, with average relative abundance of 3.78%/sample).

3.4.2 Analyses of Community Composition and Influential Factors

Samples originating from the same site, environment (*e.g.*, benthos or water column), or specific season of sampling were combined for most downstream analyses because they were most similar to each other regardless of which biological sample, DNA extraction, PCR, or sequencing run data were generated from (data not shown). Following consolidation, estimates of observed OTU richness were not saturated at depths of 30,000 sequences per sample for either the *Bacteria* V6 or the *Eukarya*-biased V9 datasets. In contrast, both Shannon and inverse Simpson diversity apparently saturated at sampling depths less than, or equal to, 10,000 sequences per sample (Appendix 3). Examination of alpha diversity metrics from V6 samples grouped by whether they were taken from the benthos or water column revealed the former as having greater OTU richness ($F_{1,468}$=45.1, p<<0.001) as well as Shannon ($F_{1,468}$=454, p<<0.001) and inverse Simpson ($F_{1,468}$=234, p<<0.001) diversities. However, when the same V6 samples were grouped by whether they were taken during wet or dry seasons, no differences were detected in these same indices (OTU richness: $F_{1,468}$=2.08, p=0.150; Shannon: $F_{1,468}$=0.009, p=0.923; inverse Simpson: $F_{1,468}$=0.116, p=0.734). Similarly, benthic V9 samples also exhibited greater OTU richness ($F_{1,464}$=38.0, p<<0.001) and Shannon ($F_{1,464}$=93.8, p<<0.001) and inverse Simpson ($F_{1,464}$=58.7, p<<0.001) diversities relative to samples from the water column. Likewise, V9 samples collected either during the wet or dry seasons exhibited no significant differences in OTU richness ($F_{1,464}$=0.203, p=0.652), Shannon diversity ($F_{1,464}$=1.96, p=0.162), or inverse Simpson diversity ($F_{1,464}$=0.599, p=0.439).

Differences between NMDS ordinations utilizing the Bray-Curtis dissimilarity metric and the Jaccard dissimilarity coefficient were minimal, hence only the Bray-Curtis ordinations for the V6 (Fig. 2a) and the V9 (Fig. 2b) OTUs are presented and discussed. Both benthic and water column samples primarily grouped by island, specifically into a cluster of Maui (*i.e.*, HM, SKIP, and QB) and Hawaii (*i.e.*, MAKA3, KBI, and PUHO3) sites (Fig. 2). Within these island-specific clusters, samples were separated by sample type (water column vs. benthos), that could be further differentiated by specific sampling site (Fig. 2a, b). Notably, the PUHO3 samples were distinctive from the other sites on Hawaii, likely due to

its historically degraded state and lack of the distinctive, laminated orange cyanobacterial-bacterial crust. The season of sampling (*i.e.,* wet vs. dry) did not appreciably influence ordinations, with significant overlap observed between samples taken from the same site and type regardless of season of sampling, with the exception of QB water column samples (Fig. 2).

The bioenv function tested 67,108,863 possible combinations among the categorical and continuous environmental variables as well as site, sample ID, season, and whether the sample originated from the benthos or water column for both datasets. From these, three and four variables were identified as significant for the V6 (r=0.835) and V9 (r=0.812) datasets, respectively. Specifically, whether a sample originated from the benthos or water column, annual rainfall (mm), and nitrite (NO_2^-) + nitrate (NO_3^-) were found as significant for both the *Bacteria* V6 and micro-*Eukarya* V9 while ammonium was identified as significant only for the V9 data. Using envfit to examine individual environmental variables, all categorical variables other than season type were identified as significantly able to explain the V6 NMDS ordination at $p<0.05$, with site accounting for the most variation (r^2=0.816) and season accounting for the least (r^2=0.0157). In a similar fashion, all categorical variables other than season and season type were identified as explanatory of the ordination for the V9 data, with site having the greatest (r^2=0.719), and goat presence and potential groundwater tied for the least (r^2=0.203), explanatory power. All of the continuous variables considered in the envfit analysis had significant explanatory power for both the V6 and V9 NMDS ordinations, with latitude, longitude, and annual rainfall having the greatest, and 15 month prior rainfall, phosphate, and TDP having the least, explanatory power (Appendix 4).

Water chemistry variables in the anchialine habitats that were examined were significantly influenced by the season of sampling (MANOVA: Wilk's Lambda $F_{8,460}$=0.369, $p<0.01$). Univariate tests with Tukey's HSD post-hoc analysis revealed the wet season as having increased habitat salinity, nitrite (NO_2^-) + nitrate (NO_3^-), ammonium, DOC, and TDN ($p<0.01$). Along with this, 39 of the 84 third-level bacterial clades (approximately class) in the V6 dataset, 12 of the 30 third-level bacterial clades in the V9 dataset, and 18 of the 28 third-level eukaryotic clades in the V9 dataset exhibited relative abundances significantly varying with season of sampling (Tables 3 and 4). When analyzed in combination, TDN,

nitrite (NO_2^-) + nitrate (NO_3^-), DOC, and salinity correlated with both the V6 and V9 clades (Tables 3 and 4) while only ammonium correlated to the V6 taxa, both those more abundant during wet seasons and those more abundant during dry seasons (Table 3).

3.5 Discussion

3.5.1 Temporal Impact on Hawaiian Anchialine Microbial Communities

This study represents the first temporal survey of *Bacteria* and micro-*Eukarya* diversity across Hawaii's anchialine ecosystems. Particular focus was dedicated to those microbial communities occupying habitats in the Cape Kinau and Kona coast regions of Maui and Hawaii, respectively, where a distinctive, laminated orange cyanobacterial-bacterial crust is endemic. Although it is likely not every member was detected, flattened rarefaction curves of Shannon and inverse Simpson diversities imply that sequencing efforts were successful in capturing the major taxonomic constituents of these communities (Appendix 3). As discussed in Chapter 2, numerous taxa previously identified in light [35] and scanning electron [34] surveys of orange cyanobacterial-bacterial crust materials from these regions of Maui and Hawaii were recovered, lending credence to this sequencing-based approach.

In contrast to the initial hypothesis, comparison of whole microbial communities found spatial factors as having a greater influence than season on diversity, composition and distribution in these communities (Fig. 2). Season of sampling was also found to have relatively little impact on OTU richness or Shannon and inverse Simpson diversities. Environmental and water quality variables, like salinity, NH_4^+, and TDN, and approximately half of the third-level *Bacteria* and micro-*Eukarya* clades, demonstrated clear variation according to season of sampling; however, any given sample was more similar to one from the same site and portion of the habitat (*i.e.*, benthos vs. water column) when considered in the context of whole community dissimilarities utilizing both presence/absence (*i.e.*, Jaccard dissimilarity coefficient) and relative abundances (*i.e.*, Bray-Curtis dissimilarity metric). Similarity in the Jaccard and Bray-Curtis ordinations suggests that distinctions between samples were likely due to both differences in relative abundance of shared OTUs and different OTU memberships

rather than only abundance-based distinctions. Indeed, neither V6 nor V9 datasets included an OTU that was present in every single sample. Taken together, the distinct and unique nature of individual laminated cyanobacterial-bacterial crust communities on the islands of Maui and Hawaii noted in Chapter 2 was maintained despite seasonal influences.

Outlying water column samples taken from QB during the dry season (summer 2010, Table 2) were uniquely dominated (*i.e.*, ~67% total sequences) by sequences belonging to the genus *Cetobacterium* in Fusobacteria; *Cetobacterium* have been identified in mammalian and fish gut microbiomes [64, 65]. QB was unique among the habitats included in this study because it appeared to function more as a wetland where the pond basin almost completely dried out during low tides and was also inhabited by the Hawaiian stilt subspecies (*Himantopus mexicanus knudseni*) (pers. obs). Thus, the *Bacteria* and micro-*Eukarya* communities in QB were distinct, likely reflecting the wetland-like nature of the habitat.

It is possible that season of sampling exerted a greater influence on the Hawaiian anchialine microbial communities examined here than indicated by these analyses, as every member of the communities was not sampled (see above, Appendix 3) and microbial taxa in complex structures have been shown to migrate with subsequent patchy distributions [66] that complicate thorough sampling. While some diversity was possibly missed, this study achieved sufficient sampling depth to capture the major players in diversity as indicated by saturation in rarefaction curves of Shannon and inverse Simpson diversity (see above). Moreover, temporal and seasonal stability has been observed in numerous other microbial assemblages and communities occupying unusual niches, including hypersaline microbial mats [67–69], cyanobacterial desert soil crusts [70], hot spring microbial mats [71], and phototrophic microbial/cyanobacterial mats found in a meromictic hypersaline lake [72, 73]. In cases where cyanobacterial-bacterial dominated communities take on a laminated mat nature or form complex structures such as crusts, the formation of micro-niches can foster greater taxonomic diversity within the community, with subsequent increases in metabolic variation and activity. In the case of the cyanobacterial component, their broad metabolic diversity enables them to survive in extreme

environments and facilitates formation of mats and crusts by driving productivity, separating oxic and anoxic microniches, and secreting the extracellular polymeric substances (EPS) that further creates structural cohesion [74]. As a result, laminated cyanobacterial-bacterial structures allow for greater community diversity and functional redundancy, characteristics which Yannarell et al. [69] noted as conducive for maintaining compositional integrity of Bahamian hypersaline microbial mats in the face of seasonal hurricane activity [68]. In the same way, lamination in the orange cyanobacterial-bacterial crust communities found in some Hawaiian anchialine habitats may also increase community resistance to seasonal fluctuations in a range of environmental factors.

On the other hand, these Hawaiian anchialine microbial communities may also be resilient to compositional fluctuations in spite of seasonal and fluctuating water chemistry if a large fraction of community members are not nutrient-limited. Land development near anchialine habitats in Hawaii can significantly increase incidents of nitrogen and phosphorus leaching into groundwater leading to levels well above natural levels [38, 75], which could pass through extant anchialine habitats on its way out to the sea via gravitational flow. Specifically, the use of treated sewage and dry fertilizers in residential developments or golf course grounds was linked in 1991 to 116% and 22% increases in nitrogen and phosphorus, respectively [75], with some anchialine pools within such developed areas in 2006 having nutrient levels >70% higher than rivers and estuaries considered heavily polluted [38]. Furthermore, experimental addition of nitrogen and phosphorus to both pristine and anthropogenically impacted anchialine habitats on Hawaii revealed that the benthic community was not nutrient-limited and was only impacted by top-down control [76]. Along with this, observed community compositional differences correlating with salinity in these habitats were found to be decoupled from co-varying nitrogen or phosphorus levels, suggesting that any bottom-up forces may be complex and/or linked to other nutrients [76]. In contrast, a survey on Hawaii of minimally to heavily impacted anchialine habitats found that greater nitrogen and phosphorus levels were associated with greater benthic biomass, autotrophy, and nutrient content as well as greater size and abundance of the endemic atyid shrimp *Halocaridina rubra*, a microbial grazer common to habitats in the islands [2]. Notably, most of the salinity and nitrogen levels

presented here were more similar to those reported by Sakihara et al. [76] rather than that of Dalton et al. [2], suggesting microbial communities from habitats with consistently lower salinity (e.g., like those surveyed by Dalton et al.) may be more nutrient-limited than those from higher salinity environments.

Additionally, the seasonal fluctuations in nutrients reported here may not have occurred for a long enough duration or been of a great enough magnitude to have a bottom-up impact on the microbial communities of the anchialine habitats examined. For example, gut content analyses of invasive poeciliids in Hawaiian anchialine habitats failed to identify seasonal influences in diet [77]. Furthermore, anchialine pools on Hawaii experience considerable fluctuations of water chemistry (specifically pH and turbidity) during the diel cycle, with lower pH and greater turbidity at night versus during the day [78]. Given this, the endemic microbial communities of the Hawaiian anchialine ecosystem may have assembled in such a way as to make the community resistant to transient fluctuations in water chemistry and thus exhibit minimal shifts due seasonal variation in factors such as nutrient availability or concentrations. Indeed, the greater explanatory power of mean annual rainfall rather than rainfall 15 months prior to sampling or rainfall during the sampling month may also be due the reduced impact of short-term fluctuations; that is, long-term alterations in environmental conditions may have greater impact on microbial communities by overcoming community resistance to short-term changes. Indeed, 2009-2010 marked a severe drought due to El Niño that was alleviated somewhat in 2011. Drought conditions, and thus annual rainfall, may have obscured seasonal impacts by applying greater environmental pressure to microbial communities over extended periods that overcame community resistance to altered conditions.

3.5.2 Temporal Impact on Water Chemistry and Relative Abundance of Taxa

Due to their simultaneous connections to both the sea and groundwater, anchialine habitats tend to exhibit both vertical stratification and water chemistry fluctuations from terrestrial sources [79]. In Hawaii, two predictable seasons – wet in the winter and spring and dry in the summer and fall [38] – are apparent. During wet seasons, significantly increased levels of $NO_2^- + NO_3^-$, ammonium, DOC, and TDN

were observed in the anchialine habitats sampled here, likely due to an increased influence by groundwater, which has greater nutrient levels than the seawater surrounding Hawaii [80]. An increase in near-surface salinity was also recorded in wet seasons, which may be induced by an increased flow of freshwater mixing the seawater and freshwater lenses, direct rainfall into the surface waters of the habitat, and/or higher levels of wind activity.

Approximately one-half of the third-level clades (approximating class) recovered in the sampling conducted here experienced seasonal shifts in their relative abundances, with about 75% of these correlating with at least one of the seasonally-varying water chemistry factors (Tables 3 and 4). Of particular note were cyanobacterial clades that were more abundant during dry seasons and correlated negatively with DOC, ammonium, and nitrite (NO_2^-) + nitrate (NO_3^-) (Table 3). Taken together with the previous evidence suggesting cyanobacteria from Hawaiian anchialine habitats might not be nutrient-limited, the lower nutrient levels and stronger sun during the dry seasons may favor the oxygenic photosynthetic portion of the microbial community, of which Cyanobacteria dominate. Further evidence for oxygenic autotrophy being favored during the dry seasons was the simultaneous increased abundance of algal chloroplasts, Rhodophyta, and Glaucophyta (Tables 3 and 4). Unsurprisingly, Glaucophyta, exclusively encompassing freshwater organisms [81], had increased abundances during the dry seasons and were correlated with reduced salinity. In contrast, the wet seasons favored organisms capable of anoxygenic photosynthesis as greater abundances of Acidobacteria, Chlorobi, and Chloroflexi were recorded (Tables 3 and 4). Anoxygenic photosynthesizers have been shown to be inhibited by increasing salinity [82, 83], and although increased salinity was measured during the wet season, increased turnover and mixing may have facilitated decreased salinities in the lower seawater lens, thus allowing greater activity by members of these groups.

Comparison of the seasonal trends in taxa identified during both this study and in other cyanobacterial-bacterial communities revealed similarities and differences. Cyanobacteria in thermophilic mats in the Philippines and China exhibited greater abundances during the wet seasons [36, 37], with both instances correlating to reduced temperatures driven by large rainwater influxes. Although

the cyanobacterial clades of this study responded differently in wet seasons, Chloroflexi were more abundant during this period both in the Hawaii anchialine ecosystem as well as in the Philippine study [36]. In this latter case, Chloroflexi abundance co-varied with increased phosphate levels, likely due to rainwater influx [36]. However, phosphate did not significantly increase during Hawaii's wet seasons, and instead greater Chloroflexi abundance was correlated with reduced salinities. In previous studies, Bacilli were greater during the dry season while Clostridia were greater during the wet season [37], whereas in this study Clostridia were found to be more abundant during Hawaii's dry season and correlated with lower DOC and salinity (Tables 3 and 4). Interestingly, the Bacilli recovered in the V6 dataset were also found to be more abundant during dry seasons despite belonging to a different order than that those found in Chinese thermophilic mats [37] whereas those in the V9 dataset included an OTU from the same order as [37] being more abundant during the wet seasons (Tables 3 and 4).

Several of the clades identified as being differentially abundant in relation to Hawaii's wet and dry seasons were apparently not correlated to any of the seasonally-varying water chemistry factors measured here. Some organisms identified as more abundant during the wet season included members of the Gemmatimonadetes and Spartobacteria, and may have been washed into the habitat from other sources, thus accounting for their lack of correlation with any of the water chemistry factors. For example, Gemmatimonadetes account for approximately 2% of soil bacterial communities [84] while the Spartobacteria are considered ubiquitous soil organisms [85], lending credence to their increased contribution to the samples during wet seasons being a result of increased groundwater influence.

3.5.3 Future Considerations for Hawaiian Anchialine Conservation Efforts

Hawaii's anchialine habitats are greatly threatened by multiple factors, including introduced species, development, and sea level change. Furthermore, introduced organisms such as poecilid and tilapia fish species and the invasive shrimp *Macrobrachium lar* cause alterations in both behavior and abundance of the atyid shrimp *H. rubra* which grazes on the benthos, leading to subsequent shifts in benthic microbial communities [86–88, 2, 32, 89, 90]. In addition, development and urbanization

continues to threaten anchialine habitats due to their presence on prime real estate along coastlines, continuing popularity of the state as a tourist destination, and continuing population growth at an average of 1% every year [91]. As an example, a single development project in 1985 destroyed over 130 habitats [92] and impacts remaining ponds by dramatically increasing nutrient levels via nonpoint sources [38, 75]. Furthermore, projected increases in sea level due to global climate change threaten the anchialine ecosystem in Hawaii since a significant portion of existing habitats will become inundated while simultaneously contributing to the spread of invasive fishes, resulting in a greater proportion of anchialine habitats being negatively impacted despite the creation of new habitats [89].

Although this study provides insight into the temporal and seasonal variation (or lack thereof) of microbial communities from the Hawaiian anchialine ecosystem, much work remains to be done. Unfortunately, this study was constrained to two wet seasons and two dry seasons, so much remains unknown concerning the generality and predictability of the observed patterns over longer time periods and whether finer-scale temporal variation in microbial community diversity, composition and distributions exists in this ecosystem. Variation in water chemistry such as pH and turbidity have been observed to occur during the diel cycle [78], but the effect, if any, on the distinctive and unique cyanobacterial-bacterial crusts was not examined. Taxa in hypersaline microbial mats were also shown to migrate vertically during a single diel period, resulting in dramatic variations in abundance [66], which may also occur in anchialine habitats. Furthermore, there are many other aspects of water chemistry that were not examined that may influence observed seasonal community fluctuations or contribute to the observed overall community stability. Indeed, measurement of chemical and physical clines within the physical community structures (*i.e.*, laminated crusts) across seasons could provide evidence of micro-niche partitioning contributing to community stability.

3.5.4 Conclusions

Here, data from high-throughput amplicon sequencing are presented which imply that seasonality minimally impacts the distinct cyanobacterial-bacterial crust communities unique to the Hawaiian

anchialine ecosystem. Although community composition as a whole appeared to be more heavily influenced by geographic and spatial factors like island and site, wet and dry seasons did significantly influence salinity, ammonium, dissolved organic carbon, total dissolved nitrogen, and nitrite (NO_2^-) + nitrate (NO_3^-). Additionally, shifts in relative abundance for approximately half of the third-level (approximately class) *Bacteria* and micro-*Eukarya* clades detected is reported, with many of these changes in OTU relative abundance being correlated with at least one of the seasonally-impacted water chemistry factors that were measured. Further work should examine both shorter- (*i.e.*, days to weeks) and longer-scale (*i.e.*, >2 years) temporal variation in Hawaii's distinct orange laminated cyanobacterial-bacterial crust communities.

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Table 1. Sampled anchialine habitats from the islands of Hawaii and Maui and their corresponding categorical and continuous environmental factors. Site abbreviations: HM- Hanamanioa, Maui; QB- Queen's Bath, Maui; Skip- Skippy's Pond, Maui; KBI- Keawaiki Bay, Hawaii; MAKA3- Makalawena Beach, Hawaii; PUHO- Pu'uhonua O Hōnaunau National Historical Park, Hawaii.

Categorical Environmental Factors	**Sites**					
	HM	**QB**	**SKIP**	**KBI**	**MAKA3**	**PUHO**
Island	Maui	Maui	Maui	Hawaii	Hawaii	Hawaii
Habitat Type	Pond	Pond	Pond	Pond	Pond	Pond
Orange Cyanobacterial-Bacterial Crust	Yes	Yes	Yes	Yes	Yes	No
Fish	No Fish	No Fish	No Fish	Tilapia	Poeciliids & Marine	No Fish
Goats	Yes	Yes	Yes	No	Yes	No
Open to Public	No	No	No	Yes	Yes	No
Aquifer [40]	Kahikinui	Central	Kahikinui	Hualalai	Hualalai	SW Mauna Loa
Watershed [40]	Kanaio	Ahihi Kinau	Ahihi Kinau	Kiholo	Kiholo	Kauna
Potential Warm Groundwater [40]	Yes	Yes	Yes	No	Yes	No
Continous Environmental Factors						
Latitude	20.58	20.6	20.6	19.89	19.79	19.01
Longitude	156.41	156.43	156.42	155.9	156.03	155.80
Annual Rainfall 2010 (mm) [40, 41]	261.1	249.9	253.5	152.9	173.0	439.8
Annual Rainfall 2011 (mm) [40, 41]	344.0	364.5	358.8	101.5	150.8	491.3
Sample Month Summer 2010 Rainfall [40, 41]	14.4	14.5	14.8	2.3	7.8	34.6

Sample Month Spring 2011 Rainfall [40, 41]	17.9	17.0	17.4	21.0	2.9	21.4
Sample Month Summer 2011 Rainfall [40, 41]	17.2	18.1	18.3	2.1	1.9	11.6
Sample Month Winter 2011 Rainfall [40, 41]	22.2	20.5	20.8	1.3	8.7	8.3
15 Month Prior Rainfall Summer 2010 [40, 41]	16.6	15.7	16.1	6.79	10.2	29.0
15 Month Prior Rainfall Spring 2011 [40, 41]	127.7	124.9	126.9	68.2	61.4	79.3
15 Month Prior Rainfall Summer 2011 [40, 41]	8.30	7.32	7.56	10.4	22.1	42.6
15 Month Prior Rainfall Winter 2011 [40, 41]	32.1	34.8	34.9	3.49	2.85	29.4
Mean Annual Solar Radiation 2014* ($Watts/m^2$) [40]	216.6	193.5	196.2	226.5	224.8	180.7
Salinity Summer 2010 (ppt)	15.0	26.5	24.0	5.0	4.0	15.5
Salinity Spring 2011 (ppt)	15.0	21.0	15.0	5.0	7.0	13.0
Salinity Summer 2011 (ppt)	5.0	23.0	12.0	2.0	5.0	9.0
Salinity Winter 2011 (ppt)	17.0	21.0	17.0	5.0	7.0	13.0
Nitrite and Nitrate Summer 2010 (NO_2+NO_3, µM)	48.1	24.6	23.9	79.8	46.3	41.1
Nitrite and Nitrate Spring 2011 (NO_2+NO_3, µM)	38.8	40.2	27.9	66.4	75.6	6.9
Nitrite and Nitrate Summer 2011 (NO_2+NO_3, µM)	32.2	22.4	13.7	60.4	54.8	5.6

Nitrite and Nitrate Winter 2011 (NO_2+NO_3, µM)	43.4	12.5	38.9	81.1	70.6	7.2
[1] Orthophosphate Summer 2010 (PO_4, µM)	1.65	ND	ND	1.24	7.24	1.88
[1] Orthophosphate Spring 2011 (PO_4, µM)	1.17	0.54	1.01	1.06	7.89	3.50
[1] Orthophosphate Summer 2011 (PO_4, µM)	1.16	0.14	0.46	1.00	6.49	1.29
[1] Orthophosphate Winter 2011 (PO_4, µM)	1.24	0.34	1.29	1.28	7.57	2.62
Silica Summer 2010 (Si, µM)	418	383	355	666	897	568
Silica Spring 2011 (Si, µM)	352	341	398	602	669	461
Silica Summer 2011 (Si, µM)	232	223	204	625	712	389
Silica Winter 2011 (Si, µM)	332	236	312	677	773	536
[2] Ammonium Summer 2010 (NH_4, µM)	1.41	ND	ND	1.41	1.16	1.73
[2] Ammonium Spring 2011 (NH_4, µM)	2.52	ND	2.69	1.69	2.06	8.07
[2] Ammonium Summer 2011 (NH_4, µM)	2.89	ND	2.11	1.90	1.72	3.97
[2] Ammonium Winter 2011 (NH_4, µM)	ND	2.67	1.16	1.23	ND	4.55
Dissolved Organic Carbon Summer 2010 (DOC, µM)	44.1	54.2	40.1	38.3	14.5	119
Dissolved Organic Carbon Spring 2011 (DOC, µM)	96.5	70.1	45.3	81.6	26.0	195

Dissolved Organic Carbon Summer 2011 (DOC, µM)	48.1	53.1	53.8	60.8	26.1	106
Dissolved Organic Carbon Winter 2011 (DOC, µM)	107	138	57.5	73.1	57.1	83.8
Total Dissolved Nitrogen Summer 2010 (TDN, µM)	49.1	24.9	23.6	73.4	42.9	9.9
Total Dissolved Nitrogen Spring 2011 (TDN, µM)	47.6	44.8	29.2	70.7	78.4	27.1
Total Dissolved Nitrogen Summer 2011 (TDN, µM)	36.0	24.0	17.4	65.2	59.0	15.9
Total Dissolved Nitrogen Winter 2011 (TDN, µM)	59.5	31.1	43.9	85.8	78.0	19.6
[3] Total Dissolved Phosphorus Summer 2010 (TDP, µM)	1.53	ND	ND	1.25	7.50	2.22
[3] Total Dissolved Phosphorus Spring 2011 (TDP, µM)	1.30	0.71	1.12	1.13	7.95	3.72
[3] Total Dissolved Phosphorus Summer 2011 (TDP, µM)	1.17	ND	ND	0.93	6.39	1.54
[3] Total Dissolved Phosphorus Winter 2011 (TDP, µM)	1.23	ND	1.09	1.27	7.60	2.62

1 Not detectable (ND) <0.10 µM

2 Not detectable (ND) <1.00 µM

3 Not detectable (ND) <0.050 µM

** 2014 Data used as approximation, data not available for sampled years*

Table 2. Number of biological samples, technical samples (produced by duplicate PCR reactions of a single biological sample), and the total number of samples sequenced from two Illumina runs analyzed in this study from sampled anchialine habitats on the islands of Hawaii and Maui.

			Summer 2010		Spring 2011		Summer 2011		Winter 2011	
			Benthos	**Column**	**Benthos**	**Column**	**Benthos**	**Column**	**Benthos**	**Column**
V6	MAKA3	Biological Samples	4	0*	4	0*	6	1	3	2
		Technical Samples	8	0*	8	0*	12	2	6	4
		Total Samples Sequenced	16	0*	16	0*	24	4	12	8
	KBI	Biological Samples	2	2	4	0*	4	2	4	2
		Technical Samples	4	4	8	0*	8	4	7	4
		Total Samples Sequenced	8	8	16	0*	16	8	14	8
	PUHO3	Biological Samples	2	0*	2	2	4	2	3	2
		Technical Samples	4	0*	4	4	8	4	6	4
		Total Samples Sequenced	8	0*	8	8	16	8	12	8
	HM	Biological Samples	2	1	4	2	4	2	4	2
		Technical Samples	4	2	8	4	8	4	8	4
		Total Samples Sequenced	8	4	16	8	16	8	16	8
	QB	Biological Samples	4	0*	3	2	4	2	4	0*
		Technical Samples	8	0*	6	4	8	4	8	0*
		Total Samples Sequenced	16	0*	12	8	16	8	16	0*
	SKIP	Biological Samples	2	2	4	2	4	1	4	2
		Technical Samples	4	4	8	4	8	2	8	4
		Total Samples Sequenced	8	8	16	8	16	4	16	8
V9	MAKA3	Biological Samples	4	0*	4	0*	6	1	3	2

	Technical Samples	8	0*	8	0*	12	2	6	4
	Total Samples Sequenced	16	0*	16	0*	24	4	12	8
KBI	Biological Samples	2	2	4	0*	4	2	4	2
	Technical Samples	4	4	8	0*	8	4	8	4
	Total Samples Sequenced	8	8	16	0*	16	8	16	8
PUHO3	Biological Samples	2	0*	2	2	2	2	3	2
	Technical Samples	4	0*	4	4	6	4	6	4
	Total Samples Sequenced	8	0*	8	8	12	8	12	8
HM	Biological Samples	2	1	4	2	4	2	4	2
	Technical Samples	3	2	8	4	8	4	8	4
	Total Samples Sequenced	6	4	16	8	16	8	16	8
QB	Biological Samples	4	0*	3	2	4	2	4	0*
	Technical Samples	8	0*	6	4	8	4	8	0*
	Total Samples Sequenced	16	0*	12	8	16	8	16	0*
SKIP	Biological Samples	2	2	4	2	4	1	4	2
	Technical Samples	4	4	8	4	8	2	8	4
	Total Samples Sequenced	8	8	16	8	16	4	16	8

**Samples were collected and sent for sequencing but failed to amplify.*

Table 3. Third-level bacterial clades identified by univariate ANOVA in the V6 dataset as exhibiting relative abundances that varied with season type and water chemistry variables that correlated with them at $p<0.05$.

Taxa	Season type when more abundant	SS	$F_{1,467}$	P	Water Chemistry Variables
Acidobacteria, BPC102	Wet	1.38E-06	9.894	1.76E-03	Ammonium, Salinity
Acidobacteria, OS.K	Wet	1.84E-04	5.314	2.16E-02	DOC, Ammonium
Acidobacteria, Sva0725	Wet	1.55E-04	4.977	2.62E-02	Ammonium, Nitrite and Nitrate
Actinobacteria, Nitriliruptoria	Wet	1.91E-06	7.646	5.92E-03	None
Armatimonadetes, 0319.6E2	Wet	4.64E-06	11.920	6.06E-04	TDN, DOC, Ammonium, Salinity, Nitrite and Nitrate
Bacteroidetes, SB.5	Wet	1.17E-06	4.189	4.13E-02	DOC, Ammonium
Chlamydiae, Chlamydiia	Wet	4.16E-06	16.050	7.19E-05	TDN, DOC, Salinity, Nitrite and Nitrate
Chlorobi, SJA.28	Wet	4.50E-05	5.060	2.50E-02	DOC, Nitrite and Nitrate
Chloroflexi, Anaerolineae	Wet	7.00E-04	5.561	1.88E-02	Salinity
Fibrobacteres, TG3	Wet	7.70E-06	5.743	1.69E-02	Salinity
Gemmatimonadetes	Wet	1.83E-06	17.111	4.18E-05	TDN, Ammonium, Nitrite and Nitrate
Gemmatimonadetes, Gemm.1	Wet	5.08E-05	10.240	1.47E-03	DOC, Ammonium
Gemmatimonadetes, Gemm.2	Wet	2.66E-04	6.327	1.22E-02	TDN, Nitrite and Nitrate
Gemmatimonadetes, Gemm.4	Wet	1.30E-03	6.640	1.03E-02	Ammonium
GN02, 3BR.5F	Wet	1.10E-05	4.355	3.75E-02	TDN, Nitrite and Nitrate
Nitrospirae, Nitrospira	Wet	7.61E-05	16.495	5.72E-05	DOC
Planctomycetes, C6	Wet	1.72E-05	6.530	1.09E-02	DOC
Proteobacteria, Gammaproteobacteria	Wet	6.44E-01	37.610	1.84E-09	None
Thermi, Deinococci	Wet	5.34E-04	15.020	1.22E-04	TDN, Nitrite and Nitrate
Verrucomicrobia, Pedosphaerae	Wet	3.91E-03	7.503	6.40E-03	DOC, Nitrite and Nitrate
Verrucomicrobia, Spartobacteria	Wet	2.43E-06	16.580	5.48E-05	TDN, Ammonium, Salinity, Nitrite and Nitrate
Verrucomicrobia, Verruco.5	Wet	1.72E-04	14.370	1.70E-04	None
WS3, PRR.12	Wet	9.80E-05	7.125	7.87E-03	Ammonium
WS6, SC72	Wet	1.00E-05	12.390	4.74E-04	TDN, Ammonium, Nitrite and Nitrate

Bacteroidetes, Rhodothermi	Dry	5.30E-04	4.372	3.71E-02	TDN, Salinity, Nitrite and Nitrate
Bacteroidetes, Saprospirae	Dry	2.37E-02	8.250	4.26E-03	DOC, Ammonium, Salinity
Caldithrix, Caldithrixae	Dry	4.60E-07	5.173	2.34E-02	DOC, Ammonium, Salinity
Cyanobacteria	Dry	7.70E-03	9.251	2.49E-03	DOC, Ammonium
Cyanobacteria, Chloroplast	Dry	5.19E-02	8.997	2.85E-03	DOC, Ammonium, Salinity, Nitrite and Nitrate
Cyanobacteria, Gloeobacterophycideae	Dry	9.70E-04	7.467	6.52E-03	DOC
Cyanobacteria, Synechococcophycideae	Dry	4.95E-02	12.560	4.33E-04	DOC, Nitrite and Nitrate
Firmicutes, Bacilli	Dry	2.10E-06	10.620	1.20E-03	TDN, DOC, Salinity, Nitrite and Nitrate
Firmicutes, Clostridia	Dry	1.03E-03	7.798	5.45E-03	Salinity
GN04, GN15	Dry	3.10E-06	8.252	4.26E-03	Ammonium, Salinity
Planctomycetes, Phycisphaerae	Dry	2.21E-04	4.739	3.00E-02	TDN, DOC, Ammonium, Nitrite and Nitrate
Planctomycetes, Pla3	Dry	9.60E-07	4.691	3.08E-02	Ammonium, Salinity
Planctomycetes, vadinHA49	Dry	8.23E-07	40.390	4.94E-10	TDN, Salinity, Nitrite and Nitrate
Proteobacteria, Epsilonproteobacteria	Dry	1.31E-06	8.280	4.19E-03	Salinity
Verrucomicrobia, Opitutae	Dry	5.60E-04	10.690	1.16E-03	DOC, Ammonium, Salinity

Table 4. Third-level bacterial and eukaryotic clades identified by univariate ANOVA in the V9 dataset as exhibiting relative abundances that varied with season type and water chemistry variables that correlated with them at $p<0.05$.

Taxa	Season type when more abundant	SS	$F_{1,463}$	P	Water Chemistry Variables
Bacteria, Firmicutes, Bacilli	Wet	3.09E-05	6.125	1.37E-02	None
Bacteria, Gemmatimonadetes, BD2.11 terrestrial group	Wet	4.90E-05	6.633	1.03E-02	None
Bacteria, Gemmatimonadetes, PAUC43f marine benthic group	Wet	2.35E-06	4.191	4.12E-02	None
Bacteria, Lentisphaerae, Lentisphaeria	Wet	2.19E-04	10.948	1.01E-03	DOC, Nitrite and Nitrate
Bacteria, Nitrospirae, Nitrospira	Wet	8.40E-05	9.180	2.58E-03	None
Bacteria, Planctomycetes, OM190	Wet	6.20E-05	8.637	3.46E-03	None
Bacteria, Planctomycetes, Planctomycetacia	Wet	4.20E-04	3.872	4.97E-02	None
Bacteria, Proteobacteria, Deltaproteobacteria	Wet	6.80E-04	4.495	3.45E-02	TDN, DOC, Nitrite and Nitrate
Bacteria, Proteobacteria, SPOTSOCT00m83	Wet	4.73E-07	13.584	2.55E-04	TDN, DOC, Salinity, Nitrite and Nitrate,
Bacteria, Verrucomicrobia, OPB35 soil group	Wet	9.40E-04	9.863	1.79E-03	TDN, DOC, Nitrite and Nitrate
Bacteria, Verrucomicrobia, Opitutae	Wet	7.10E-05	4.992	2.59E-02	Salinity
Bacteria, Verrucomicrobia, Spartobacteria	Wet	4.10E-04	4.766	2.95E-02	None
Eukaryota, Amoebozoa, Conosa	Wet	1.34E-03	37.169	2.29E-09	Nitrite and Nitrate
Eukaryota, Amoebozoa, Discosea	Wet	2.06E-05	4.159	4.20E-02	None
Eukaryota, Archaeplastida, Chloroplastida	Wet	1.05E-01	11.850	6.29E-04	TDN, DOC, Nitrite and Nitrate
Eukaryota, Incertae Sedis, Apusomonadidae	Wet	4.69E-07	8.986	2.87E-03	DOC
Eukaryota, SAR, Rhizaria	Wet	4.10E-04	4.168	4.18E-02	TDN, DOC, Nitrite and Nitrate
Bacteria, Firmicutes, Clostridia	Dry	4.98E-04	16.815	4.87E-05	DOC

Bacteria, Proteobacteria, Betaproteobacteria	Dry	6.10E-05	12.510	4.45E-04	None
Eukaryota, Amoebozoa, Lobosa	Dry	1.19E-03	23.310	1.88E-06	Salinity
Eukaryota, Archaeplastida, Glaucophyta	Dry	3.35E-05	6.657	1.02E-02	Salinity
Eukaryota, Archaeplastida, Rhodophyceae	Dry	7.50E-06	4.375	3.70E-02	TDN, DOC, Nitrite and Nitrate
Eukaryota, Centrohelida, Heterophryidae	Dry	4.58E-05	10.170	1.52E-03	None
Eukaryota, DH147.EKD10.uncultured marine eukaryote	Dry	9.80E-07	8.992	2.86E-03	None
Eukaryota, Excavata, Discoba	Dry	2.79E-04	25.140	7.63E-07	None
Eukaryota, Incertae Sedis, Palpitomonas	Dry	1.38E-06	10.003	1.67E-03	TDN, DOC, Nitrite and Nitrate
Eukaryota, Kathablepharidae, Roombia	Dry	5.59E-06	19.478	1.27E-05	None
Eukaryota, SA1.3c06. uncultured eukaryote	Dry	6.37E-06	9.536	2.14E-03	None
Eukaryota, SAR, Alveolata	Dry	1.84E-01	5.515	1.93E-02	None
Eukaryota, Zeuk77, uncultured Eimeriidae	Dry	5.40E-06	4.810	2.88E-02	None

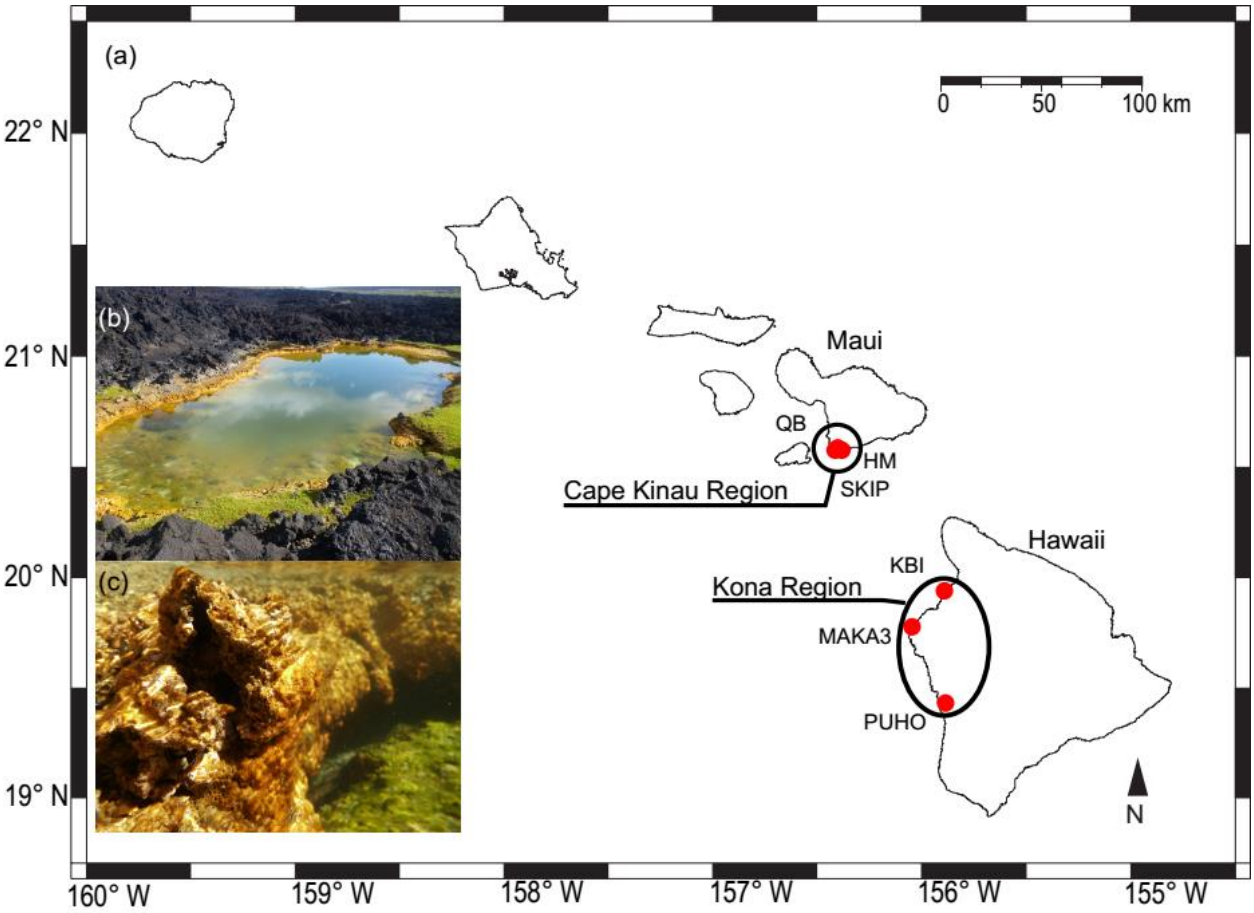

Fig. 1. a Map depicting sampling sites of anchialine habitats on the islands of Maui and Hawaii with the regions of Cape Kinau, Maui and Kona, Hawaii indicated (open circles). **b** Example of a Hawaiian anchialine open pool habitat (*i.e.*, site SKIP) with the orange cyanobacterial-bacterial crust found in Cape Kinau, Maui and Kona, Hawaii. **c** Close-up of orange cyanobacterial-bacterial crust found exclusively in the Cape Kinau region of Maui and the Kona region of Hawaii.

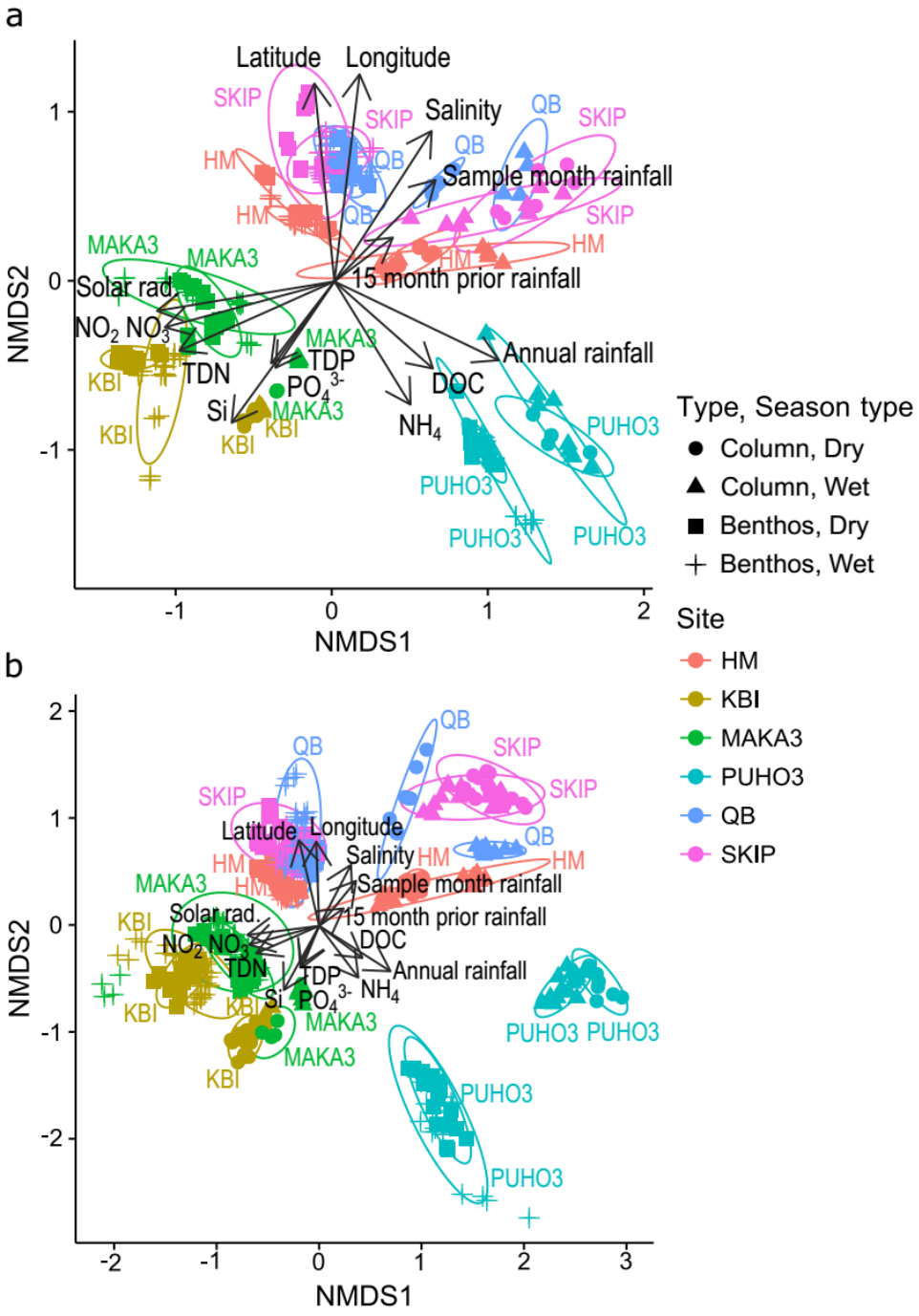

Fig. 2. Non-metric Multidimensional Scaling (NMDS) ordination using the Bray-Curtis Dissimilarity Index of samples grouped by benthic or water column microbial communities within seasons and anchialine sites that were surveyed. Environmental factors were fitted to and overlaid on ordinations, and represent environmental gradients in ordination space. **a** Samples generated using the *Bacteria*-specific

V6 hypervariable region of the 16S-rDNA gene. **b** Samples generated using the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene.

Chapter 4. Comparison of microbial consortia composition in the layers of laminated cyanobacterial-bacterial mats found in select Hawaiian anchialine habitats

4.1 Abstract

Laminated microbial mats have been frequently studied since they offer insight to processes such as the evolution of life, community assembly, and ecosystem functions. However, some examples of these communities have received little attention, including the distinct, laminated orange cyanobacterial-bacterial crusts found in the Cape Kinau and Kona regions of Maui and Hawaii, respectively, of the Hawaiian Islands. These microbial consortia are responsible for most, if not all, primary productivity in the region's anchialine habitats, defined as nearshore bodies of water with subsurface freshwater and seawater connections. To develop insight into the potential functional roles of microbes in these crusts, orange laminated crust samples were collected from six anchialine habitats on Maui and Hawaii and their four distinct layers (*i.e.*, top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL)) separated for high-throughput amplicon fsequencing of ribosomal DNA (rDNA) hypervariable regions (*i.e.*, *Bacteria*-specific V6 and *Eukarya*-biased V9). Increasing microbial richness with depth into the crust structure was documented, with the bottom layer having the greatest and the top layer the least richness. Overall, samples of a given layer were more similar to different layers from the same site than to those of the analogous layer from different sites, and samples from sites on the same island were more similar to each other, regardless of which layer they originated from, than to those from the same layer in sites from the other island. Furthermore, Cyanobacteria and algae were abundant in both the surface and bottom layers, suggesting the crust is oxygenated from both above and below. Correspondingly, anaerobic and chemoautotrophic taxa were concentrated in the middle two layers of the

crust. Thus, the orange crust in select Hawaiian anchialine habitats are distinct from other laminated cyanobacterial-bacterial communities in arrangement of oxygenated versus anoxygenated niches.

4.2 Introduction

Laminated microbial mats, in which taxa exhibit vertically stratified distributions in response to environmental or chemical gradients, have proven to be a source of valuable biological and technological discoveries. For example, our understanding of the evolution of biological and community assembly, as well as ecosystem function, has grown from studying laminated microbial mats, as fossilized mats known as stromatolites have helped us estimate when life first evolved [1–4] and understand how life may evolve on other planets [5]. Furthermore, current genetic techniques would not have been possible without the discovery of *Taq* polymerase from *Thermus aquaticus* [6, 7], isolated from microbial mats in the hot springs of Yellowstone National Park, Montana, USA [8]. Microbial mats have also proved useful in exploring more efficient means for bioremediation of contamination such as aquaculture effluent [9, 10] and mine drainages [11, 12]. While some laminated microbial mats, particularly from ecosystems of specific interest, have been extensively studied, others have largely remained ignored or have been underexplored, including the unique, orange laminated cyanobacterial-bacterial crust endemic to particular habitats of the Hawaiian anchialine ecosystem (Fig. 1b, c).

First defined in 1973, the anchialine ecosystem encompasses near-shore bodies of water with fluctuating volumes and salinities receiving subsurface input of both fresh groundwater and seawater [13–16]. Habitats fitting this description are located primarily in the tropics [13, 14, 17, 16, 18–22] and occur within a variety of basin substrates, including karst caves, cenotes, natural wells and springs, fossilized coral reefs, and coastal basalt (*i.e.*, lava) fields [13, 14, 16]. Notably, the simultaneous influence of fresh- and seawater can result in complex physical and chemical clines within the water column of anchialine habitats [15, 18, 19]. While these habitats have been found to host high levels of species richness and endemism when it comes to macroorganisms [13, 23–29], relatively little work has been done to explore the microbial communities within them [30–34], including the only laminated microbial crust communities (Fig. 1) reported for this ecosystem [35, 36].

Previous microscopy-based studies of the endemic laminated microbial crust communities from the Hawaiian anchialine ecosystem have reported their structure as being comparable to that of

stromatolites, being primarily composed of filamentous cyanobacteria and algae colonized by diatoms and other microorganisms [35, 36]. In stromatolites and other laminated microbial mats, the overall structure can be divided into zones based on physical and environmental properties such as the presence or absence of oxygen and the penetration of light. Typically, starting at the surface to the bottom, there are: 1) an oxic photic zone; 2) an anoxic photic zone, and; 3) an anoxic aphotic zone [3]. In these distinct niches, oxygenic phototrophs and aerobic heterotrophs most often occur near the surface while anaerobic phototrophs such as Chromatiales, Rhodobacterales, and Rhodospirilalles and sulfate-reducing bacteria like Syntrophobacterales occupy the bottom [3, 37–46]. Although Cyanobacteria are thought to be instrumental to mat formation, they typically contribute just 10-20% of the total microbial population [3, 40]. In spite of this, Cyanobacteria are thought to be major contributors to primary production as photoautotrophs, and are also vital to the structure's integrity as contributors of filamentous taxa and secretors of extracellular polymeric substances [3]. The physical complexity provided by Cyanobacteria in combination with the resulting oxygen and light gradients ultimately allows for greater niche diversity [47] that in turn fosters greater species richness further into the structure [41, 43–45, 48].

In the above context, this chapter reports on the diversity, composition, and functional group partitioning among the four discernable layers within the distinct orange laminated cyanobacterial-bacterial crust communities endemic to anchialine habitats within the Cape Kinau and Kona regions of Maui and Hawaii, respectively, in the Hawaiian Islands (Fig. 1). Given the robust island- and site-based distinctions in the whole crust community reported in Chapter 2, it was hypothesized that such distinctions would also occur between analogous layers from different sites and islands. Furthermore, as these orange crusts exhibit a laminated phenotype, it was hypothesized that similar stratifications of taxa and functional groups as found in other laminated microbial mats would be recovered, with concentrations of oxygenic phototrophs and aerobic heterotrophs near the surface and anaerobic and sulfur-cycling organisms at the bottom. This study represents the first to examine the microbial community composition of the different layers present in these orange crusts from select anchialine habitats in Hawaii.

4.3 Materials and Methods

4.3.1 Sites and Sampling

Sampling of orange laminated cyanobacterial-bacterial crust communities occurred within an 8-day span during the spring of 2011 from six anchialine habitats on the islands of Maui and Hawaii (Fig. 1a). On Maui, sites were located at Cape Hanamanioa (HM) and within the Ahihi-Kinau Natural Area Reserve at Skippy's Pond (SKIP) and Queen's Bath (QB). Sites on Hawaii were located at Makalawena Beach ponds #2 and #3 (MAKA2 and MAKA3, respectively) and Kiawaiki Bay (KBI). At all sites, the crust was composed of four distinct layers: a top orange layer (TOL), a second orange layer (2OL), a pink layer (PL), and a bottom green layer (GL) (Fig. 1c). Although all six habitats were open ponds occurring in basalt basins, they differed in degree of impact by invasive fish, goats, and public accessibility (Table 1). Data from the Hawaii Statewide GIS Program was used to identify additional environmental factors, such as aquifer, watershed, and annual rainfall [49, 50], for each site. Whole crust samples were collected from three locations within the habitat per site and preserved in RNALater (Thermo Fisher Scientific, MA, USA) as specified in Chapter 2. Archival samples were also collected, preserved, and submitted to the Hawaiian Anchialine Microbial Repository with The Ocean Genome Legacy (http://www.oglf.org) under accession numbers S23033-S23083 as in Chapter 2. Additionally, ~ 0.25 L of water was filtered per site with a sterile 0.2 μm Sterivex (Millipore, MA, USA) unit before being submitted to the University of Hawaii Hilo Analytical Laboratory for water chemistry analysis. Specifically, dissolved organic carbon (DOC), ammonium (NH_4^+), nitrite (NO_2^-) + nitrate (NO_3^-), total dissolved nitrogen (TDN), orthophosphate (PO_4^{3-}), total dissolved phosphorus (TDP), silica (Si), and salinity were quantified from these water samples.

4.3.2 Sequence Data Generation

Crust samples preserved in RNALater were dissected using sterile razors into their four differently colored layers (Fig. 1c) prior to DNA extraction using MoBio PowerSoil DNA Isolation Kits

(MOBIO, CA, USA) according to Chapter 2. Extracted DNA was shipped to the HudsonAlpha Institute for Biotechnology, Inc. Genomic Services Laboratory (Huntsville, AL, USA) where they were amplified in duplicate by the polymerase chain reaction (PCR) and sequenced on two independent runs of an Illumina HiSeq 2500. Details on the PCR set-up were consistent with those in Chapters 2 and 3. Sequencing was performed on dual-barcoded amplicons to obtain 100 bp paired-end (PE) reads of the V6 and V9 hypervariable regions of small subunit ribosomal DNA (rDNA) genes within the microbial community under examination. Specifically, the V6 hypervariable region of the 16S rDNA was amplified using the *Bacteria*-specific primers 967-985F and 1078-1061R [51] and the V9 hypervariable region of the 18S-rDNA using the *Eukarya*-biased primers 1389F and 1510R [52]. These *Bacteria*- and *Eukarya*-biased primers were selected to detect the greatest proportion of microbial diversity present with a sample. Replicate PCR reactions and sequencing runs were employed to minimize the potential for sample handling errors. All raw high-throughput sequencing reads used in this study are available through the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Accession Number SAMN05833379-SAMN05833402, BioProject ID Number PRJNA325159).

4.3.3 Operational Taxonomic Unit (OTU) Clustering

Alignment of forward and reverse sequencing reads and removal of primers, as well as sequences with uncalled bases, were done in PandaSeq v.2.5 [53]. Reads were further filtered using the FASTQ Quality Filter in the FASTX-Toolkit v.13.2 [54], with a conservative quality score cut-off >30 over at least 75% of the nucleotides in a read. Within the QIIME v.1.8 pipeline [55], USEARCH61 [56] was employed to remove potentially chimeric sequences prior to clustering into operational taxonomic units (OTUs) via UCLUST [56] in the `pick_open_reference_otus.py` script. Sequences were clustered at 95% sequence similarity and 0.005% abundance using the 99% clustered GreenGenes 13.8 [57] and Silva 111 [58] databases as initial references for the *Bacteria* V6 and Eukarya-biased V9 hypervariable regions, respectively. The 0.005% OTU abundance filter was utilized to improve clustering results [59], and the most abundant sequence from each cluster was designated as the reference sequence.

Sequence clusters were then assigned taxonomic identities with megaBLAST v.2.2.26 [60] and the appropriate above-mentioned curated database at a sequence identity of ≥90% and e-value of $1x10^{-6}$. Finally, any OTUs failing to align with default parameters against the appropriate database by PYNAST v.1.2.2 [61] were removed from the final OTU tables.

4.3.4 Analyses of Layer Consortia Composition

Estimates of community richness were calculated as the number of observed OTUs as well as the Shannon [62] and inverse Simpson [63] diversity indices using the package PhyloSeq v.1.10.0 [64] in the R v.3.1.13 statistical environment [65]. Shannon diversity is influenced by the evenness of the distribution of OTUs/taxa such that it quantifies the uncertainty in predicting what group the next sampled sequence would belong to and returns a higher index to reflect greater diversity [62]. In contrast, Simpson diversity is more heavily impacted by the presence of very dominant OTUs/taxa because it measures the probability that two randomly-selected sequences belong to the same group [63]. Taking the inverse of the Simpson index results in a more intuitive interpretation in which greater diversity results in higher indices. These three metrics (*i.e.*, richness, Shannon index, and inverse Simpson index) were used to produced rarefaction curves in R with ten replicates at sequencing depths of 1, 10, 1,000, 10,000, 20,000, and 30,000 sequences/sample to assess the thoroughness of our sampling. Additionally, the three metrics were tested with one-way analysis of variances (ANOVAs) and Tukey's Honest Significant Difference (HSD) post-hoc tests to determine whether layer, island, and presence of introduced fish in the sites influenced these metrics. Introduced fish have been previously documented as inducing shifts toward algal monocultures in the microbial communities in Hawaiian anchialine habitats and were thus expected to decrease richness [25, 36, 66–68]. ANOVAs and Tukey's HSD post hoc tests were performed using the R package agricolae v.1.2.3 [65, 69], with $p<0.05$ considered significant.

The final OTU tables produced by the `pick_open_reference_otus.py` script in the QIIME pipeline were transformed to even sampling depths before the Bray-Curtis dissimilarity metric and Jaccard dissimilarity coefficient were applied in the R package vegan v.2.3.1 [65, 70]. As common

ecological metrics, the Bray-Curtis dissimilarity metric assesses the abundance of OTUs shared between samples [71], while the Jaccard dissimilarity coefficient solely considers the presence or absence of OTUs when estimating the proportion of unshared taxa between samples [72]. Ordinations for the layer consortia were created using non-metric multidimensional scaling (NMDS) plots with 95% confidence ellipses in the R package PhyloSeq v.1.10.0 [64, 65] from the Bray-Curtis dissimilarity and Jaccard dissimilarity data matrices. The envfit function (999 permutations) was utilized in the R package vegan v.2.3.1 [65, 70] to identify environmental variables, including sample site and crust layer, with significant explanatory power (α=0.05) of the sample ordinations. Environmental variables identified as explanatory were overlaid on the ordinations as vectors scaled by explanatory power (r).

Core OTUs, or those present in all samples for a particular category (*i.e.*, layer), were identified using the QIIME v.1.8 pipeline script `compute_core_microbiome.py` [55]. Tables of second-level (*i.e.*, phylum and approximately phylum in GreenGenes and Silva, respectively) and third-level (*i.e.*, class and approximately class in GreenGenes and Silva, respectively) clades were created using the `summarize_taxa_through_plots.py` script in the QIIME v.1.8 pipeline [55]. One-way ANOVAs with Tukey's HSD post-hoc tests were then utilized to identify clades with relative abundances that varied between the layers. Identified clades with varying relative abundances were then classified by performing a literature search for the closest characterized relatives into one of the following metabolic groups: aerobic chemoautotroph, aerobic heterotroph, anaerobic chemoautotroph, anaerobic heterotroph, anaerobic photoautotroph, anaerobic photoheterotroph, fermenter, oxygenic photoautotroph, and parasite. We also utilized PICRUSt v.1.0.0 [73] and Tax4Fun v.1.0.0 [74] to examine the relative abundance of predicted gene families using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [75] and compared those results to the above metabolic groups. The final V6 OTU table was filtered using the QIIME script `filter_otus_from_otu_table.py` to remove *de novo* OTUs and only keep those with GreenGene identifiers [57] before using the PICRUSt prediction workflow. First, the `normalize_by_copy_number.py` script was utilized to account for known and predicted ribosomal

DNA copy number abundance using the GreenGenes database as a reference. After normalization, the `predict_metagenomes.py` script was used to predict functional gene abundance and calculate Nearest Sequenced Taxon Index (NSTI) values. Nearest Sequenced Taxon Index (NSTI) values were also calculated for samples; these values reflect how closely related sample microbes are to sequenced and characterized taxa such that lower values indicate a closer relationship. These scores correspond to percent nucleotide substitutions per site such that a NSTI score of 0.03 corresponds to 97% sequence identity. Tax4Fun requires OTUs created using the Silva database [58]; however, Tax4Fun requires use of a more updated version of the Silva database than Silva 111 for creation of OTUs with Silva identifiers. Therefore, the most updated supported version, Silva 123, was utilized to create closed reference OTUs with 94% sequence similarity before application of Tax4Fun to predict functional gene abundance. Only functions belonging to the KEGG functional gene subgroup "Energy Metabolism" were considered for comparison with the above metabolic groups via one-way ANOVAs with Tukey's HSD post hoc tests to identify potential differences in relative abundance and function between crust layers. All R code, QIIME scripts, and other commands utilized in this study can be downloaded from http://www.auburn.edu/santosr/XXXXXX.htm.

4.4 Results

4.4.1 Data Generation and OTU Clustering

Each of the four phenotypically distinct layers present in crusts from each site were successfully amplified in duplicate for the *Bacteria*-specific V6 and *Eukarya*-biased V9 hypervariable regions via PCR and sequenced with two independent sequencing runs, resulting in 96 samples originating from six habitats. A total of 15,869,028 demultiplexed V6 Illumina reads were obtained in each paired-end direction, averaging ($\bar{x}$) 82,651 reads/sample. For V9, a total of 9,578,684 demultiplexed V9 reads were returned ($\bar{x}$=49,888 reads/sample). Following alignment, quality-filtering, removal of potential chimeric sequences, and abundance filtering, 1,674,332 *Bacteria* V6 reads (an 89.4% reduction overall, $\bar{x}$=17,440 reads/sample) and 1,281,037 *Eukarya*-biased V9 reads (an 86.6% reduction overall, $\bar{x}$=13,344

reads/sample) remained. Such stringent filtering parameters were utilized to reduce the noise-to-signal ratio as well as mitigate potential issues due to the short read lengths produced by Illumina instruments. Reads from the V6 and V9 regions ranged in length from 69-80 bp ($\bar{x}$=75 bp) and 65-162 bp ($\bar{x}$=120bp), respectively. PYNAST identified two V6 and ten V9 OTUs as failing to align and these were subsequently removed from the final datasets. Of the 1,222 V6 OTUs in the final dataset, 132 (181,034 reads, 11.5% of total) were not assigned taxonomic identities using the GreenGenes 13.8 database [57] while 12 of the final 1,083 V9 OTUs (10,283 reads, 0.85% of total) were not assigned identities using the Silva 111 database [58]. When compared to NCBI's GenBank repository [75] using BLASTN v.2.3.0 [60], the unassigned V6 OTUs primarily revealed affiliations with uncultured members of the Acidobacteria, Actinobacteria, Alphaproteobacteria, Bacteroidetes, candidate division NC10, Chlamydiae, Chlorobi, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Deltaproteobacteria, Firmicutes, Mollicutes, Planctomycetes, and Verrucomicrobia at low e-values (data not shown). The 12 V9 OTUs which were not assigned taxonomies most closely matched members of Ciliophora, Amoebozoa, Bacillariophyceae, Alphaproteobacteria, and uncultured Archaea in the GenBank repository, also at low e-values (data not shown). Seven V6 OTUs (0.34% of total) were identified as most likely originating from eukaryotic chloroplasts of haptophytes and/or stramenopiles.

4.4.2 Analyses of Layer Consortia Composition

Samples of the analogous (*i.e.*, same position and color) crust layer from the same site were combined for most downstream analyses because they were most similar to each other regardless of which PCR or sequencing reaction data were generated from (data not shown). At 30,000 reads/sample, OTU richness did not saturate for either the V6 or V9 datasets while both the Shannon and inverse Simpson diversity indices appeared saturated at sampling depths less than or equal to 10,000 reads/sample (Appendix 5). Comparison of alpha diversity metrics between V6 samples grouped by crust layer identified the bottom-most green layer as having the greatest OTU richness while the top orange layer had the least ($F_{3,92}$=3.11, p=0.030) (Fig. 2a). However, no significant differences in Shannon or inverse

Simpson diversities were found between these layers. In the *Eukarya*-biased V9 data, a trend toward greater OTU richness was observed in the bottom green vs. top orange layers, though it was not significant at $p \leq 0.05$ ($F_{3,92}=2.27$, $p=0.085$) (Fig. 2b). Additionally, significantly greater V6 and V9 OTU richness was observed in samples from Maui than in those from Hawaii ($F_{1,94}=102$, $p<<0.001$ and $F_{1,94}=235$, $p<<0.001$, respectively) (Fig. 2a). While the V6 samples from Maui also had a significantly greater Shannon diversity than those from Hawaii ($F_{1,94}=28.0$, $p<<0.001$), island did not have a significant impact on inverse Simpson diversity for either the *Bacteria* V6 or *Eukarya*-biased V9 datasets (Fig. 2). Presence of fish, regardless of species, also was linked to a reduction in OTU richness compared to fishless habitats in both V6 and V9 datasets ($F_{3,92}=34.4$, $p<<0.01$ and $F_{3,92}=78.3$, $p<<0.01$, respectively). The presence of fish also impacted V6 and V9 Shannon diversity and V9 inverse Simpson diversity; for Shannon diversity, sites without fish and those with tilapia (*Oreochromis* spp.) tended to have greater diversity than those with only poeciliid guppies (*Poecilia* spp. and *Gambusia affinis*) and those with poeciliid guppies and marine fish (V6: $F_{3,92}= 10.3$, $p<<0.01$ and V9: $F_{3,92}=14.6$, $p<<0.01$). With regard to inverse Simpson diversity, impact of fish had no effect in the V6 dataset ($F_{3,92}=1.75$, $p=0.16$). However, sites with tilapia or without any fish had greater V9 inverse Simpson diversity than sites with only poeciliid guppies or with poeciliids and marine fish ($F_{1,94}=14.2$, $p<<0.01$). Sites visited by goats had greater OTU richness in both V6 and V9 datasets ($F_{1,94}=17.7$, $p<<0.01$ and $F_{1,94}=21.8$, $p<<0.01$, respectively) but lower *Eukarya*-biased V9 Shannon and inverse Simpson diversity ($F_{1,94}=18.1$, $p<<0.01$ and $F_{1,94}=24.6$, $p<<0.01$, respectively).

Minimal differences were observed between NMDS ordinations generated from the abundance-based Bray-Curtis dissimilarity metric or the binary Jaccard dissimilarity coefficient. Given this, only the Bray-Curtis ordinations for the V6 (Fig. 3a) and the V9 (Fig. 3b) data are presented and discussed. Crust layer samples primarily grouped by island, such that those from all Maui sites clustered separately from the Hawaii sites (Fig. 3). Within these island-specific clusters, samples were further grouped into site-specific clusters that included all four layers from their laminated cyanobacterial-bacterial crust (Fig. 3). While samples from the analogous layer were distinct on a site-specific basis, there was no clear pattern

in the arrangement of different layers within a site (Fig. 3). With the exception of originating layer within the crust, all categorical and continuous environmental variables were explanatory of the NMDS ordination at $p<0.05$ (Appendix 6), with site accounting for the most variation among the categorical variables (V6 $r^2=0.95$, V9 $r^2=0.95$) and salinity accounting for the most among the continuous variables (V6 $r^2=0.96$, V9 $r^2=0.96$).

Examination of the relative abundance of *Bacteria* phyla, proteobacterial classes (due to the great metabolic and physiological diversity within Proteobacteria), and *Eukarya* second-level (approximately phyla) clades between the four layers revealed that all shared the same clades, but in different abundances (Fig. 4b and c). For example, while Cyanobacteria were present in all four layers, they were most abundant in both orange layers at the top of the crust and in the bottom green layer (Fig. 4b). The ability of some cyanobacterial taxa to produce heterocysts, which would facilitate nitrogen fixation in oxygenic environmental conditions, did not appear to influence their distributions among the various layers. Additionally, though algae were ubiquitous in all four layers, they were most abundant in the second orange layer and least abundant in the top orange layer (Fig. 4c). Bacteroidetes was most abundant in the top orange layer and decreased in abundance with increasing depth into the crust (Fig. 4b). Gammaproteobacteria also increased in abundance with increasing depth in the crust in both V6 and V9 datasets (Fig. 4b, c), while differing patterns were observed for the members of Alphaproteobacteria. Specifically, alphaproteobacterial taxa detected by the *Bacteria*-specific V6 primers increased in abundance with increasing crust depth while those detected with the *Eukarya*-biased V9 primers generally decreased with increasing depth (Fig. 4b, c).

As previously noted, oxygenic photoautotrophs like the Cyanobacteria exhibited greater relative abundance in the *Bacteria* V6 dataset within the two orange layers as well as the bottom green layer (Fig. 5a). A peak in aerobic heterotrophs and anaerobic photoautotrophs in the V9 *Eukarya*-biased data (Fig. 5b) also further characterized the second orange layer. These aerobic heterotrophs were primarily members of the Alpha- and Gammaproteobacteria, Gemmatimonadetes, and Verrucomicrobia, and anaerobic photoautotrophs belonging to the gammaproteobacterial Chromatiales. An increased

abundance of fermenters characterized the pink layer in both datasets (Fig. 5a, b), as well as an increased abundance of aerobic heterotrophs and anaerobic photoautotrophs in the *Bacteria* V6 data (Fig. 5a). Fermenters in both datasets belonged to the groups Acidobacteria, Chloroflexi, Fibrobacteres, Firmicutes, Fusobacteria, Lentisphaerae, Planctomycetes, Beta-, Delta-, and Gammaproteobacteria, Spirochaetes and Verrucomicrobia. Aerobic heterotrophs in the *Bacteria* V6 belonged to the Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Planctomycetes, Alpha- and Gammaproteobacteria, Deinococcus-Thermus, and Verrucomicrobia. Anaerobic photoautotrophs in the V6 data belonged to Chloroflexi and the gammaproteobacterial Chromatiales. Finally, the green layer had the greatest relative abundance of anaerobic photoheterotrophs, members of the alphaproteobacterial Rhodospirillales (Fig. 5b), together with many fermenters and oxygenic photoautotrophs (Fig. 5a and b).

Among the numerous cladal constituents listed above, a core set of *Bacteria* V6 and *Eukarya* V9 OTUs were identified as being present in each of the four Hawaiian anchialine cyanobacterial-bacterial crust layers (Appendix 7). Each layer consortium shared 18-26 OTUs in each of the V6 and V9 datasets; however, due to some OTUs belonging to core consortia for more than one layer, there were only 49 and 38 unique core V6 and V9 OTUs, respectively. Seventeen OTUs, 9 among the V6 and 8 in the V9 datasets, were observed in all four core consortia and were identified as members of Alphaproteobacteria, Bacteroidetes (Saprospiraceae), Cyanobacteria, Firmicutes (Carnobacteriaceae), Planctomycetes (Pirellulaceae), and Ciliophora (*Metanophrys sinensis*). Alphaproteobacterial OTUs were identified as members of BD7-3, Rhizobiales, Rhodobacterales, Rhodospirillales, and Sphingomonadales. One of the two core cyanobacterial OTUs identified in all four layer consortia was not assigned any taxonomic levels below Cyanobacteria and the other was identified as *Halomicronema*. The top orange layer was the only core consortium to contain a member of the Euglenozoa while the pink layer was unique in containing an actinobacterial OTU. Only the green layer consortium contained a gammaproteobacterial OTU, and Deltaproteobacterial OTUs were only identified as core consortia members in the bottom three layers (*i.e.*, 2nd orange layer, pink layer, and green layers).

Six hundred and thirty-three *de novo* OTUs were removed from the final V6 data, leaving 589 OTUs for the PICRUSt analysis. Nearest Sequenced Taxon Index (NSTI) values for our samples ranged from 0.11-0.24 ($\bar{x}$=0.17±0.03). Tax4Fun was run on 3,233 Silva OTUs. Of the eight energy metabolism functions identified in the PICRUSt KEGG analysis, six had relative abundances that differed between the four crust layers, with greater abundance correlating with depth into the crust (Fig. 6a). Energy metabolism functions that did not differ in abundance between the layers included photosynthesis and photosynthesis-antenna proteins. On the other hand, sulfur metabolism ($F_{3,87}$=5.87, p<0.01), oxidative phosphorylation ($F_{3,87}$=4.97, p<0.01), nitrogen metabolism ($F_{3,87}$=6.40, p<0.01), methane metabolism ($F_{3,87}$=5.209, p<0.01), carbon fixation pathways in prokaryotes ($F_{3,87}$=5.693, p<0.01), and carbon fixation pathways in photosynthetic organisms ($F_{3,87}$=4.264, p<0.01) were all most abundant in the green layer and pink layer than the top orange layer. In contrast, of the eight energy metabolism functions identified in the Tax4Fun KEGG analysis, six had relative abundances that differed between the four crust layers (Fig. 6b). Photosynthesis-antenna proteins and methane metabolism did not differ in relative abundance between layers in the Tax4Fun data. Sulfur metabolism ($F_{3,87}$=12.90, p<0.01), photosynthesis ($F_{3,87}$=2.765, p=0.04), oxidative phosphorylation ($F_{3,87}$=4.29, p<0.01), nitrogen metabolism ($F_{3,87}$=5.730, p<0.01), carbon fixation pathways in prokaryotes ($F_{3,87}$=7.901, p<0.01), and carbon fixation in photosynthetic organisms ($F_{3,87}$=17.694, p<0.01) did differ in relative abundance between the four layers. While all of the PICRUSt KEGG pathways that differed between layers were more abundant in the green and pink layer, only carbon fixation in photosynthetic organisms and photosynthesis were most abundant in the green and pink layer in the Tax4Fun data. The other pathways were more abundant in the top orange layer and least abundant in the green and pink layers.

4.5 Discussion

4.5.1 Comparison of Layer Consortia Diversity

While previous studies of the endemic cyanobacterial-bacterial crusts found in anchialine habitats of the Cape Kinau and Kona coast regions of Maui and Hawaii, respectively, examined whole samples

[35, 36], this is the first work to specifically target the individual layers composing these unique crusts. Rarefaction analyses suggest that while sampling depth may have failed to recover all of the microbial richness in these consortia, the taxa that contribute to diversity were captured in this study (Appendix 5). The recovery of greater OTU richness at the bottom of these crusts (Fig. 2) aligns well with previous work from laminated microbial mats, including those found in hypersaline environments [41, 44, 45, 48] and salt marshes [43]. Although laminated microbial mats or cyanobacterial-bacterial crusts have not been described from other habitats consistent with the anchialine definition, increasing microbial richness and abundance has been observed with increased depth in this ecosystem [22, 76]. Due to their seawater and freshwater influences, increasing depth results in dynamic environmental conditions in anchialine habitats [13–16, 18, 19], thus allowing different taxa to occupy niches meeting their metabolic needs. Similarly, microbial mats and crusts can create and maintain many niches that can compress high levels of microbial diversity into a relatively small spatial distance [3, 47]. Greater richness was also recovered from samples collected on Maui compared to Hawaii (Fig. 2); however, the apparent impacts of invasive fish, goats and humans on microbial richness may explain these island-based differences. For example, higher OTU richness, but not diversity, was correlated with feral goats frequenting all of the sampled habitats on Maui and the two Makalawena sites on Hawaii to obtain drinking water from the pools. Furthermore, all of the sampled habitats on Hawaii impacted by some combination of tilapia, poeciliid guppies, and marine fish species, and the presence of any type of fish was found to have a negative impact on OTU richness. It is possible that goats on Maui introduce microbes into the anchialine habitats they visit that have little impact on diversity (*i.e.*, rare taxa) but inflated richness while fish (and potentially humans) reduced microbial richness (and diversity in the case of poeciliid guppies) on Hawaii through a variety of mechanisms. Indeed, previous studies investigating the impacts of poeciliids on Hawaiian anchialine habitats have noted reduced abundances and grazing by the endemic atyid shrimp *H. rubra* [66, 67, 77, 78] that precipitates shifts in the microbial crust community composition towards an algal-monoculture-dominated benthos [66, 77, 79]. As the algal-dominated benthos lacks the niche

diversity present in the laminated crust, decreased microbial richness and diversity would be an expected outcome in such habitats.

Although a relationship between OTU richness and layer was observed, NMDS ordinations using the binary Jaccard dissimilarity coefficient (data not shown) and abundance-based Bray-Curtis dissimilarity metric were not structured or easily interpreted by the layer of sample origin (Fig. 3). However, the island- and site-based distinctiveness in whole crust communities previously observed in Chapter 2 was maintained when examined at the level of the crust's individual layers (Fig. 3). Furthermore, salinity exhibited a strong explanatory power in the ordinations, consistent with previous results of its impacts on benthic and water column microbial communities across the spatial and environmental range of Hawaiian anchialine habitats in Chapter 2. This is not surprising since salinity has been shown to be influential in structuring microbial communities of high-altitude Tibetan lakes [80, 81] and riverine bacterioplankton communities [82], and was previously identified as a likely determinant of the dominant algal and cyanobacterial taxa of anchialine habitats on Cape Kinau, Maui [35]. The lack of clustering difference between the binary Jaccard and abundance-based Bray-Curtis ordinations suggests differences between sites, islands, and samples were not due to abundance-based differences of shared OTUs but were most likely due to both different OTU memberships and differences in abundances of shared OTUs. Both datasets included ~9 OTUs which were present in all 96 samples and ~9 OTUs that were present in only a few samples, implying most sample distinctions were due to differing OTU memberships.

4.5.2 Comparison of Layer Consortia Composition and Metabolism

Though present throughout all four layers, oxygenic phototrophs, in the form of Cyanobacteria, were observed to have the greatest relative abundances in both the top and bottom of the crust (Fig. 4b, 5a). Specifically, OTUs identified as *Halomicronema*, a nonheterocystous filamentous cyanobacterium previously observed in laminated mats [38, 83], was ubiquitous to every sample. While the abundance of Cyanobacteria at, and near, the surface of the mat is consistent with laminated cyanobacterial-bacterial

mats [3, 37–40, 43–46, 48, 84], the finding of an almost equal abundance of Cyanobacteria at the bottom of crusts such as these has not been previously documented. In addition, V6 OTUs identified as algal chloroplasts and algal V9 OTUs were more abundant in the pink and green layers than the top orange layer, with the exception of two Haptophyta V9 OTUs which were more abundant in the top orange layer. Although the two studies previously examining these orange cyanobacterial-bacterial crusts compared them to stromatolites [35, 36], stromatolites and most laminated mats exhibit strict oxygen gradients with anoxic conditions at the bottom of the mat [3, 37, 38, 40, 44–46, 84], which is in contrast to the microbial community composition reported here. One reason for this is that the orange cyanobacterial-bacterial crust in Hawaii's anchialine ecosystem only loosely adheres to the benthic substrate and often grows in shelves and protuberances unattached to the substrate below (pers. obs.), which is distinctive relative to other laminated mats. Furthermore, seawater typically enters an anchialine habitat through the porous basin, thus potentially providing oxygenation to the bottom of the mat, and may be aided in circulation by the pockets of water between the crust structure and the substrate and cracks and holes in the crust itself. Despite imperfect dissection of layers, the abundance of Cyanobacteria and algae in the bottom green layer was unlikely to be an artifact of contamination as previous light microscopy of such crusts identified the green color of the bottom layer as due to the presence of Cyanobacteria and green algae [35]. Thus, the orange laminated cyanobacterial-bacterial crust present in anchialine habitats in the Cape Kinau and Kona coast regions of Maui and Hawaii seem to be unique in that the top and bottom of the crusts appears oxygenated, in contradiction to the initially proposed hypothesis.

Given the apparent occurrence of oxic zones at the surface and bottom of the crust, increased abundances of anaerobic taxa were observed in the middle two layers, phenotypically identifiable as a second orange and a pink layer (Fig. 5 and 6). Heterotrophy was also concentrated in the middle two layers and chemoautotrophy to the lower three, although chemoautotrophs were primarily concentrated in the pink layer (Fig. 6). Many chemoautotrophs require anoxygenic conditions, implying appropriate niches were concentrated in the pink layer. In contrast, a laminated mat community from Kiritimati Atoll was found to exhibit increased abundance of aerobic heterotrophs in the oxic layers near the top that

decreased as fermenters increased in the transitional center layers, with relatively low abundances of either at the bottom anoxic layers where chemoautotrophs were localized [45]. Whereas typical laminated mats exhibit vertical gradients that stratify anaerobic metabolic niches across the lower layers of the mat, the orange crust communities examined here appear to concentrate anaerobic conditions in the center of the structure. Consistent with this is the enrichment within the lower two layers (*i.e.*, pink and green layers) for PICRUSt sulfur, nitrogen, and methane metabolism pathways approximately corresponding with the observed increase in chemoautotrophic taxa in the pink layer. In direct contrast, Tax4Fun identified sulfur and nitrogen metabolism pathways as enriched in the top orange layer over the bottom green layer. Functional pathways belonging to photosynthetic organisms, however, were found to exhibit contradictory patterns with the bottom two layers being enriched for carbon fixation in photosynthetic organisms in both PICRUSt and Tax4Fun and photosynthesis in Tax4Fun, while there was no difference between layers for pathways involved with photosynthesis, photosynthesis proteins, or photosynthesis antenna proteins. Such contradictions serve to highlight the limitations of PICRUSt and Tax4Fun when examining environmental samples, particularly those from poorly-characterized habitats that may contain novel taxa. The NSTI scores for the samples presented in this study were relatively large and indicated PICRUSt results may not be accurate. Indeed, Langille et al. [73] found that PICRUSt analysis accuracy decreased as NSTI scores increased with greatest predictive accuracy for samples with mean NSTI scores <0.05 and approximately 50% accuracy for samples with NSTI scores comparable to those found here. Tax4Fun utilizes only OTUs with close relatives in the Silva database and characterized KEGG pathways, making it a more conservative approach than PICRUSt, but the identification of nitrogen and sulfur metabolism enrichment in the upper-most layer suggests prediction of functional pathways using short 16S rDNA sequences remains of limited value.

4.5.3 Concerns Regarding Conservation of Hawaiian Anchialine Habitats

Maintenance of the unique laminated crust instead of algal monocultures has been attributed to the endemic atyid shrimp, *H. rubra*, acting as a keystone grazer [36, 66, 67, 25, 68]. The introduction of

poeciliids and tilapia, however, induces diel migratory behavior as a predator-avoidance strategy in *H. rubra* [66, 77, 78, 85, 86], which results in changes in the biomass and productivity of the microbial community and increases overall habitat nutrient load [2, 66]. In this study, introduced fish, regardless of species, were identified as having a negative impact on the richness of the orange laminated crust community, supporting the idea that alterations in the abundance and behavior of *H. rubra* due to fish presence has negative impacts on the stability of these crusts [66, 77, 85–88]. *Halocaridina rubra* also faces predation by the invasive shrimp *Macrobrachium lar*, which also results in altered *H. rubra* behavior and abundance [89] that might have additional impacts. Additionally, projected increases in sea level due to climate change are expected to exacerbate the situation by providing fish and other invasive species access to a greater proportion of Hawaiian anchialine habitats than are currently impacted [87]. A further threat to these unique habitats is from coastal development that both destroys entire habitats and degrades those nearby through non-point source pollution [79, 90, 91]. Though a number of the endemic shrimp species found in Hawaii's anchialine ecosystem are listed as candidates for protection with the State of Hawaii and the federal government (http://dlnr.hawaii.gov/wildlife/files/2013/09/Fact-Sheet-anchialine-shrimps.pdf), no conservation efforts currently exist to protect or preserve the unique *Bacteria* and micro-*Eukarya* diversity present within these ecosystems.

Though the advancement of sequencing technologies enabled this study to examine the unique consortia present in the colored layers of the orange crust endemic to particular Hawaiian anchialine habitats, it is recognized that this approach is limited in its accuracy and that future work is necessary to understand how these microbial communities function. A conservative approach was adopted to compensate for the limited taxonomic resolution resulting from relatively short length of Illumina sequence reads as well as the fact that identifications were limited to rDNA genes. However, this removed both potentially novel taxa in OTUs that failed to align with the reference databases as well as rare OTUs that may represent the controversial 'rare biosphere' [84, 92, 93]. Additionally, the great metabolic and physiological diversity present in *Bacteria*, evenly among closely related taxa, limits the accuracy at which predictions can be made on the functional role these organisms fill in this ecosystem.

Measurement of chemical gradients in the crust, more precise dissection or separation of the colored layers coupled with culture-based, and/or genomic sequencing may provide a better understanding of niche partitioning in the crust, which taxa are present in each layer, and what metabolic and functional roles those taxa fill.

4.5.4 Conclusions

Comparison of microbial consortia from the four colored layers present in the endemic orange laminated cyanobacterial-bacterial crust of the Cape Kinau and Kona coast regions of Maui and Hawaii revealed that both the top and bottom of the crust appeared oxygenated, in direct contrast with typical laminated microbial mats where oxygen is depleted at the bottom of the mat. Supporting this, anaerobic and chemoautotrophic taxa were found to be concentrated in the middle two layers. Furthermore, introduced fish, regardless of species, appear to negatively impact taxonomic richness of microbes in these laminated crusts. We advise that future efforts examine the chemical gradients present within the mat and incorporate techniques with more accurate identification of metabolism and physiology to better understand the niche partitioning within this unique microbial community. Finally, any conservation plans to preserve the Hawaiian anchialine ecosystem should consider the unique orange laminated crust community and the *Bacteria* and micro-*Eukarya* diversity within it.

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conducted in this study comply with current laws of the United States and the State of Hawaii. Funding support for this work came from the National Science Foundation (DEB #0949855 to S.R.S).

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Table 1. Sampled anchialine habitats from the islands of Maui and Hawaii and their corresponding categorical and continuous environmental factors. Site abbreviations: HM – Hanamanioa, Maui; QB – Queen's Bath, Maui; SKIP – Skippy's Pond, Maui; KBI – Keawaiki Bay, Hawaii; MAKA2 – Makalawena Beach, Pond 2, Hawaii; MAKA3 – Makalawena Beach, Pond 3.

Categorical Environmental Factors	**Sites**					
	HM	**QB**	**SKIP**	**KBI**	**MAKA2**	**MAKA3**
Island	Maui	Maui	Maui	Hawaii	Hawaii	Hawaii
Fish	No Fish	No Fish	No Fish	Tilapia	Poeciliids	Poeciliids & Marine
Goats	Yes	Yes	Yes	No	Yes	Yes
Open to Public	No	No	No	Yes	Yes	Yes
Aquifer [49]	Kahikinui	Central	Kahikinui	Hualalai	Hualalai	Hualalai
Watershed [49]	Kanaio	Ahihi Kinau	Ahihi Kinau	Kiholo	Kiholo	Kiholo
Potential Warm Groundwater [49]	Yes	Yes	Yes	No	No	Yes
Continuous Environmental Factors						
Latitude	20.58	20.6	20.6	19.89	19.79	19.79
Longitude	156.41	156.43	156.42	155.9	156.03	156.03
Annual Rainfall (mm) [49, 94]	364.7	363.9	366.4	242.9	320.4	320.4
Mean Annual Solar Radiation (Watts/m^2) [49]	216.6	193.5	196.2	226.5	226.5	224.8
Salinity (ppt)	15	21	15	5	7	7
Nitrite and Nitrate (NO_2+NO_3, µM)	38.8	40.1	27.9	66.4	67.8	75.6
Orthophosphate (PO_4, µM)	1.17	0.54	1.01	1.06	7.06	7.89
Silica (Si, µM)	352.0	341.1	398.3	601.9	714.1	669.2
[1] Ammonium (NH_4, µM)	2.52	ND	2.68	1.69	2.33	2.06
Dissolved Organic Carbon (DOC, µM)	96.5	70.1	45.3	81.6	78.0	25.9
Total Dissolved Nitrogen (TDN, µM)	47.57	44.8	29.1	70.7	75.9	78.3
Total Dissolved Phosphorus (TDP, µM)	1.3	0.7	1.1	1.1	7.4	7.9

1 Not detectable (ND) <1.00 µM

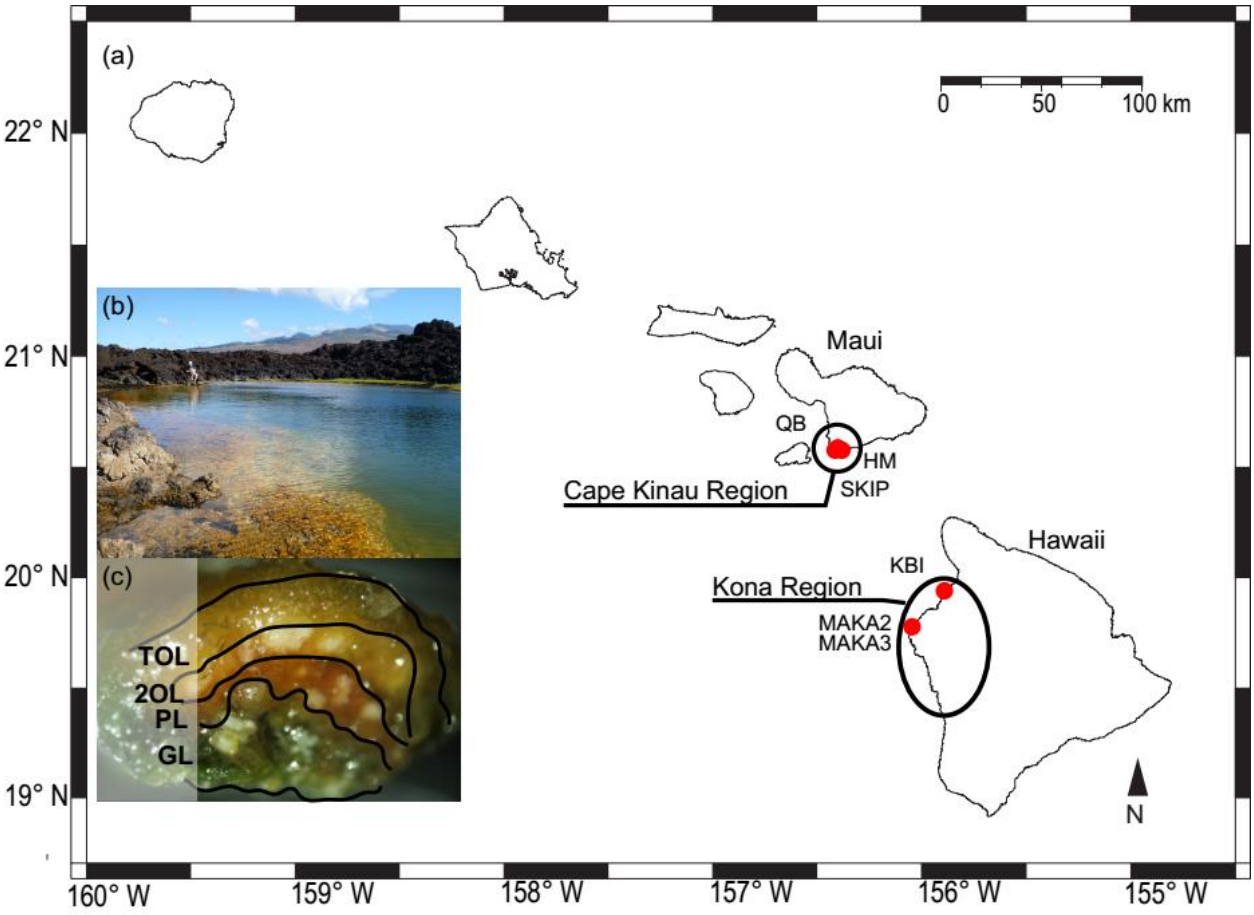


Fig. 1. a Map depicting sampling sites of anchialine habitats on the islands of Maui and Hawaii with the regions of Cape Kinau, Maui and Kona, Hawaii indicated (open circles). **b** Example of a Hawaiian anchialine open pool habitat (*i.e.*, site SKIP) with the orange cyanobacterial-bacterial crust found in Cape Kinau, Maui and Kona, Hawaii. **c** Close-up of laminated orange cyanobacterial-bacterial crust with four layers found exclusively in the Cape Kinau region of Maui and the Kona region of Hawaii. In order from top to bottom, the layers are top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL).

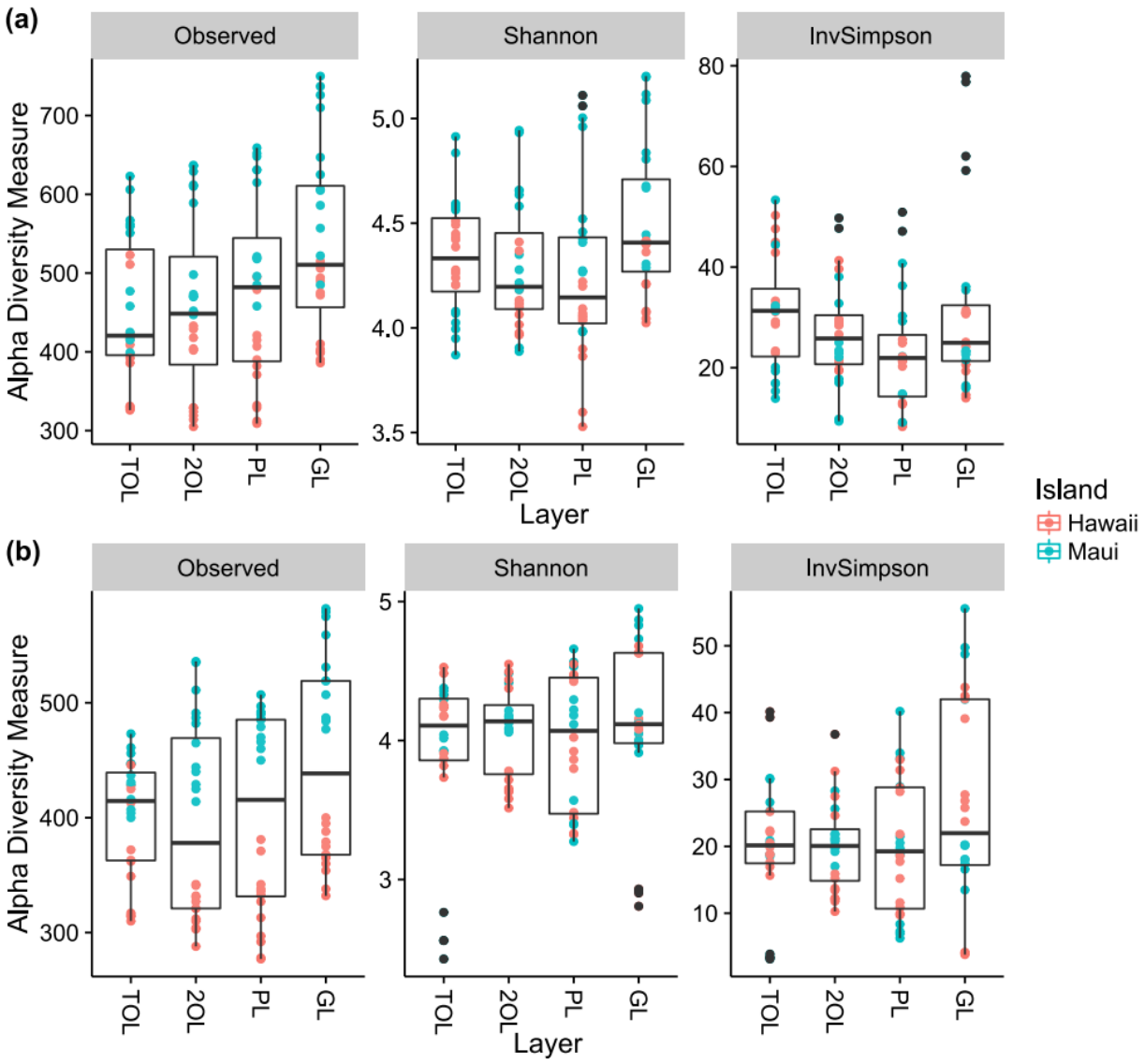


Fig. 2. Diversity estimates, specifically number of observed OTUs, Shanon diversity, and Inverse Simpson diversity, of the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene **(a)**, and the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene **(b)**. Samples were colored by island of origin and grouped by layer within the laminated orange cyanobacterial-bacterial crust. In order from top to bottom, the layers are top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL).

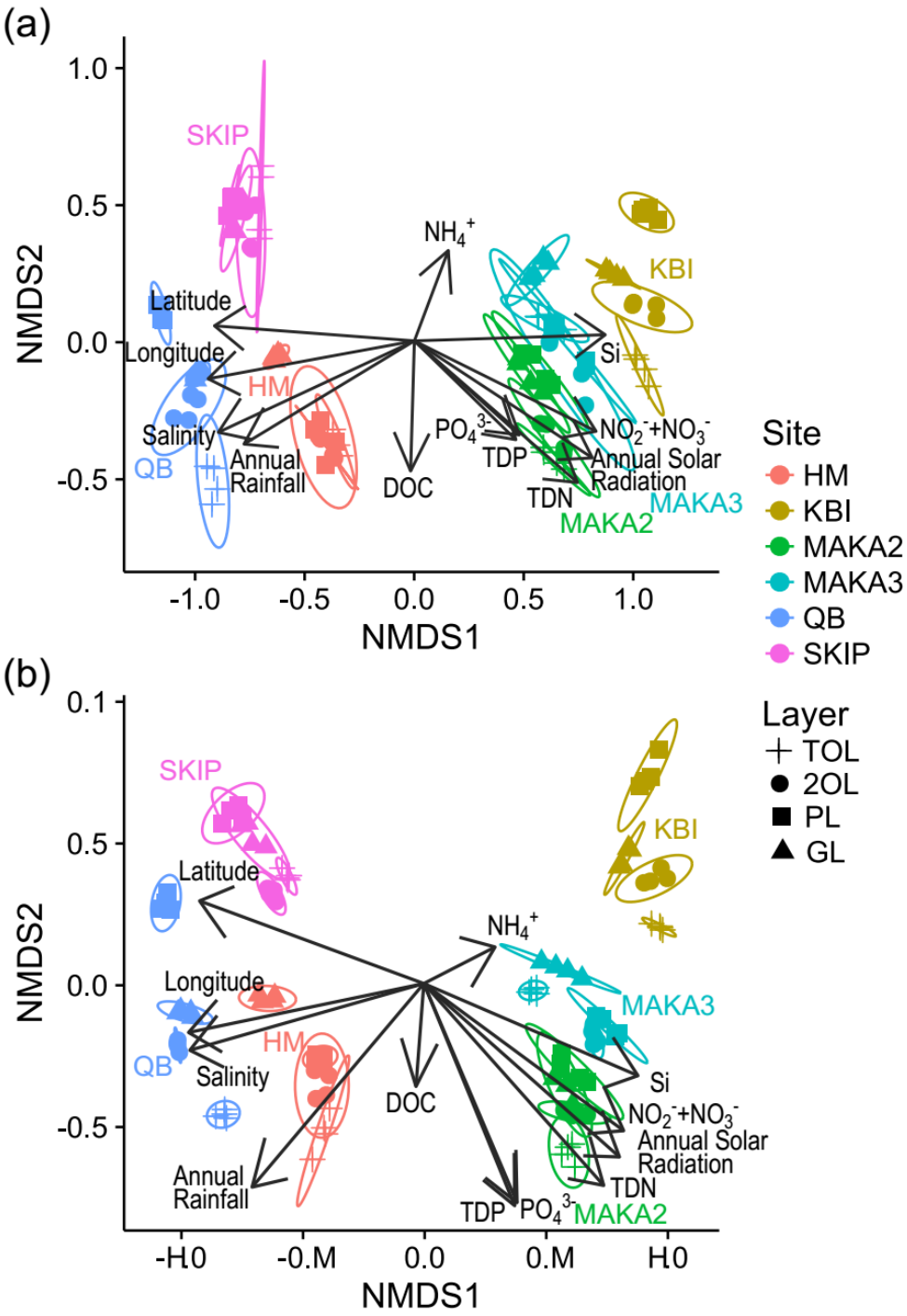


Fig. 3. Non-metric Multidimensional Scaling (NMDS) ordination using the Bray-Curtis Dissimilarity Index of samples grouped by crust layer within anchialine sites that were surveyed. In order from top to bottom, the layers are top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL). Environmental factors were fitted to and overlaid on ordinations, and represent

environmental gradients in ordination space. **a** Samples generated using the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene. **b** Samples generated using the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene.

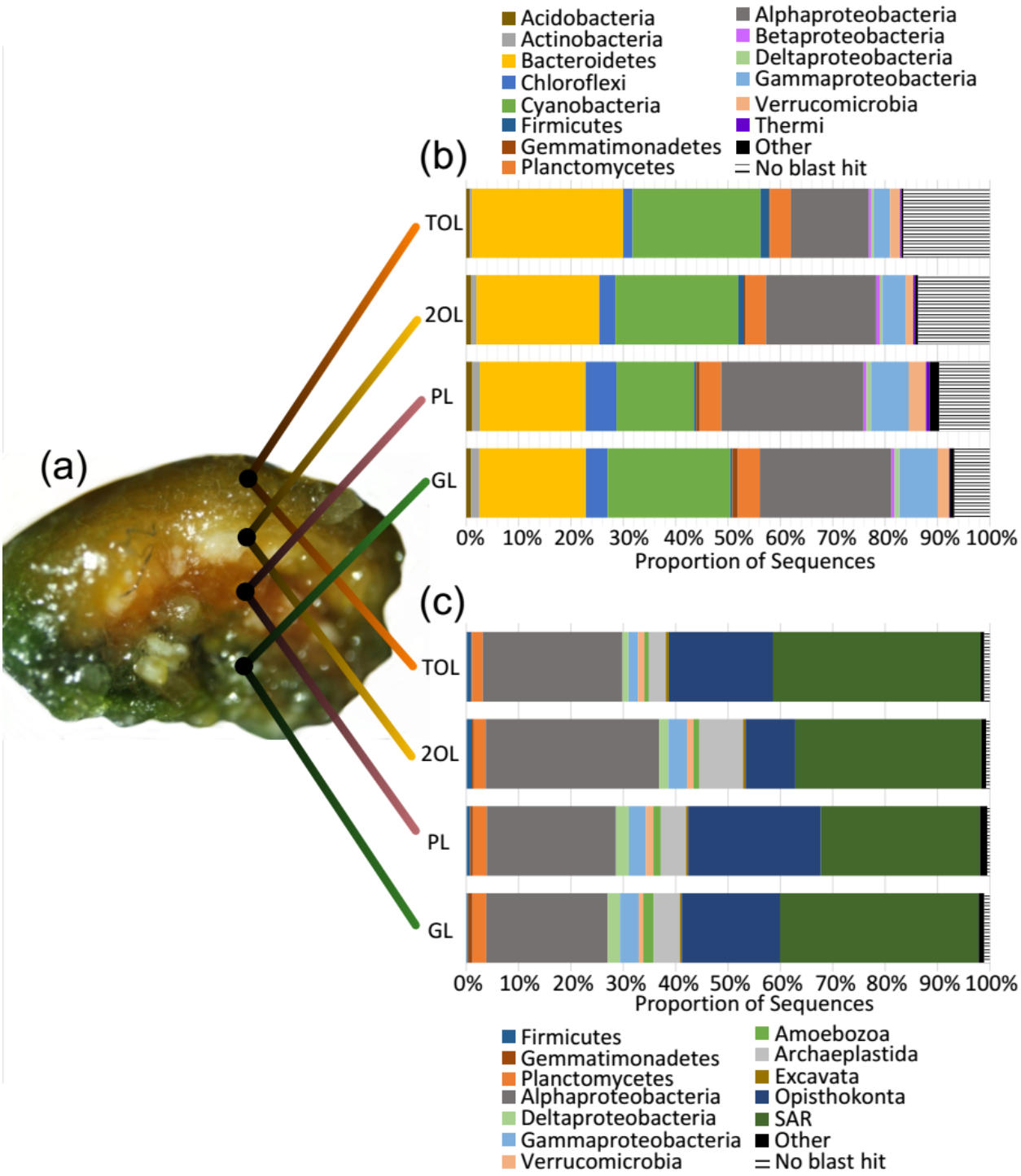


Fig. 4. Relative abundance of taxa identified in samples grouped by layer within the crust. Taxa present at less than 0.5% relative abundance in every layer were summarized in the artificial group "Other." **a** In order from top to bottom, the layers are top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL). **b** Bacterial phyla and proteobacterial classes identified by the *Bacteria-*

specific V6 hypervariable region of the 16S-rDNA gene using the GreenGenes 13.8 database. The artificial "Other" category included Armatimonadetes, BRC1, Chlamydiae, Chlorobi, FCPU426, Fibrobacteres, GOUTA4, Lentisphaerae, NKB19, Nitrospirae, OP3, OP8, Other Proteobacteria, Zetaproteobacteria, Spirochaetes, and WS6. **c** Approximately phyla-level bacterial clades, proteobacterial classes, and approximately phyla-level eukaryotic clades identified by the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene using the Silva 111 database. The artificial "Other" category included Acidobacteria, Actinobacteria, Bacteroidetes, Candidate division OD1, Chloroflexi, Fibrobacteres, Fusobacteria, Lentisphaerae, Nitrospirae, Betaproteobacteria, Other Proteobacteria, Cryptophyceae, Haptophyta, Incertae_Sedis (Protista), and Kathablepharidae.

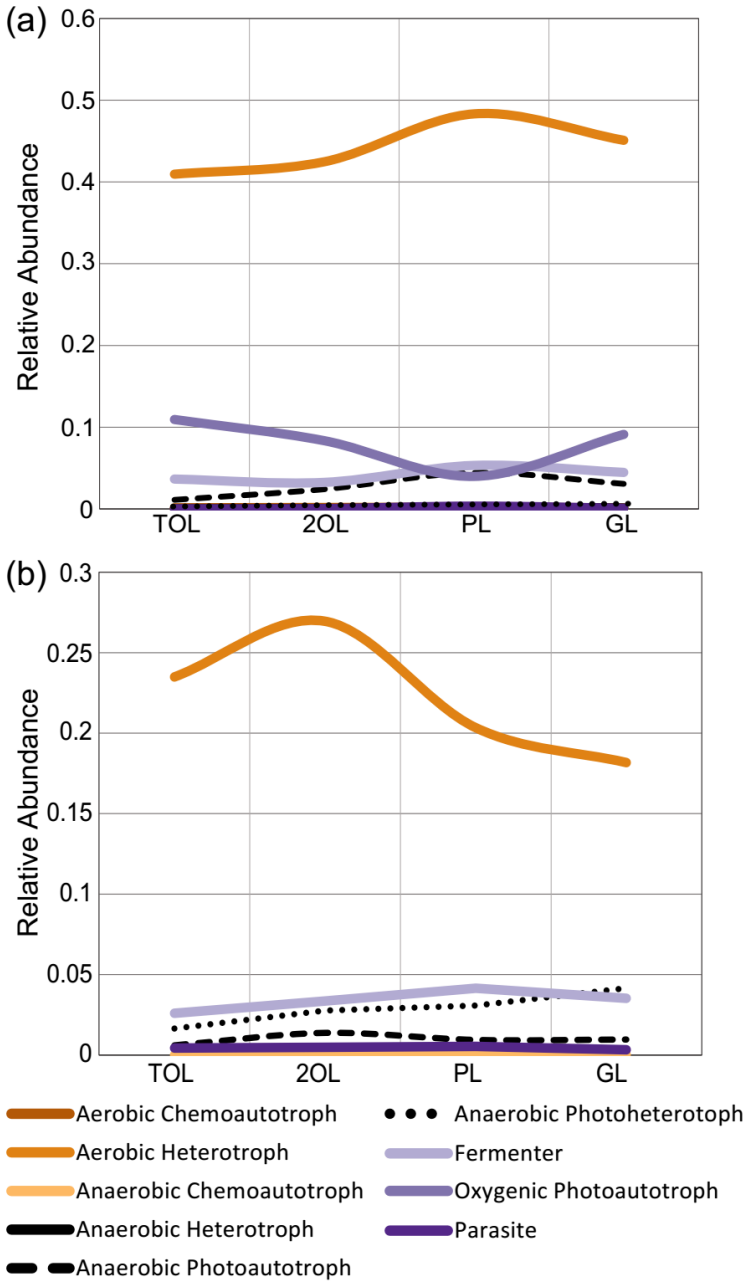
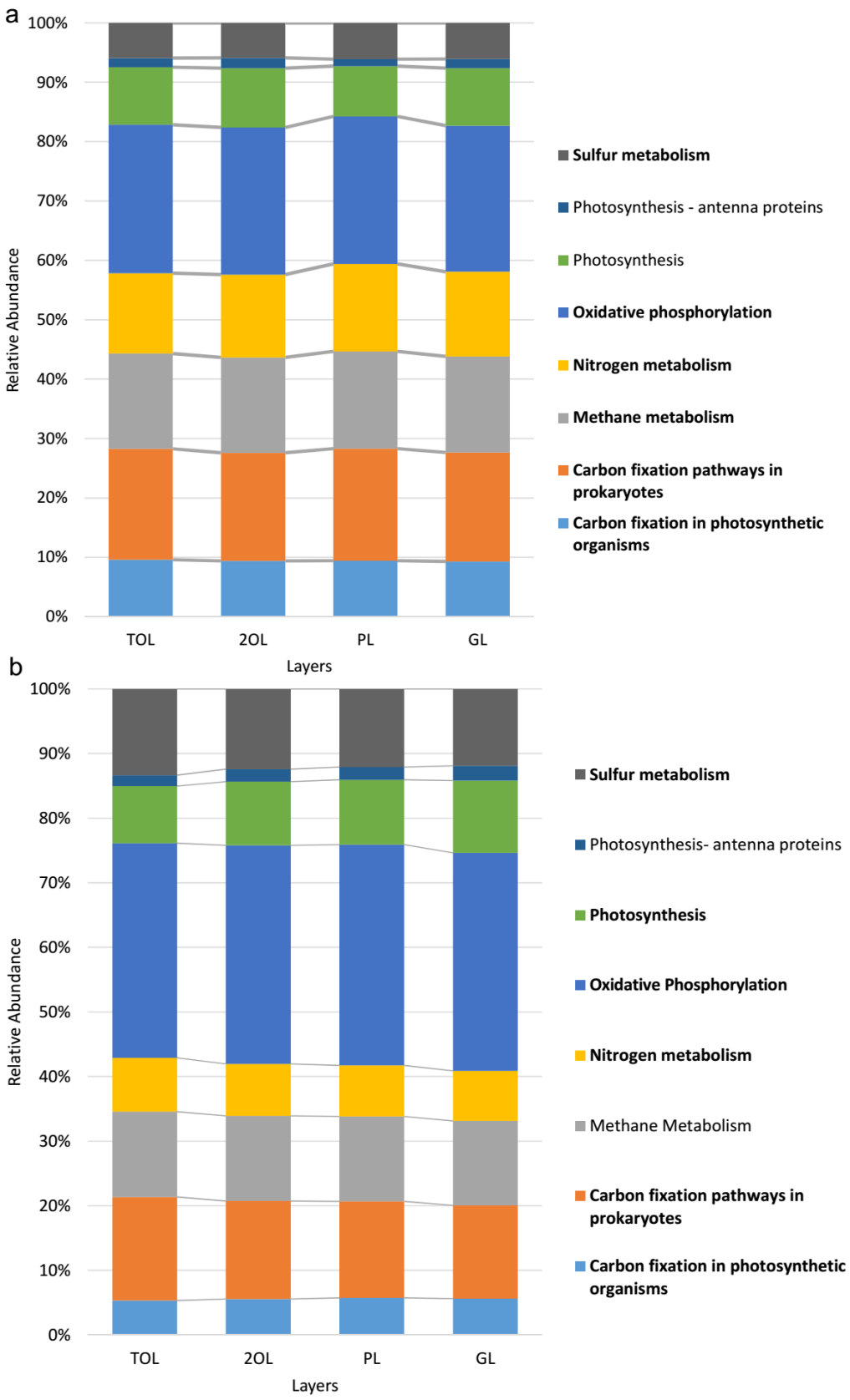


Fig. 5. Relative abundance of functional groups identified in samples grouped by layer within the crust. In order from top to bottom, the layers are top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL). **a** Bacterial functional groups identified by the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene using the GreenGenes 13.8 database. **b** Bacterial functional groups identified by the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene using the Silva 111 database.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Layers
Sulfur metabolism
Photosynthesis- antenna proteins
Photosynthesis
Oxidative Phosphorylation
Nitrogen metabolism
Methane Metabolism
Carbon fixation pathways in prokaryotes
Carbon fixation in photosynthetic organisms

Fig. 6. Relative abundance of predicted gene families belonging to the KEGG functional gene subgroup "Energy Metabolism" in the four layers of the crust as identified using PICRUSt (**a**) and Tax4Fun (**b**). In order from top to bottom, the layers are top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL). Bolded gene families listed in the key had significantly different abundances in the layers at $p \leq 0.05$.

Chapter 5. Conclusions and future directions

5.1 General conclusions

As demonstrated here, anchialine habitats are part of a uniquely complex ecosystem that are tied to both freshwater and seawater influences and are home to great endemic diversity. Although the invertebrate fauna from such habitats has been the focus of prior study [1–12], relatively little work had been previously done to document the microbial communities that form the base of food webs in these habitats [2, 13–21]. Unfortunately, these distinctive habitats are more threatened than ever before due in large part to their tropical and coastal settings that are prime development locations. In the Hawaiian Islands, home to the world's greatest concentration of anchialine habitats, development has both destroyed known habitats and contributed to significant nutrient loading in many of those that remain. Furthermore, the introduction of invasive species such as poeciliids (*Poecilia* spp. and *Gambusia affinis*), tilapia (*Oreochromis* spp.), and the Tahitian prawn (*Macrobrachium lar*) have been found to alter benthic microbial communities through impacts on the endemic atyid shrimp and keystone grazer, *Halocaridina rubra* [2, 11, 22–25]. Global climate change will also challenge the anchialine ecosystem; predictions for Hawaii include inundation of existing habitats and the continuing spread of invasive fishes, resulting in a net loss of pristine sites despite the creation of new habitats [26].

While serious challenges face Hawaiian anchialine habitats, conservation efforts have begun in earnest. For example, six of the eight shrimp species found in Hawaiian anchialine habitats have been listed as federal and state candidates for protection (http://dlnr.hawaii.gov/wildlife/files/2013/09/Fact-Sheet-anchialine-shrimps.pdf). Additionally, anchialine habitats have been protected in state-managed Natural Area Reserves (http://dlnr.hawaii.gov/ecosystems/nars/) and federally-managed National Parks that also serve to preserve native Hawaiian culture and culturally-important anchialine sites [3, 8, 9, 11,

22, 27–29]. Documentation of genetic diversity within these habitats has been a priority, including archiving samples collected during the course of this dissertation research with the Hawaiian Anchialine Microbial (HAM) repository in conjunction with Ocean Genome Legacy (http://www.northeastern.edu/ogl/). The goal of this dissertation was to provide the first, in-depth examination of the diversity, composition, and structure of microbial communities within Hawaiian anchialine habitats in order to begin developing a better understanding of the base of the food web and how it may be affected by spatial and seasonal factors. Specifically, spatial and seasonal variations in Hawaiian anchialine microbial communities were investigated and the consortia and metabolic niches present in the four distinct layers found in the unique orange cyanobacterial-bacterial crusts from particular regions of the islands characterized using high-throughput amplicon sequencing of environmental small subunit ribosomal DNA (rDNA). Ideally, this research can serve as a reference for future efforts as well as provide compelling evidence for considering microbial community diversity, especially the unique orange cyanobacterial-bacterial crust, in future conservation and management efforts.

A major finding of this dissertation was the distinctiveness of the microbial communities from each of the examined anchialine habitats. However, when the conditions under which these communities assemble are considered, such distinctions are expected. Hawaiian anchialine habitats on the islands of Maui and Hawaii, where most such habitats are found, occur within relatively young lava flows (<200 yrs old), the temperatures of which would have initially resulted in sterile conditions. The microbial communities observed in this dissertation, therefore, assembled relatively rapidly and independently multiple times, as Maui and Hawaii have never been geologically connected. Given independent assembly and the great diversity in environmental conditions under which Hawaiian anchialine habitats are found, each habitat appears to harbor a robustly distinct microbial community. As found in Chapter 2, even Hawaiian anchialine habitats that share watersheds and aquifers and occur in close geographic proximity do not have identical water chemistry profiles or microbial communities.

Many of the water chemistry factors measured in the course of this dissertation were found to be

apparently important in microbial community structure as well as varying with season. Of these, salinity appeared particularly important. Indeed, salinity was identified as having great explanatory power for both spatial community structure and seasonality. Previous studies of freshwater systems have documented salinity as highly influential in structuring microbial communities [30–32], as well as inhibitive of anoxygenic photosynthesis [33, 34]. Salinity varied by island, such that sites on Hawaii had generally lower salinity than those on Maui, and also with season, with greater salinity measured during wet season samples. Though anchialine habitats are under greater groundwater influence during wet seasons, increased salinity was measured near the surface of the habitat, likely reflecting greater mixing between the upper freshwater and lower saltwater lens that would be expected to result in lower salinity near the bottom of the habitat. Thus, anoxygenic photosynthesizers near the bottom of the habitat may be released from saline inhibition during wet seasons.

As mentioned above, greater relative abundance of anoxygenic photosynthesizing taxa was observed during wet seasons, perhaps due to seasonal changes in habitat salinity. Correspondingly, oxygenic photosynthesizers were observed as more abundant during dry season samplings, reflecting the resistance of the laminated cyanobacterial-bacterial crust communities. The diversity of niches created and maintained in laminated microbial communities likely provides stability and an ability to be self-sustaining in extreme environments [35, 36]. Thus, the ability of the orange laminated cyanobacterial-bacterial crust to support both oxygenic and anoxygenic photosynthesis likely contributes to the lack of large seasonal shifts in community structure as noted in Chapter 3.

Specific examination of consortia present in the distinct layers of the cyanobacterial-bacterial crust in Chapter 4 identified both similarities and differences with other laminated microbial mats from the scientific literature. This dissertation documented the presence of anaerobic taxa within the crust, including purple bacteria, a polyphyletic group of anoxygenic phototrophs common in laminated mats. Furthermore, increasing taxonomic richness with increasing depth in the laminations was observed, a trend attributable to greater niche diversity within the crust structure. However, unlike other laminated microbial communities, the Hawaiian anchialine crust has oxygenated zones at both the top and bottom of

the crust, allowing increased cyanobacterial abundance and confining anaerobic taxa to the middle two layers buried within the structure. Typically, laminated mat communities only receive oxygenation from the top of the structure, creating a decreasing oxygen gradient with depth that results in the bottom of the mat being anoxic. Oxygenation of the bottom layer of the orange cyanobacterial-bacterial crust likely comes from the combination of oxygenated water intrusion through the basin substrate beneath the crust, circulation of water in pockets between the crust and substrate, and circulation of water through cracks and openings in the crust. Thus, the orange cyanobacterial-bacterial crust communities found in anchialine ponds in the Cape Kinau region of Maui and Kona region of Hawaii represent novel laminated microbial communities worthy of further study and conservation.

5.2 Future directions

Although this dissertation has significantly increased scientific understanding of Hawaiian anchialine microbial communities and the environmental factors influencing them, many questions remain. For example, anchialine microbes on other islands in the Pacific Ocean have not been examined, and the similarity in benthic substrates (*i.e.*, lava flows and fossilized coral) could provide insight into how environmental conditions shape these resident microbial communities. Furthermore, there are other water chemistry factors that were not measured by the research in this dissertation but which may be important in also structuring these community, including dissolved oxygen, pH, and calcium. Additionally, although anchialine habitats from a range of environmental conditions observed in Hawaii were included in this dissertation research, many others were not sampled. Of particular interest would be those occurring at the periphery of the regions where laminated crust communities are found and where those crust communities have been described as thinner, less developed, and lacking lamination [13]. This might help to better identify what environmental factors contribute to the formation of these crust communities. Also concerning these crust communities, this dissertation was able to provide the first examination of the microbial consortia present in each of the four distinct layers. However, identification of the physiological and metabolic processes occurring within them are speculative. Measurements of

oxygen and other chemical gradients within the crust could provide further evidence of niche partitioning, as would including culture-based approaches or sequencing of functional genes. Sequencing of longer DNA spans would also offer better taxonomic resolution while enabling more confident identification of organisms in these habitats, as well as allow for more precise physiology and metabolism predictions.

An area that will prove interesting and informative is in regards to the temporal influences on Hawaiian anchialine microbial communities. Chapter 3 provided a limited first examination, as the survey covered just two wet/dry seasonal cycles. In this case, longer-term seasonal monitoring would have either given more robust evidence to support the observed relative stability of these communities or suggested that such stability resulted from small sample size. The impact of El Niño drought years was also a potentially confounding factor in Chapter 3, as the first dry season sampling occurred during a severe drought year while the remaining samples were taken the following year as drought conditions eased. Indeed, it could be that a seasonal effect only impacts these microbial communities during drought years (or vice versa); thus, continued and long-term sampling would be needed to address this. Furthermore, other cyanobacterial-bacterial mats have been documented to undergo community shifts during the diel period as taxa move vertically through the mat to meet their physiological and metabolic needs [37], a phenomenon that has not been examined in Hawaiian anchialine microbial communities. Indeed, while changes in water chemistry have been observed in Hawaiian anchialine habitats over the diel period [38], the impact of such rapid changes on the microbial community composition and structure have not been studied.

Little is known regarding the "life cycle" of Hawaiian anchialine habitats and how their creation and senescence alter resident microbial communities. For example, anchialine lakes in Bermuda appear to lose marine influence as a result of sedimentation [39], a process also hypothesized and observed in the anchialine habitats of Hawaii [13, 25]. However, more than sedimentation may influence senescence, as one of the sites sampled for this dissertation suggests. In March 2011, the Tōhoku tsunami inundated one site, Makalawena Pond #2, hours after it was sampled, filling it completely with sediment. When the site was revisited in July 2011, it was still completely dry and filled with sand. However, by December 2011,

the pond basin had partially emptied and contained water as well as green benthic growth with small patches of orange, suggesting the anchialine habitat and microbial community were reforming following disturbance by the tsunami. Finally, the impact and "success" of anchialine restoration efforts, including removal of invasive fish and Tahitian prawns, on the microbial community, is unknown. Evidence that invasive species alter the Hawaiian anchialine food web and microbial community has been collected in other studies and here in this dissertation, but how the ecosystem reacts to the removal of these threats needs to be further, and rigorously, quantified. While the community would be expected to shift to more closely resemble that of its pre-invasion state, the length of time for this recovery and whether there would be lasting impacts are unknown.

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Appendix 1. Core *Bacteria* (V6) and micro-*Eukarya* (V9) OTUs, that is those present in all samples, and their taxonomic identities for benthic and water column communities belonging to orange cyanobacterial-bacterial crusts and caves from sampled anchialine habitats on the islands of Hawaii and Maui.

V6 Maui Crust Benthos

OTU ID	Taxonomic Identity
3127356	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, C111
4444043	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, Microthrixaceae
1110041	Bacteria, Bacteroidetes, Rhodothermi, Rhodothermales, Rhodothermaceae, Rubricoccus
New.ReferenceOTU1593	Bacteria, Bacteroidetes, Saprospirae, Saprospirales
1938968	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.ReferenceOTU4511	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.ReferenceOTU7219	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, Lewinella
New.ReferenceOTU5256	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Flammeovirgaceae
4385825	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Flammeovirgaceae, Fulvivirga
New.ReferenceOTU1208	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Flammeovirgaceae, Marivirga, tractuosa
1109032	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales
136105	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, Owenweeksia
165148	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
New.ReferenceOTU2558	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
New.ReferenceOTU3221	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Myroides, odoratimimus
4435279	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Nonlabens, sediminis
New.ReferenceOTU6898	Bacteria, Chloroflexi, Anaerolineae, DRC31
4334035	Bacteria, Chloroflexi, Anaerolineae, SBR1031, A4b
New.ReferenceOTU1846	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Xenococcaceae, Chroococcidiopsis,
278809	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
New.ReferenceOTU3811	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
New.ReferenceOTU2332	Bacteria, Planctomycetes, Phycisphaerae, S-70
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
New.ReferenceOTU3803	Bacteria, Planctomycetes, vadinHA49
2501082	Bacteria, Proteobacteria, Alphaproteobacteria

900969	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
3371208	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4336993	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4345424	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4413994	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
New.Reference OTU5452	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
353953	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4471228	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
New.Reference OTU3197	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Hyphomonadaceae, Hirschia
814074	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
1864705	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
4399537	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae
834330	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
4384896	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
151374	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, aquimaris
4041621	Bacteria, Proteobacteria, Gammaproteobacteria
4449964	Bacteria, Proteobacteria, Gammaproteobacteria, Marinicellales, Marinicellaceae
4455390	Bacteria, Proteobacteria, Gammaproteobacteria, Marinicellales, Marinicellaceae
313062	Bacteria, Proteobacteria, Gammaproteobacteria, Chromatiales, Ectothiorhodospiraceae
880783	Bacteria, Proteobacteria, Gammaproteobacteria, HTCC2188, HTCC2089
361994	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
25719	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae
4370744	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.Reference OTU3485	No blast hit
New.Reference OTU4467	No blast hit
New.Reference OTU5124	No blast hit
New.Reference OTU7811	No blast hit
New.Reference OTU7843	No blast hit

V9 Maui Crust Benthos

OTU ID	**Taxonomic Identity**
New.Reference OTU1582	Bacteria, Chloroflexi, Caldilineae, Caldilineales, Caldilineaceae, uncultured, uncultured Chloroflexi bacterium

EU369136	Bacteria, Gemmatimonadetes, BD2-11 terrestrial group, uncultured bacterium
New.ReferenceOTU4865	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales, Phycisphaeraceae, Phycisphaera, uncultured bacterium
New.ReferenceOTU2145	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales, Phycisphaeraceae, Phycisphaera, uncultured Planctomycetales bacterium
JF769543	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Blastopirellula, uncultured bacterium
FJ624355	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Pirellula, uncultured Planctomycetaceae bacterium
JF769736	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured bacterium
AF234756	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, Woodsholea, uncultured sludge bacterium S36
DQ395841	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured organism
DQ395877	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured organism
EU603455	Bacteria, Proteobacteria, Alphaproteobacteria, E6aD10, Bradyrhizobiaceae bacterium PTG4-2
GU302454	Bacteria, Proteobacteria, Alphaproteobacteria, Parvularculales, Parvularculaceae, Parvularcula, uncultured bacterium
JF514231	Bacteria, Proteobacteria, Alphaproteobacteria, Parvularculales, Parvularculaceae, Parvularcula, uncultured bacterium
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
AACY02053469	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, Cohaesibacter, marine metagenome
JF514232	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, uncultured, uncultured bacterium
JN178071	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Tepidamorphus, uncultured Rhizobiales bacterium
FJ152760	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Gaetbulicola, uncultured bacterium
JN637794	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Loktanella, uncultured marine microorganism
DD437360	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, Paracoccus sp. 101
JN683955	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Ruegeria, uncultured bacterium
JN874361	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Ruegeria, uncultured bacterium
EF202612	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Thalassobacter, uncultured bacterium
JN685454	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Thioclava, uncultured bacterium
FJ467624	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
New.ReferenceOTU4158	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium

JN157655	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured bacterium
EU802813	Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, SAR116 clade, uncultured bacterium
DQ396170	Bacteria, Proteobacteria, Alphaproteobacteria, SAR11 clade, Deep 1, uncultured organism
JN178837	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Altererythrobacter, uncultured bacterium
AB035544	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, Erythrobacter sp. MBIC4117
JF769550	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, uncultured bacterium
JN874356	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, uncultured bacterium
DQ396035	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, uncultured organism
HM030990	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, marine bacterium KS-9-10-4
JN024028	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, uncultured bacterium
JF727692	Bacteria, Proteobacteria, Deltaproteobacteria, Bdellovibrionales, Bacteriovoracaceae, Peredibacter, uncultured delta proteobacterium
New.Reference OTU24	Bacteria, Proteobacteria, Deltaproteobacteria, Bdellovibrionales, Bacteriovoracaceae, Peredibacter, uncultured delta proteobacterium
JF272215	Bacteria, Proteobacteria, Deltaproteobacteria, Desulfuromonadales, GR-WP33-58, uncultured bacterium
GQ274154	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Nannocystineae, Nannocystaceae, Nannocystis, uncultured deep-sea bacterium
JN178401	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, Sandaracinus, uncultured delta proteobacterium
FJ425609	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, uncultured Myxococcales bacterium
New.Reference OTU3097	Bacteria, Proteobacteria, Deltaproteobacteria, SAR324 clade(Marine group B), uncultured bacterium
GU083688	Bacteria, Proteobacteria, Gammaproteobacteria, Chromatiales, Granulosicoccaceae, Granulosicoccus, uncultured bacterium
HQ190493	Bacteria, Proteobacteria, Gammaproteobacteria, Order Incertae Sedis, Family Incertae Sedis, Marinicella, uncultured bacterium
HQ190555	Bacteria, Proteobacteria, Gammaproteobacteria, Order Incertae Sedis, Family Incertae Sedis, Marinicella, uncultured bacterium
EU735671	Bacteria, Proteobacteria, Gammaproteobacteria, Order Incertae Sedis, Family Incertae Sedis, Thiohalophilus, uncultured sediment bacterium
AY753620	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Neobodo, Neobodo designis
JN542576	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Neobodo, uncultured kinetoplastid
New.Reference OTU3801	Eukaryota, Opisthokonta, Metazoa, Cnidaria, Anthozoa, Ricordea, Ricordea florida
New.Reference OTU4843	Eukaryota, Opisthokonta, Metazoa, Nematoda, Chromadorea, Siphonolaimidae, Astomonema, Astomonema sp. NCM-2006

New.Reference OTU2662	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Colpodea, Cyrtolophosidida, Aristerostoma, Aristerostoma marinum
New.Reference OTU699	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Scuticociliatia, Metanophrys, Metanophrys sinensis
New.Reference OTU5627	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Spirotrichea, Euplotia, Euplotes, Euplotes raikovi
New.Reference OTU3489	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Spirotrichea, Hypotrichia, Parabirojimia, Parabirojimia similis
New.Reference OTU1789	Eukaryota, SAR, Stramenopiles, Bicosoecida, Bicosoecidae, Bicosoeca, Stramenopile, Stramenopile sp. MESS21
New.Reference OTU1799	Eukaryota, SAR, Stramenopiles, Bicosoecida, Cafeteriidae, Cafeteria, Cafeteria roenbergensis
New.Reference OTU2676	Eukaryota, SAR, Stramenopiles, Bicosoecida, Siluaniidae, Caecitellus, Caecitellus pseudoparvulus
AY179995	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Navicula, uncultured stramenopile
GQ452862	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Phaeodactylum, Phaeodactylum tricornutum
New.Reference OTU4821	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Mediophyceae, Attheya, uncultured stramenopile

V6 Hawaii Crust Benthos

OTU ID	**Taxonomic Identity**
3312256	Bacteria, Acidobacteria, Solibacteres, Solibacterales, Solibacteraceae
4471428	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, C111
2773722	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales
New.Reference OTU2197	Bacteria, Bacteroidetes, Saprospirae, Saprospirales
New.Reference OTU5702	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae
1938968	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.Reference OTU1198	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.Reference OTU4888	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.Reference OTU792	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.Reference OTU99	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.Reference OTU5326	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, Lewinella, cohaerens
3934741	Bacteria, Bacteroidetes, Cytophagia, Cytophagales
New.Reference OTU141	Bacteria, Bacteroidetes, Cytophagia, Cytophagales
New.Reference OTU5278	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Cyclobacteriaceae
102384	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Flammeovirgaceae, Roseivirga
278327	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
4396051	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
4408391	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae

4471839	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, Fluviicola
4347492	Bacteria, Cyanobacteria, Gloeobacterophycideae, Gloeobacterales, Gloeobacteraceae, Gloeobacter
4460895	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Cyanobacteriaceae
New.ReferenceOTU862	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Cyanobacteriaceae, Cyanothece
4472222	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae
240428	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
New.ReferenceOTU6009	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
New.ReferenceOTU6309	Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, Faecalibacterium, prausnitzii
New.ReferenceOTU3540	Bacteria, Firmicutes, Clostridia, Clostridiales, Syntrophomonadaceae, Syntrophomonas
628974	Bacteria, Planctomycetes, OM190, CL500-15
New.ReferenceOTU8351	Bacteria, Planctomycetes, OM190, CL500-15
816440	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
3846383	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
4343962	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
New.ReferenceOTU2952	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
New.ReferenceOTU775	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
New.ReferenceOTU3282	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae, A17
787856	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
3639072	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4345424	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
New.ReferenceOTU5502	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae
New.ReferenceOTU1823	Bacteria, Proteobacteria, Alphaproteobacteria, Kordiimonadales, Kordiimonadaceae
737645	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales
808124	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Cohaesibacteraceae
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4479751	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
New.ReferenceOTU7971	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
211231	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium
1722553	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Hyphomonadaceae

257210	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
2995140	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
3721058	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
4355400	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
4474732	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
4476430	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
New.Reference OTU5498	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
New.Reference OTU8524	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
736504	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Rhodobacter
834330	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
New.Reference OTU4429	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae
114102	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
4445754	Bacteria, Proteobacteria, Deltaproteobacteria, GMD14H09
New.Reference OTU661	Bacteria, Proteobacteria, Gammaproteobacteria, Pasteurellales
361994	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
263149	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae
353183	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae
291608	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Luteimonas
310512	Bacteria, Verrucomicrobia, Opitutae
New.Reference OTU5021	Bacteria, Verrucomicrobia, Opitutae
New.Reference OTU8312	Bacteria, Verrucomicrobia, Opitutae, Puniceicoccales, Puniceicoccaceae
4370744	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.Reference OTU2856	No blast hit
New.Reference OTU3566	No blast hit
New.Reference OTU5124	No blast hit
New.Reference OTU7225	No blast hit

V9 Hawaii Crust Benthos

OTU ID	**Taxonomic Identity**
EF203205	Bacteria, Gemmatimonadetes, Gemmatimonadales, Gemmatimonadaceae, Gemmatimonas, uncultured bacterium
New.Reference OTU4865	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales, Phycisphaeraceae, Phycisphaera, uncultured bacterium
New.Reference OTU4817	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales, Phycisphaeraceae, SM1A02, uncultured bacterium

FJ624355	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Pirellula, uncultured Planctomycetaceae bacterium
FJ516864	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured Hyphomonadaceae bacterium
AF234756	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, Woodsholea, uncultured sludge bacterium S36
DQ856507	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured bacterium
JF272159	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured bacterium
DQ395935	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured organism
FN667474	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Devosia, uncultured bacterium
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
JN178526	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, JG34-KF-361, uncultured bacterium
JN177997	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium, uncultured bacterium
AACY020302266	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, Phyllobacterium, marine metagenome
JN391734	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Rhodobium, uncultured bacterium
JN178071	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Tepidamorphus, uncultured Rhizobiales bacterium
HQ706108	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Xanthobacteraceae, Azorhizobium, Azorhizobium caulinodans
FJ152760	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Gaetbulicola, uncultured bacterium
JN637794	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Loktanella, uncultured marine microorganism
JF769718	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Loktanella, uncultured marine microorganism
JN178732	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Palleronia, uncultured bacterium
FJ153021	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Pannonibacter, uncultured bacterium
DD437360	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, Paracoccus sp. 101
JF905624	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, Paracoccus sp. 101
JN082658	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, uncultured Paracoccus sp.
FM956479	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Roseovarius, uncultured bacterium
EU360293	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Thalassobius, uncultured Thalassobius sp.
FJ152971	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
FJ467624	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium

New.Reference OTU4158	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
New.Reference OTU4815	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured Rhodobacteraceae bacterium
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium
New.Reference OTU491	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Candidatus Alysiosphaera, uncultured alpha proteobacterium
EF100150	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, Azospirillum, Azospirillum lipoferum
EU360296	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, wr0007, uncultured alpha proteobacterium
JN717193	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Altererythrobacter, uncultured marine bacterium
JN178666	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Novosphingobium, uncultured bacterium
JN024028	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, uncultured bacterium
JN869013	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, uncultured, uncultured bacterium
New.Reference OTU4176	Bacteria, Proteobacteria, Deltaproteobacteria, Desulfuromonadales, GR-WP33-58, uncultured bacterium
EF636835	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, uncultured bacterium
JN684010	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, DEV007, uncultured bacterium
New.Reference OTU2468	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Prosthecobacter, uncultured bacterium
JN542576	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Neobodo, uncultured kinetoplastid
New.Reference OTU699	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Scuticociliatia, Metanophrys, Metanophrys sinensis
New.Reference OTU2944	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Phyllopharyngea, Cyrtophoria, Chilodonella, Chilodonella uncinata
New.Reference OTU5751	Eukaryota, SAR, Stramenopiles, Bicosoecida, Cafeteriidae, Cafeteria, Cafeteria roenbergensis
EF165110	Eukaryota, SAR, Stramenopiles, Chrysophyceae, Ochromonadales, Ochromonas, Ochromonas sp. CCMP2767

V6 All Crust Benthos

OTU ID	**Taxonomic Identity**
1938968	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4345424	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
834330	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
361994	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae

4370744	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.Reference OTU5124	No blast hit

V9 All Crust Benthos

OTU ID	**Taxonomic Identity**
New.Reference OTU4865	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales, Phycisphaeraceae, Phycisphaera, uncultured bacterium
FJ624355	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Pirellula, uncultured Planctomycetaceae bacterium
AF234756	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, Woodsholea, uncultured sludge bacterium S36
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
JN178071	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Tepidamorphus, uncultured Rhizobiales bacterium
FJ152760	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Gaetbulicola, uncultured bacterium
JN637794	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Loktanella, uncultured marine microorganism
DD437360	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, Paracoccus sp. 101
FJ467624	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
New.Reference OTU4158	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium
JN024028	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, uncultured bacterium
JN542576	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Neobodo, uncultured kinetoplastid
New.Reference OTU699	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Scuticociliatia, Metanophrys, Metanophrys sinensis

V6 Maui Crust Water Column

OTU ID	**Taxonomic Identity**
1109043	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales, Microbacteriaceae
268664	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales, Microbacteriaceae, Candidatus Aquiluna, rubra
4372360	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
New.Reference OTU8191	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, Fluviicola
348807	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
165148	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
144373	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
812759	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
826143	Bacteria, Cyanobacteria, Chloroplast, Haptophyceae
837283	Bacteria, Fusobacteria, Fusobacteriia, Fusobacteriales, Fusobacteriaceae, Cetobacterium, somerae

787856	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4345424	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4336993	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
New.Reference OTU6117	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
324499	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
4390055	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
4410373	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Roseovarius, aestuarii
151374	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, aquimaris
326429	Bacteria, Proteobacteria, Betaproteobacteria, Methylophilales, Methylophilaceae
4327205	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Glaciecola
216695	Bacteria, Proteobacteria, Gammaproteobacteria, HTCC2188
4331228	Bacteria, Proteobacteria, Gammaproteobacteria, Legionellales, Francisellaceae, Francisella,
4451498	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales
589202	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter
4343217	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Vibrio
4409846	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Vibrio
4472884	Bacteria, Verrucomicrobia, Opitutae, Puniceicoccales, Puniceicoccaceae, Coraliomargarita,
4409489	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium,
New.Reference OTU5441	No blast hit

V9 Maui Crust Water Column

OTU ID	**Taxonomic Identity**
AB530182	Bacteria, NPL-UPA2, uncultured bacterium
JQ337896	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Devosia, Devosia sp. G-He10
JF514232	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, uncultured, uncultured bacterium
AB491860	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Dinoroseobacter, uncultured alpha proteobacterium
EF471669	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Marivita, uncultured alpha proteobacterium
DD437360	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, Paracoccus sp. 101
FJ535115	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Rhodobacter, uncultured alpha proteobacterium
JN683955	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Ruegeria, uncultured bacterium

JN874361	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Ruegeria, uncultured bacterium
GQ274281	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Sulfitobacter, uncultured bacterium
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium
HM030990	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, marine bacterium KS-9-10-4
HQ697709	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Alcanivoracaceae, Alcanivorax, uncultured bacterium
HM041920	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillaceae, Marinobacterium, uncultured Marinobacterium sp.
GU474845	Bacteria, Verrucomicrobia, Opitutae, Puniceicoccales, Puniceicoccaceae, Lentimonas, uncultured bacterium
New.Reference OTU3512	Eukaryota, Amoebozoa, Conosa, Variosea, Varipodida, Flamella, Flamella arnhemensis
AB425959	Eukaryota, Archaeplastida, Chloroplastida, Chlorophyta, Ulvophyceae, Ulva, Ulva sp. P36
AM491021	Eukaryota, Haptophyta, Prymnesiophyceae, Prymnesiales, Chrysochromulina, Chrysochromulina simplex
New.Reference OTU1860	Eukaryota, Opisthokonta, Holozoa, Choanomonada, Craspedida, Lagenoeca, uncultured choanoflagellate
AM503930	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU2497	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU2612	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU2186	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU4496	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU1585	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU942	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU1789	Eukaryota, SAR, Stramenopiles, Bicosoecida, Bicosoecidae, Bicosoeca, Stramenopile, Stramenopile sp. MESS21
New.Reference OTU2682	Eukaryota, SAR, Stramenopiles, Chrysophyceae, Ochromonadales, Ochromonas, uncultured marine eukaryote
AF525663	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Navicula, Pseudogomphonema, Pseudogomphonema sp. LM-2002
AY179995	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Navicula, uncultured stramenopile
AB183646	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Nitzschia, Bacillariophyta, Bacillariophyta sp. MBIC10816
GQ452862	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Phaeodactylum, Phaeodactylum tricornutum
New.Reference OTU1707	Eukaryota, SAR, Stramenopiles, Peronosporomycetes, Halodaphnea, Halocrusticida, Halocrusticida parasitica

V6 Hawaii Crust Water Column

OTU ID	Taxonomic Identity
1956921	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, JdFBGBact
175311	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, wb1 P06
New.ReferenceOTU6920	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales, Sporichthyaceae, Sporichthya
New.ReferenceOTU5363	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae
1938968	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
2540	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
740498	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.ReferenceOTU6734	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.ReferenceOTU6727	Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae
199016	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Cyclobacteriaceae
New.ReferenceOTU1512	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Flammeovirgaceae, Reichenbachiella
New.ReferenceOTU7816	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales
4408391	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
303477	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
316494	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
4156020	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, Crocinitomix
4471839	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, Fluviicola
1962473	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, Wandonia
4385994	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
812759	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
New.ReferenceOTU5414	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
141302	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
838871	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
308318	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
997342	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
4467414	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
4385507	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
161828	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium, gelidilacus
154970	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium, gelidilacus
1118322	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Myroides

4435279	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Nonlabens, sediminis
New.ReferenceOTU1024	Bacteria, Cyanobacteria
1105902	Bacteria, Cyanobacteria, Chloroplast, Stramenopiles
New.ReferenceOTU1619	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
4310401	Bacteria, Fibrobacteres, Fibrobacteria, 258ds10
837283	Bacteria, Fusobacteria, Fusobacteriia, Fusobacteriales, Fusobacteriaceae, Cetobacterium, somerae
4387300	Bacteria, Nitrospirae, Nitrospira, Nitrospirales, Nitrospiraceae, Nitrospira
327521	Bacteria, Planctomycetes, OM190, CL500-15
628974	Bacteria, Planctomycetes, OM190, CL500-15
4406062	Bacteria, Planctomycetes, OM190, CL500-15
816440	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
New.ReferenceOTU775	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
New.ReferenceOTU3282	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae, A17
808797	Bacteria, Proteobacteria, Alphaproteobacteria
249034	Bacteria, Proteobacteria, Alphaproteobacteria
47933	Bacteria, Proteobacteria, Alphaproteobacteria
New.ReferenceOTU884	Bacteria, Proteobacteria, Alphaproteobacteria
New.ReferenceOTU6693	Bacteria, Proteobacteria, Alphaproteobacteria
New.ReferenceOTU1245	Bacteria, Proteobacteria, Alphaproteobacteria
New.ReferenceOTU7334	Bacteria, Proteobacteria, Alphaproteobacteria
787856	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
900969	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4393213	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
817491	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
3371208	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4355539	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
3639072	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
345859	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4345424	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4413994	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
268436	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4336993	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4388413	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3

1068111	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
New.ReferenceOTU4958	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
New.ReferenceOTU5452	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
New.ReferenceOTU8177	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4418989	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales
808124	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Cohaesibacteraceae
4479751	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
211231	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium
4482023	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Rhodoplanes
344370	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, Mesorhizobium
167289	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
567410	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
New.ReferenceOTU620	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
New.ReferenceOTU5498	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
3711798	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Anaerospora
3889679	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae
4458420	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae
New.ReferenceOTU1274	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae
New.ReferenceOTU7708	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae
New.ReferenceOTU5467	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Novosphingobium
114102	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
2801319	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Methylibium
610527	Bacteria, Proteobacteria, Deltaproteobacteria, Desulfobacterales, Desulfobulbaceae
4445754	Bacteria, Proteobacteria, Deltaproteobacteria, GMD14H09
New.ReferenceOTU7603	Bacteria, Proteobacteria, Deltaproteobacteria, GMD14H09
New.ReferenceOTU7958	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales
New.ReferenceOTU8449	Bacteria, Proteobacteria, Deltaproteobacteria, Spirobacillales
4369210	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae
4383202	Bacteria, Proteobacteria, Gammaproteobacteria, 34P16
828124	Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae
559104	Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae
4412902	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae

201358	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae, Alishewanella
4465421	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae, Rheinheimera
4327385	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae, Rheinheimera
4390688	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae, Rheinheimera
534869	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, 211ds20
822382	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae
77437	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae
356014	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae
4353369	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae
8892	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Alteromonas
2718805	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio
4260142	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio
52677	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio
2594460	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Ferrimonadaceae, Ferrimonas
2000018	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, OM60
358229	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Shewanellaceae, Shewanella
4379510	Bacteria, Proteobacteria, Gammaproteobacteria, Chromatiales, Halothiobacillaceae, Halothiobacillus
362155	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae
4062645	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae
1146947	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Erwinia
4371014	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Erwinia
684433	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Klebsiella
211586	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Providencia
New.ReferenceOTU6726	Bacteria, Proteobacteria, Gammaproteobacteria, HTCC2188, HTCC2089
266502	Bacteria, Proteobacteria, Gammaproteobacteria, Legionellales, Coxiellaceae
4482664	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillaceae
568874	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillaceae, Marinomonas
4421705	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillaceae, Oceanospirillum
4175062	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Saccharospirillaceae, ML110J-20

New.ReferenceOTU661	Bacteria, Proteobacteria, Gammaproteobacteria, Pasteurellales
4335515	Bacteria, Proteobacteria, Gammaproteobacteria, Pasteurellales, Pasteurellaceae, Haemophilus, parainfluenzae
4394920	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae
589202	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter
3905357	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae
4364205	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Azomonas, agilis
103107	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Pseudomonas
722635	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Pseudomonas, stutzeri
New.ReferenceOTU6817	Bacteria, Proteobacteria, Gammaproteobacteria, PYR10d3
3726190	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
3185360	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
4373421	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
4303359	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
554223	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
837313	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Pseudoalteromonadaceae
4355281	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Pseudoalteromonadaceae, Vibrio, alginolyticus
816715	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae
4409846	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Vibrio
111152	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Vibrio, splendidus
1125638	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae, Steroidobacter
263149	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae
New.ReferenceOTU6073	Bacteria, Spirochaetes, Spirochaetes, Spirochaetales, Spirochaetaceae, Spirochaeta
4477307	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales, auto67 4W
New.ReferenceOTU946	Bacteria, Verrucomicrobia, Verruco-5, LD1-PB3
252314	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae
4458675	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Luteolibacter
4444335	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Prosthecobacter
3996106	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
4409489	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.ReferenceOTU3620	No blast hit

New.Reference OTU5441	No blast hit
New.Reference OTU7225	No blast hit
New.Reference OTU2723	No blast hit
New.Reference OTU7359	No blast hit
New.Reference OTU4202	No blast hit
New.Reference OTU7986	No blast hit
New.Reference OTU362	No blast hit
New.Reference OTU1473	No blast hit
New.Reference OTU5109	No blast hit
New.Reference OTU5630	No blast hit
New.Reference OTU7618	No blast hit

V9 Hawaii Crust Water Column

OTU ID	**Taxonomic Identity**
DQ432398	Bacteria, Firmicutes, Clostridia, Halanaerobiales, Halanaerobiaceae, Halarsenatibacter, uncultured low G+C Gram-positive bacterium
FJ624355	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Pirellula, uncultured Planctomycetaceae bacterium
AF234756	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, Woodsholea, uncultured sludge bacterium S36
JF272159	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured bacterium
DQ395935	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured organism
DQ395877	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured organism
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
JN177997	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium, uncultured bacterium
D14513	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, Rhizobium tropici
HQ706108	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Xanthobacteraceae, Azorhizobium, Azorhizobium caulinodans
FJ152760	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Gaetbulicola, uncultured bacterium
JN637794	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Loktanella, uncultured marine microorganism
FJ153021	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Pannonibacter, uncultured bacterium
JN082658	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, uncultured Paracoccus sp.

JN869165	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Rhodobacter, uncultured bacterium
JN874361	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Ruegeria, uncultured bacterium
FJ467624	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
JN874356	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, uncultured bacterium
JN024028	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, uncultured bacterium
EF188429	Bacteria, Proteobacteria, Deltaproteobacteria, Bdellovibrionales, Bacteriovoracaceae, Peredibacter, uncultured delta proteobacterium
New.Reference OTU1772	Bacteria, Proteobacteria, Deltaproteobacteria, Bdellovibrionales, Bdellovibrionaceae, Bdellovibrio, uncultured delta proteobacterium
JN977244	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, uncultured bacterium
HQ397578	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, uncultured, uncultured delta proteobacterium
New.Reference OTU1202	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, uncultured, uncultured delta proteobacterium
DQ659451	Bacteria, Proteobacteria, Gammaproteobacteria, 34P16, uncultured marine bacterium
GQ388987	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, BD1-7 clade, uncultured bacterium
AF130897	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Pantoea, Pantoea agglomerans
New.Reference OTU3935	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Alcanivoracaceae, Alcanivorax, halophilic bacterium TAG F1
JN178325	Bacteria, Verrucomicrobia, Opitutae, Opitutales, Opitutaceae, Opitutus, uncultured bacterium
New.Reference OTU3049	Eukaryota, Amoebozoa, Conosa, Variosea, Varipodida, Flamella, Flamella arnhemensis
U42426	Eukaryota, Archaeplastida, Chloroplastida, Charophyta, Phragmoplastophyta, Streptophyta, Embryophyta, Tracheophyta, Spermatophyta, Magnoliophyta, Cucurbitales, Datisca, Datisca glomerata
New.Reference OTU3914	Eukaryota, Archaeplastida, Chloroplastida, Chlorophyta, Ulvophyceae, Pseudendoclonium, Pseudendoclonium submarinum
New.Reference OTU2359	Eukaryota, Cryptophyceae, Cryptomonadales, Cryptomonas, uncultured eukaryote
New.Reference OTU4276	Eukaryota, Cryptophyceae, Cryptomonadales, Geminigera, Geminigera cryophila
New.Reference OTU1001	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Diplonemea, Rhynchopus, Rhynchopus sp. SH-2004-I
AY753601	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Neobodo, Bodonidae, Bodonidae sp. Dev1
JN542576	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Neobodo, uncultured kinetoplastid
DQ465524	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Rhynchomonas, Rhynchomonas nasuta
New.Reference OTU1389	Eukaryota, Haptophyta, Prymnesiophyceae, Prymnesiales, Chrysochromulina, uncultured marine eukaryote

HM161743	Eukaryota, Opisthokonta, Fungi, Ascomycota, Pezizomycotina, Dothideomycetes, Phoma, Phoma sp. Y3 EG-2010
New.Reference OTU4926	Eukaryota, Opisthokonta, Fungi, Ascomycota, Pezizomycotina, Dothideomycetes, Pleosporomycetidae, Pleosporales, Mytilinidiaceae, Mytilinidion, Mytilinidion mytilinellum
HM009314	Eukaryota, Opisthokonta, Fungi, Ascomycota, Saccharomycotina, Saccharomycetes, Saccharomycetales, Saccharomycetaceae, Saccharomyces, "Saccharomyces cerevisiae bakers yeast"
New.Reference OTU2241	Eukaryota, Opisthokonta, Fungi, Basal fungi, Blastocladiomycota, uncultured eukaryote
DQ322624	Eukaryota, Opisthokonta, Fungi, Basal fungi, Chytridiomycota, Olpidium, Olpidium brassicae
New.Reference OTU5302	Eukaryota, Opisthokonta, Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Trichaptum, Trichaptum biforme
EU868735	Eukaryota, Opisthokonta, Metazoa, Arthropoda, Crustacea, Malacostraca, Typhlatya, Typhlatya mitchelli
New.Reference OTU3467	Eukaryota, Opisthokonta, Metazoa, Arthropoda, Crustacea, Maxillopoda, Parastenhelia, Parastenhelia sp. New Caledonia-RJH-2007
New.Reference OTU4151	Eukaryota, Opisthokonta, Metazoa, Arthropoda, Crustacea, Maxillopoda, Parastenhelia, Parastenhelia sp. New Caledonia-RJH-2007
New.Reference OTU2169	Eukaryota, Opisthokonta, Metazoa, Arthropoda, Crustacea, Maxillopoda, Sinelobus, Sinelobus sp. KK-2011-1
New.Reference OTU92	Eukaryota, Opisthokonta, Metazoa, Mollusca, Gastropoda, Neritopsina, Theodoxus, Theodoxus fluviatilis
New.Reference OTU5419	Eukaryota, Opisthokonta, Metazoa, Nematoda, Chromadorea, Monhysteridae, Diplolaimelloides, Diplolaimelloides environmental sample
New.Reference OTU2697	Eukaryota, Opisthokonta, Metazoa, Nematoda, Chromadorea, Monhysteridae, Monhysteridae environmental sample
New.Reference OTU4924	Eukaryota, Opisthokonta, Metazoa, Platyhelminthes, Trematoda, Digenea, Maritrema, Maritrema oocysta
GQ922318	Eukaryota, Opisthokonta, Metazoa, Rotifera, Philodinidae, uncultured bdelloid rotifer
AB505576	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Litostomatea, Mesodiniidae, uncultured marine eukaryote
AM503930	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701
New.Reference OTU2482	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, uncultured marine eukaryote
New.Reference OTU5564	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Suessiaceae, Symbiodinium, uncultured marine eukaryote
New.Reference OTU3066	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Suessiaceae, Woloszynskia, uncultured alveolate
New.Reference OTU4076	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Suessiaceae, Woloszynskia, uncultured alveolate
New.Reference OTU4678	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Peridiniphycidae, Thoracosphaeraceae, Pfiesteria, Pfiesteria sp. B112456
New.Reference OTU4760	Eukaryota, SAR, Alveolata, FV18-2D11, uncultured eukaryote
New.Reference OTU2189	Eukaryota, SAR, Alveolata, Protalveolata, Perkinsidae, A31, uncultured alveolate
New.Reference OTU2123	Eukaryota, SAR, Alveolata, Protalveolata, Syndiniales, Amoebophrya, uncultured Amoebophrya

New.Reference OTU1346	Eukaryota, SAR, Alveolata, Protalveolata, Syndiniales, Syndiniales Group I, uncultured dinoflagellate
New.Reference OTU1387	Eukaryota, SAR, Rhizaria, Cercozoa, Clathrulinidae, Hedriocystis, Clathrulina, Clathrulina elegans
New.Reference OTU1590	Eukaryota, SAR, Rhizaria, Foraminifera, Tubothalamea, Miliolida, Archaias, Archaias angulatus
New.Reference OTU516	Eukaryota, SAR, Stramenopiles, Bicosoecida, Cafeteriidae, Cafeteria, Cafeteria roenbergensis
New.Reference OTU1898	Eukaryota, SAR, Stramenopiles, Bicosoecida, Cafeteriidae, Pseudobodo, Pseudobodo tremulans
New.Reference OTU1600	Eukaryota, SAR, Stramenopiles, C2-E039, uncultured eukaryote
New.Reference OTU2765	Eukaryota, SAR, Stramenopiles, Chrysophyceae, Chromulinales, Poterioochromonas, Poterioochromonas malhamensis
EF165110	Eukaryota, SAR, Stramenopiles, Chrysophyceae, Ochromonadales, Ochromonas, Ochromonas sp. CCMP2767
New.Reference OTU279	Eukaryota, SAR, Stramenopiles, Chrysophyceae, Ochromonadales, Ochromonas, Ochromonas sp. TCS-2004
New.Reference OTU4790	Eukaryota, SAR, Stramenopiles, Chrysophyceae, Ochromonadales, Ochromonas, uncultured stramenopile
New.Reference OTU1288	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Achnanthes, Achnanthidium, Achnanthidium coarctatum
AJ535148	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Cocconeis, Cocconeis cf. molesta
New.Reference OTU2770	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Gyrosigma, Gyrosigma acuminatum
EU050967	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Navicula, uncultured eukaryote
New.Reference OTU5770	Eukaryota, SAR, Stramenopiles, Dictyochophyceae, Pedinellales, Pteridomonas, uncultured eukaryote
New.Reference OTU1442	Eukaryota, SAR, Stramenopiles, Labyrinthulomycetes, Labyrinthula, Labyrinthula sp. 01-Jy-1b
New.Reference OTU4467	Eukaryota, SAR, Stramenopiles, Labyrinthulomycetes, Thraustochytriaceae, Aplanochytrium, Aplanochytrium sp. S1a
New.Reference OTU5179	Eukaryota, SAR, Stramenopiles, Labyrinthulomycetes, Thraustochytriaceae, Thraustochytrium, Thraustochytrium aureum
New.Reference OTU1886	Eukaryota, SAR, Stramenopiles, MAST-12, Stramenopile, Stramenopile sp. MAST-12 KKTS D3
New.Reference OTU567	Eukaryota, SAR, Stramenopiles, Peronosporomycetes, Haliphthoros, Haliphthoros sp. NJM 0034
EF418924	Eukaryota, SAR, Stramenopiles, Peronosporomycetes, Phytium, Pythium, Pythium ostracodes
New.Reference OTU5583	Eukaryota, Zeuk77, uncultured Eimeriidae

V6 All Crust Water Column

OTU ID	**Taxonomic Identity**
812759	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
837283	Bacteria, Fusobacteria, Fusobacteriia, Fusobacteriales, Fusobacteriaceae, Cetobacterium, somerae
787856	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3

4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4345424	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4336993	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
589202	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter
4409846	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Vibrio
4409489	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.Reference OTU5441	No blast hit

V9 All Crust Water Column

OTU ID	**Taxonomic Identity**
JN874361	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Ruegeria, uncultured bacterium
AM503930	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, Naked, Naked dinoflagellate UDNSW0701

V6 Cave Benthos

OTU ID	**Taxonomic Identity**
3269318	Bacteria, Acidobacteria, Chloracidobacteria, PK29
New.Reference OTU7823	Bacteria, Acidobacteria, Chloracidobacteria, RB41
4448087	Bacteria, Acidobacteria, Chloracidobacteria, RB41, Ellin6075
4400369	Bacteria, Acidobacteria, Chloracidobacteria, RB41, Ellin6075
4442075	Bacteria, Acidobacteria, Acidobacteria-6, CCU21
4117488	Bacteria, Acidobacteria, Acidobacteria-6, CCU21
587792	Bacteria, Acidobacteria, Acidobacteria-6, CCU21
4260701	Bacteria, Acidobacteria, Acidobacteria-6, iii1-15
810967	Bacteria, Acidobacteria, Acidobacteria-6, iii1-15, mb2424
801421	Bacteria, Acidobacteria, RB25
New.Reference OTU4225	Bacteria, Acidobacteria, Solibacteres, Solibacterales, PAUC26f
270614	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales
11418	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales
365923	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales
4405403	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales
245756	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales
4471428	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, C111
3127356	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, C111
4418306	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, C111
1130769	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, C111, Ilumatobacter, fluminis
1956921	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, JdFBGBact
175311	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, wb1 P06
1056445	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales, Kineosporiaceae

4472103	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales, Nocardioidaceae, Kribbella
551895	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae
New.ReferenceOTU8493	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.ReferenceOTU6734	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
199016	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Cyclobacteriaceae
104298	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Cytophagaceae
349159	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales
4156020	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, Crocinitomix
838871	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
308318	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
4467414	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
2815573	Bacteria, Bacteroidetes, Sphingobacteriia, Sphingobacteriales
710866	Bacteria, Chloroflexi, Anaerolineae, S0208
4483859	Bacteria, Chloroflexi, Anaerolineae, S0208
New.ReferenceOTU3172	Bacteria, Chloroflexi, Anaerolineae, SHA-20
4455477	Bacteria, Chloroflexi, Ellin6529
102760	Bacteria, Chloroflexi, Ellin6529
593363	Bacteria, Chloroflexi, Ellin6529
New.ReferenceOTU2353	Bacteria, Cyanobacteria, Chloroplast
1012766	Bacteria, Cyanobacteria, Chloroplast, Stramenopiles
807561	Bacteria, Cyanobacteria, Chloroplast, Stramenopiles
1105902	Bacteria, Cyanobacteria, Chloroplast, Stramenopiles
837283	Bacteria, Fusobacteria, Fusobacteriia, Fusobacteriales, Fusobacteriaceae, Cetobacterium, somerae
4428033	Bacteria, Gemmatimonadetes, Gemm-1
336425	Bacteria, Gemmatimonadetes, Gemm-1
4356025	Bacteria, Gemmatimonadetes, Gemm-1
227014	Bacteria, Gemmatimonadetes, Gemm-2
4352971	Bacteria, Gemmatimonadetes, Gemm-2
4436238	Bacteria, Gemmatimonadetes, Gemmatimonadetes, KD8-87
76374	Bacteria, Gemmatimonadetes, Gemmatimonadetes, KD8-87
4387300	Bacteria, Nitrospirae, Nitrospira, Nitrospirales, Nitrospiraceae, Nitrospira
4365596	Bacteria, Nitrospirae, Nitrospira, Nitrospirales, Nitrospiraceae, Nitrospira
2160921	Bacteria, Nitrospirae, Nitrospira, Nitrospirales, Nitrospiraceae, Nitrospira
4316523	Bacteria, Planctomycetes, C6, d113
811450	Bacteria, Planctomycetes, C6, d113
327521	Bacteria, Planctomycetes, OM190, CL500-15
4454320	Bacteria, Planctomycetes, OM190, CL500-15

695617	Bacteria, Planctomycetes, OM190, CL500-15
628974	Bacteria, Planctomycetes, OM190, CL500-15
4406062	Bacteria, Planctomycetes, OM190, CL500-15
New.ReferenceOTU304	Bacteria, Planctomycetes, Pla3
1083816	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
4445752	Bacteria, Proteobacteria, Alphaproteobacteria
4394196	Bacteria, Proteobacteria, Alphaproteobacteria
816404	Bacteria, Proteobacteria, Alphaproteobacteria
4464051	Bacteria, Proteobacteria, Alphaproteobacteria
4393213	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4336993	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
191415	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4471228	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4341675	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium
New.ReferenceOTU4465	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium
4451543	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Pedomicrobium, australicum
4482023	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Rhodoplanes
4434021	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae
344370	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, Mesorhizobium
4484310	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Hyphomonadaceae
4365850	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Hyphomonadaceae
906323	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Hyphomonadaceae
New.ReferenceOTU7694	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
3889679	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae
4454486	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae
4453083	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales
4323160	Bacteria, Proteobacteria, Betaproteobacteria
4406766	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
2801319	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Methylibium
4433654	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Methylibium
1115097	Bacteria, Proteobacteria, Deltaproteobacteria, NB1-j
New.ReferenceOTU5594	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Sulfurimonas
800619	Bacteria, Proteobacteria, Gammaproteobacteria
4041621	Bacteria, Proteobacteria, Gammaproteobacteria

4390688	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae, Rheinheimera
4452444	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae
2718805	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio
4315336	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, HTCC2188, HTCC
2000018	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, OM60
313062	Bacteria, Proteobacteria, Gammaproteobacteria, Chromatiales, Ectothiorhodospiraceae
566976	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae
4338666	Bacteria, Proteobacteria, Gammaproteobacteria, HTCC2188, HTCC2089
New.ReferenceOTU6726	Bacteria, Proteobacteria, Gammaproteobacteria, HTCC2188, HTCC2089
589202	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter
New.ReferenceOTU6817	Bacteria, Proteobacteria, Gammaproteobacteria, PYR10d3
3726190	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
4373421	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
4421345	Bacteria, Proteobacteria, Gammaproteobacteria, Thiotrichales, Piscirickettsiaceae
834418	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae
4357767	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae
4415230	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae
4371709	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae
4379538	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae
4446651	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae
291608	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Luteimonas
561970	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Luteimonas
570109	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Pseudoxanthomonas, mexicana
849553	Bacteria, Verrucomicrobia, Pedosphaerae
4419418	Bacteria, Verrucomicrobia, Pedosphaerae
3029735	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales
1131351	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales
807678	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales
4417923	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales
4319881	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales
4388032	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales
New.ReferenceOTU8025	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales, Pedosphaeraceae
4477307	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales, auto67 4W
4329822	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales, auto67 4W
161448	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales, Ellin515
3849648	Bacteria, Verrucomicrobia, Pedosphaerae, Pedosphaerales, R4-41B
236458	Bacteria, Verrucomicrobia, Spartobacteria, Chthoniobacterales, Chthoniobacteraceae

247767	Bacteria, Verrucomicrobia, Opitutae
959419	Bacteria, Verrucomicrobia, Opitutae, Opitutales, Opitutaceae
4444335	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Prosthecobacter
4370744	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium

V6 Cave Water Column

OTU ID	**Taxonomic Identity**
New.Reference OTU8493	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
139618	Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales
4327032	Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae, Prevotella,
364651	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales
316494	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
4385994	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
643716	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae
4467414	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
249417	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
39169	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
4385507	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
4395488	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium
108074	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Flavobacterium, succinicans
New.Reference OTU5968	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Microcystaceae, Microcystis
3540195	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Xenococcaceae
2990433	Bacteria, Fusobacteria, Fusobacteriia, Fusobacteriales
837283	Bacteria, Fusobacteria, Fusobacteriia, Fusobacteriales, Fusobacteriaceae, Cetobacterium, somerae
New.Reference OTU17	Bacteria, Lentisphaerae, Lentisphaeria, Victivallales, Victivallaceae
4434021	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae
4453923	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Rhodobacter
320516	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
324220	Bacteria, Proteobacteria, Betaproteobacteria, Methylophilales
4438992	Bacteria, Proteobacteria, Betaproteobacteria, Neisseriales, Neisseriaceae
161369	Bacteria, Proteobacteria, Deltaproteobacteria, Desulfobacterales, Desulfobulbaceae
237963	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales
New.Reference OTU2036	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Campylobacteraceae, Arcobacter

New.ReferenceOTU7232	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Sulfuricurvum, kujiense
559104	Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae
4356823	Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, sharmana
4465421	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae, Rheinheimera
4390688	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Chromatiaceae, Rheinheimera
822382	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae
4353369	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae
2718805	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio
4260142	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio
52677	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Alteromonadaceae, Cellvibrio
358229	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, Shewanellaceae, Shewanella
362155	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae
297311	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Enterobacter, cloacae
4371014	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Erwinia
684433	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Klebsiella
211586	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Providencia
New.ReferenceOTU403	Bacteria, Proteobacteria, Gammaproteobacteria, Legionellales, Francisellaceae
New.ReferenceOTU241	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Hahellaceae
568874	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillaceae, Marinomonas
4335515	Bacteria, Proteobacteria, Gammaproteobacteria, Pasteurellales, Pasteurellaceae, Haemophilus, parainfluenzae
589202	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter
533635	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter
821653	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter, lwoffii
4364205	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Azomonas, agilis
722635	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Pseudomonas, stutzeri
New.ReferenceOTU6817	Bacteria, Proteobacteria, Gammaproteobacteria, PYR10d3
837313	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Pseudoalteromonadaceae
816715	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae

4407228	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Photobacterium
4301591	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Vibrio
4347599	Bacteria, Proteobacteria, Gammaproteobacteria, Vibrionales, Vibrionaceae, Vibrio, metschnikovii
269231	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae
1125638	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Sinobacteraceae, Steroidobacter
4361345	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae
570109	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Pseudoxanthomonas, mexicana
New.Reference OTU903	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Stenotrophomonas
959419	Bacteria, Verrucomicrobia, Opitutae, Opitutales, Opitutaceae
New.Reference OTU3545	No blast hit
New.Reference OTU2349	No blast hit

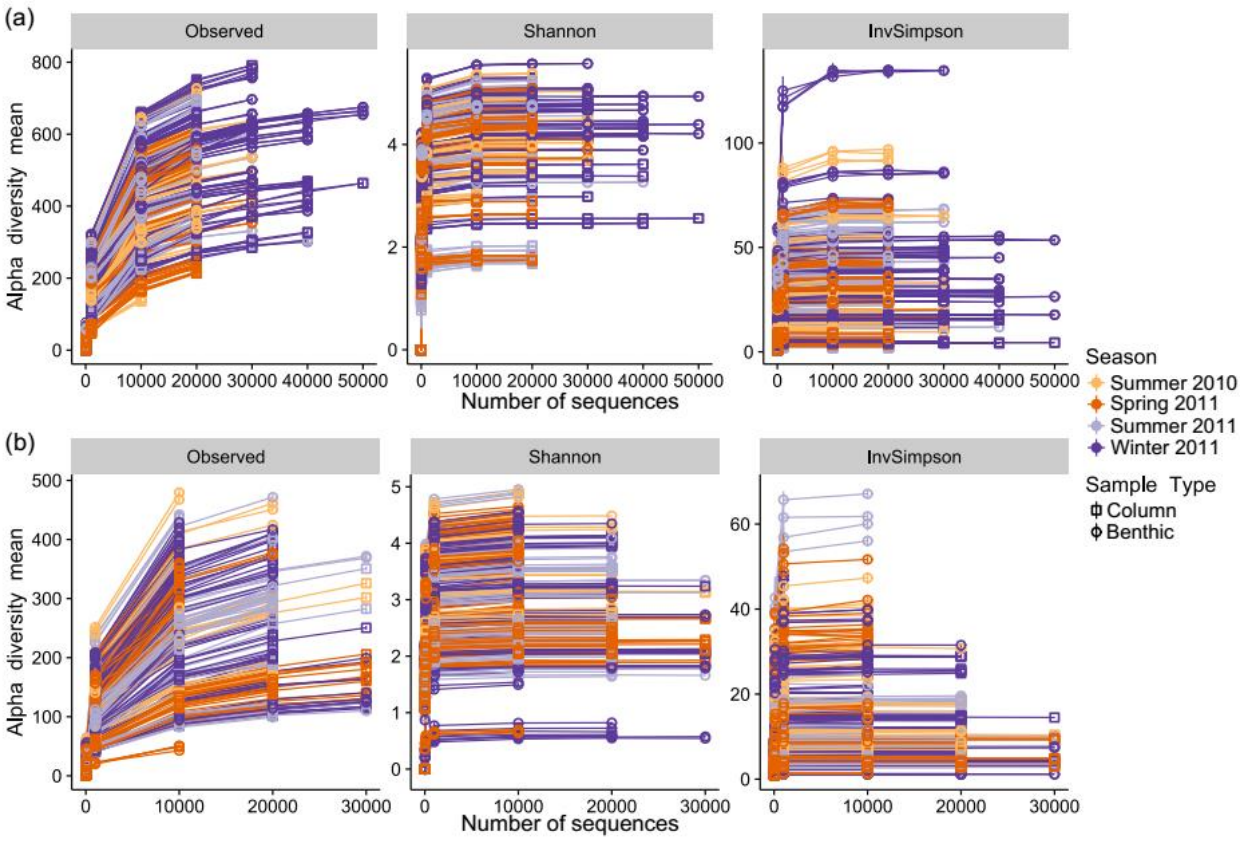
V9 Cave Water Column

OTU ID	**Taxonomic Identity**
AF130897	Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Pantoea, Pantoea agglomerans
U42426	Eukaryota, Archaeplastida, Chloroplastida, Charophyta, Phragmoplastophyta, Streptophyta, Embryophyta, Tracheophyta, Spermatophyta, Magnoliophyta, Cucurbitales, Datisca, Datisca glomerata
New.Reference OTU834	Eukaryota, Cryptophyceae, Cryptomonadales, Cryptomonas, Cryptomonas paramecium
New.Reference OTU1389	Eukaryota, Haptophyta, Prymnesiophyceae, Prymnesiales, Chrysochromulina, uncultured marine eukaryote
New.Reference OTU4926	Eukaryota, Opisthokonta, Fungi, Ascomycota, Pezizomycotina, Dothideomycetes, Pleosporomycetidae, Pleosporales, Mytilinidiaceae, Mytilinidion, Mytilinidion mytilinellum
New.Reference OTU1367	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Peniculia, Paramecium, Paramecium bursaria
AB505576	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Litostomatea, Mesodiniidae, uncultured marine eukaryote
New.Reference OTU4309	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, uncultured marine eukaryote
New.Reference OTU2482	Eukaryota, SAR, Alveolata, Dinoflagellata, Dinophyceae, Gymnodiniphycidae, Gyrodinium, uncultured marine eukaryote
New.Reference OTU4760	Eukaryota, SAR, Alveolata, FV18-2D11, uncultured eukaryote
New.Reference OTU5308	Eukaryota, SAR, Alveolata, Protalveolata, Syndiniales, Amoebophrya, Amoebophrya sp. ex Gonyaulax polygramma

Appendix 2. Environmental factor (vectors and factors) correlations with NMDS ordination of anchialine *Bacteria* (V6) and micro-*Eukarya* (V9) community composition from sampled anchialine habitats on the islands of Hawaii, Maui and Oahu.

Environmental Factors		V6		V9	
		r^2	Pr(>*r*)	r^2	Pr(>*r*)
Vectors	Annual Rainfall	0.4797*	0.000999	0.6367*	0.001
	Mean Annual Solar Radiation	0.1794*	0.000999	0.0638*	0.02
	Salinity	0.7885*	0.000999	0.6451*	0.001
	Nitrite & Nitrate	0.1382*	0.000999	0.0449	0.075
	Orthophosphate	0.2278*	0.000999	0.1888*	0.001
	Silica	0.111*	0.000999	0.0452	0.068
	Ammonium	0.5935*	0.000999	0.4445*	0.001
	Dissolved Organic Carbon (DOC)	0.3889*	0.000999	0.5544*	0.001
	Total Dissolved Nitrogen (TDN)	0.0949*	0.002997	0.0286	0.187
	Total Dissolved Phosphorus (TDP)	0.2104*	0.000999	0.1716*	0.001
	Latitude	0.4331*	0.000999	0.5015*	0.001
	Longitude	0.4868*	0.000999	0.6096*	0.001
Factors	Site	0.8568*	0.000999	0.858*	0.001
	Island	0.3864*	0.000999	0.3131*	0.001
	Crust Presence	0.4308*	0.000999	0.3741*	0.001
	Benthic Substrate	0.2263*	0.000999	0.2011*	0.001
	Pond or Cave	0.2366*	0.000999	0.3543*	0.001
	Fish Presence	0.4414*	0.000999	0.4994*	0.001
	Goat Presence	0.4028*	0.000999	0.3256*	0.001
	Public Accessibility	0.2419*	0.000999	0.2135*	0.001
	Sample type	0.1463*	0.000999	0.1888*	0.001
	DLNR Aquifer	0.8061*	0.000999	0.8243*	0.001
	Watershed	0.8203*	0.000999	0.8205*	0.001
	Potential Warm Groundwater	0.4028*	0.000999	0.3256*	0.001

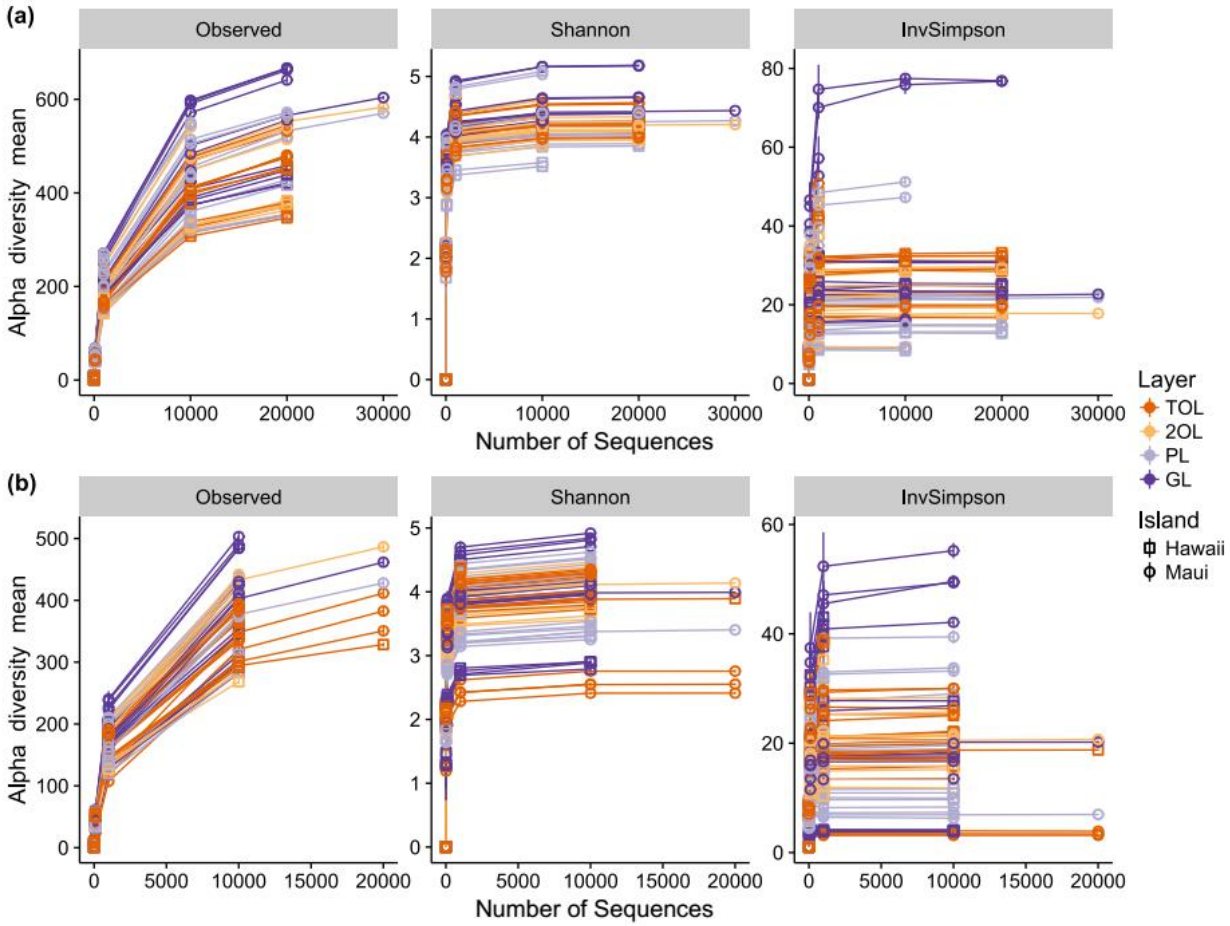
** Significance at* $P < 0.05$ *based on 999 permutations.*



Appendix 3. Diversity estimates, specifically number of observed OTUs, Shanon diversity, and Inverse Simpson diversity, of the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene (**a)**, and the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene (**b**). Samples were grouped by benthos and water column communities within sites and seasons.

Appendix 4. Environmental variable (vectors and factors) correlations with NMDS ordination of anchialine microbial community composition. Significance at $p<0.05$ based on 999 permutations is indicated by an asterix.

Environmental Variables		V6		V9	
		r^2	Pr(>*r*)	r^2	Pr(>*r*)
Vectors	Annual Rainfall	0.6884*	0.001	0.5953*	0.001
	Sample Month Rainfall	0.4260*	0.001	0.3302*	0.001
	15 Month Prior Rainfall	0.1119*	0.001	0.0870*	0.001
	Mean Annual Solar Radiation	0.6709*	0.001	0.5123*	0.001
	Salinity	0.6672*	0.001	0.4611*	0.001
	Nitrite & Nitrate	0.6402*	0.001	0.5114*	0.001
	Phosphate	0.2221*	0.001	0.1870*	0.001
	Si	0.6333*	0.001	0.5214*	0.001
	Ammonium	0.4371*	0.001	0.4094*	0.001
	DOC	0.3550*	0.001	0.2819*	0.001
	TDN	0.5968*	0.001	0.4597*	0.001
	TDP	0.2263*	0.001	0.1978*	0.001
	Latitude	0.8199*	0.001	0.7539*	0.001
	Longitude	0.9029*	0.001	0.6925*	0.001
Factors	Site	0.8157*	0.001	0.7186*	0.001
	Island	0.3583*	0.001	0.2343*	0.001
	Season	0.0157*	0.028	0.0110	0.109
	Season Type	0.0045	0.115	0.0030	0.246
	Fish Presence	0.4524*	0.001	0.2701*	0.001
	Goat Presence	0.2684*	0.001	0.2032*	0.001
	Public Accessibility	0.4346*	0.001	0.2634*	0.001
	Sample Type	0.1593*	0.001	0.2242*	0.001
	DLNR Aquifer	0.7807*	0.001	0.6352*	0.001
	Watershed	0.7976*	0.001	0.6679*	0.001
	Potential Warm Groundwater	0.2684*	0.001	0.2032*	0.001



Appendix 5. Diversity estimates, specifically number of observed OTUs, Shanon diversity, and Inverse Simpson diversity, of the *Bacteria*-specific V6 hypervariable region of the 16S-rDNA gene (**a)**, and the *Eukarya*-biased V9 hypervariable region of the 18S-rDNA gene (**b**). Samples were grouped by layer within the orange crust structure and island. In order from top to bottom, the layers are top orange layer (TOL), second orange layer (2OL), pink layer (PL), and green layer (GL).

Appendix 6. Environmental factor (vectors and factors) correlations with NMDS ordination of anchialine *Bacteria* (V6) and micro-*Eukarya* (V9) layer consortia composition from sampled anchialine habitats on the islands of Maui and Hawaii.

Environmental Factors		**V6**		**V9**	
		r^2	**Pr(>*r*)**	r^2	**Pr(>*r*)**
Vectors	Annual Rainfall	0.7829*	0.001	0.8298*	0.001
	Mean Annual Solar Radiation	0.8866*	0.001	0.8878*	0.001
	Salinity	0.9635*	0.001	0.9625*	0.001
	Nitrite & Nitrate	0.8380*	0.001	0.8490*	0.001
	Orthophosphate	0.3639*	0.001	0.5298*	0.001
	Silica	0.8036*	0.001	0.8441*	0.001
	Ammonium	0.1422*	0.003	0.0965*	0.009
	Dissolved Organic Carbon (DOC)	0.2455*	0.001	0.0855*	0.021
	Total Dissolved Nitrogen (TDN)	0.8611*	0.001	0.8728*	0.001
	Total Dissolved Phosphorus (TDP)	0.3610*	0.001	0.5335*	0.001
	Latitude	0.8829*	0.001	0.9060*	0.001
	Longitude	0.9551*	0.001	0.9566*	0.001
Factors	Site	0.9532*	0.001	0.9567*	0.001
	Layer	0.0198	0.620	0.0201	0.657
	Island	0.7922*	0.001	0.7828*	0.001
	Fish Presence	0.8394*	0.001	0.8631*	0.001
	Goat Presence	0.2998*	0.001	0.3049*	0.001
	Public Accessibility	0.7922*	0.001	0.7828*	0.001
	DLNR Aquifer	0.8319*	0.001	0.8174*	0.001
	Watershed	0.8492*	0.001	0.8334*	0.001
	Potential Warm Groundwater	0.4515*	0.001	0.4473*	0.001

** Significance at* $P < 0.05$ *based on 999 permutations.*

Appendix 7. Core *Bacteria* (V6) and micro-*Eukarya* (V9) OTUs, that is those present in all samples, and their taxonomic identities for the constoria belonging to each of the four layers observed in orange cyanobacterial-bacterial crusts from sampled anchialine habitats on the islands of Hawaii and Maui.

V6 Top Orange Layer

OTU ID	**Taxonomic Identity**
4453882	Bacteria, Proteobacteria, Alphaproteobacteria
278809	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
4345424	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
808124	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Cohaesibacteraceae
278327	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
4336993	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
834330	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
1938968	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
4474732	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
3371208	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4467345	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4471228	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4370744	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.ReferenceOTU703	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Hyphomonadaceae, Hirschia
New.ReferenceOTU2499	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Xenococcaceae, Chroococcidiopsis
New.ReferenceOTU2673	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.ReferenceOTU212	No blast hit
New.ReferenceOTU1565	Bacteria, Cyanobacteria
New.ReferenceOTU2915	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Cyanobacteriaceae, Cyanothece
New.ReferenceOTU3402	No blast hit
New.ReferenceOTU1288	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3

V9 Top Orange Layer

OTU ID	**Taxonomic Identity**

JN082658	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, uncultured Paracoccus sp.
JN024028	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, uncultured bacterium
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
JN637794	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Loktanella, uncultured marine microorganism
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium
AB183646	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Nitzschia, Bacillariophyta, Bacillariophyta sp. MBIC10816
FJ152760	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Gaetbulicola, uncultured bacterium
GU302454	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, uncultured, uncultured bacterium
JN178071	Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, Trichococcus, uncultured Trichococcus sp.
AB035544	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, Erythrobacter sp. MBIC4117
JN178703	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured Rhodobacteraceae bacterium
EF100150	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, Azospirillum, Azospirillum lipoferum
DD437360	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, Paracoccus sp. 101
FJ516864	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured Hyphomonadaceae bacterium
EU360293	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Thalassobius, uncultured Thalassobius sp.
FJ716891	Bacteria, Proteobacteria, Alphaproteobacteria, SB1-18, uncultured bacterium
FM873449	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Candidatus Alysiosphaera, uncultured bacterium
New.ReferenceOTU1069	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured bacterium
New.ReferenceOTU1939	Eukaryota, Excavata, Discoba, Discicristata, Euglenozoa, Kinetoplastea, Metakinetoplastina, Neobodonida, Rhynchomonas, Rhynchomonas nasuta
New.ReferenceOTU3073	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Phyllopharyngea, Cyrtophoria, Chlamydodon, uncultured eukaryote
New.ReferenceOTU763	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Scuticociliatia, Metanophrys, Metanophrys sinensis
New.ReferenceOTU2852	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales, Phycisphaeraceae, SM1A02, uncultured Planctomycetales bacterium
New.ReferenceOTU2966	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
New.ReferenceOTU2996	Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, Trichococcus, uncultured Trichococcus sp.

V6 2nd Orange Layer

OTU ID	Taxonomic Identity
278809	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
808124	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Cohaesibacteraceae
278327	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae
4460895	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Cyanobacteriaceae
4472222	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae
834330	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
3846383	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
1938968	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
4474732	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
4467345	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
4471228	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
New.ReferenceOTU2673	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
New.ReferenceOTU212	No blast hit
New.ReferenceOTU1565	Bacteria, Cyanobacteria

V9 2nd Orange Layer

OTU ID	Taxonomic Identity
JN082658	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, uncultured Paracoccus sp.
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
JN637794	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Loktanella, uncultured marine microorganism
JN178401	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, Sandaracinus, Sandaracinus amylolyticus
EF636835	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, uncultured delta proteobacterium
EF208657	Bacteria, Planctomycetes, OM190, uncultured bacterium
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium
AB183646	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Nitzschia, Bacillariophyta, Bacillariophyta sp. MBIC10816

GQ452862	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Phaeodactylum, Phaeodactylum tricornutum
FJ152760	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Gaetbulicola, uncultured bacterium
GU302454	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, uncultured, uncultured bacterium
JN178071	Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, Trichococcus, uncultured Trichococcus sp.
AB035544	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, Erythrobacter sp. MBIC4117
JN178703	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured Rhodobacteraceae bacterium
FJ516864	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured Hyphomonadaceae bacterium
EU360293	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Thalassobius, uncultured Thalassobius sp.
New.ReferenceOTU766	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Candidatus Alysiosphaera, uncultured alpha proteobacterium
New.ReferenceOTU763	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Scuticociliatia, Metanophrys, Metanophrys sinensis
New.ReferenceOTU2966	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
New.ReferenceOTU2996	Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, Trichococcus, uncultured Trichococcus sp.

V6 Pink Layer

OTU ID	**Taxonomic Identity**
4453882	Bacteria, Proteobacteria, Alphaproteobacteria
278809	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
4460895	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Cyanobacteriaceae
834330	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
3846383	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
4479751	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
206913	Bacteria, Bacteroidetes, Cytophagia, Cytophagales, Flammeovirgaceae
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
628974	Bacteria, Planctomycetes, OM190, CL500-15
3127356	Bacteria, Actinobacteria, Acidimicrobiia, Acidimicrobiales, C111
4467345	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
1115987	Bacteria, Proteobacteria, Alphaproteobacteria

4471228	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4370744	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.ReferenceOTU3636	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
New.ReferenceOTU1565	Bacteria, Cyanobacteria
New.ReferenceOTU2915	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Cyanobacteriaceae, Cyanothece
New.ReferenceOTU3402	No blast hit

V9 Pink Layer

OTU ID	**Taxonomic Identity**
JN082658	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, uncultured Paracoccus sp.
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
JN178401	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, Sandaracinus, Sandaracinus amylolyticus
EF636835	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, uncultured delta proteobacterium
EF208657	Bacteria, Planctomycetes, OM190, uncultured bacterium
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium
GU302454	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, uncultured, uncultured bacterium
JN178071	Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, Trichococcus, uncultured Trichococcus sp.
FJ467624	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium
FN667474	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Rhodobium, uncultured bacterium
FJ624355	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Pirellula, uncultured Planctomycetaceae bacterium
EU360293	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Thalassobius, uncultured Thalassobius sp.
JN391734	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Rhodobium, uncultured bacterium
New.ReferenceOTU917	No blast hit
New.ReferenceOTU766	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Candidatus Alysiosphaera, uncultured alpha proteobacterium
New.ReferenceOTU763	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Scuticociliatia, Metanophrys, Metanophrys sinensis
New.ReferenceOTU2844	Eukaryota, Opisthokonta, Metazoa, Nematoda, Chromadorea, Monhysteridae, Monhysteridae environmental sample
New.ReferenceOTU2966	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium

V6 Green Layer

OTU ID	**Taxonomic Identity**

278809	Bacteria, Cyanobacteria, Synechococcophycideae, Pseudanabaenales, Pseudanabaenaceae, Halomicronema
4454320	Bacteria, Planctomycetes, OM190, CL500-15
4460895	Bacteria, Cyanobacteria, Oscillatoriophycideae, Chroococcales, Cyanobacteriaceae
4399225	Bacteria, Proteobacteria, Gammaproteobacteria, Alteromonadales, OM60, Congregibacter
4336993	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
834330	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter
151374	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, aquimaris
353953	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4329245	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
3846383	Bacteria, Planctomycetes, Phycisphaerae, Phycisphaerales
1938968	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
4435809	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4421632	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
3057523	Bacteria, Planctomycetes, Planctomycetia, Pirellulales, Pirellulaceae
4474732	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae
3371208	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
628974	Bacteria, Planctomycetes, OM190, CL500-15
4467345	Bacteria, Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae
191415	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4477805	Bacteria, Proteobacteria, Alphaproteobacteria, BD7-3
1115987	Bacteria, Proteobacteria, Alphaproteobacteria
4471228	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae
4370744	Bacteria, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium
New.ReferenceOTU1565	Bacteria, Cyanobacteria
New.ReferenceOTU3402	No blast hit
New.ReferenceOTU3298	Bacteria, Planctomycetes, OM190, CL500-15

V9 Green Layer

OTU ID	**Taxonomic Identity**
JN082658	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, uncultured Paracoccus sp.
Y14302	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium, Hyphomicrobium vulgare
HM030990	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Sphingomonas, marine bacterium KS-9-10-4

EF636835	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Sorangiineae, Sandaracinaceae, uncultured delta proteobacterium
EF208657	Bacteria, Planctomycetes, OM190, uncultured bacterium
JN790962	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Wenxinia, uncultured alpha proteobacterium
AB183646	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Nitzschia, Bacillariophyta, Bacillariophyta sp. MBIC10816
GQ452862	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Phaeodactylum, Phaeodactylum tricornutum
FJ152760	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Gaetbulicola, uncultured bacterium
GU302454	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, uncultured, uncultured bacterium
JN178071	Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, Trichococcus, uncultured Trichococcus sp.
AB035544	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, Erythrobacter sp. MBIC4117
EF100150	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, Azospirillum, Azospirillum lipoferum
JF834543	Eukaryota, SAR, Stramenopiles, Diatomea, Bacillariophytina, Bacillariophyceae, Amphora, Amphora sp. PP-2011
FN667474	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Rhodobium, uncultured bacterium
FJ624355	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Pirellula, uncultured Planctomycetaceae bacterium
DD437360	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Paracoccus, Paracoccus sp. 101
FJ516864	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured Hyphomonadaceae bacterium
EU360293	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Thalassobius, uncultured Thalassobius sp.
JN391734	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhodobiaceae, Rhodobium, uncultured bacterium
FM873449	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Candidatus Alysiosphaera, uncultured bacterium
New.ReferenceOTU2558	Bacteria, Proteobacteria, Alphaproteobacteria, DB1-14, uncultured alpha proteobacterium
New.ReferenceOTU3073	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Phyllopharyngea, Cyrtophoria, Chlamydodon, uncultured eukaryote
New.ReferenceOTU766	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Candidatus Alysiosphaera, uncultured alpha proteobacterium
New.ReferenceOTU763	Eukaryota, SAR, Alveolata, Ciliophora, Intramacronucleata, Conthreep, Oligohymenophorea, Scuticociliatia, Metanophrys, Metanophrys sinensis
New.ReferenceOTU2966	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium