Vol. 82: 87–104, 2018
https://doi.org/10.3354/ame01884

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online September 20

Comparing the community structure of *Bacteria* and micro-*Eukarya* from the Hawaiian anchialine ecosystem during wet and dry seasons

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ABSTRACT: The anchialine ecosystem, defined as tidally influenced, nearshore bodies of water with subterranean freshwater and seawater connections, has been relatively unstudied regarding its microbial communities. Notably, anchialine habitats of the Hawaiian Archipelago, specifically the Cape Kinau (Maui) and Kona (Hawaii) regions, can possess distinctive, laminated orange cyanobacterial–bacterial crusts found nowhere else worldwide. However, nearly nothing is known about the degree to which seasonal fluctuations in water chemistry might influence shifts in their community composition. To test the hypothesis that taxonomic diversity and relative sequence abundance exhibit dynamics correlating to particular seasonal environmental factors, benthic and water column microbial communities from 6 habitats in these 2 geographical regions were examined during 2 wet and 2 dry seasons via high-throughput sequencing of hypervariable V6 (*Bacteria*-specific) and V9 (micro-*Eukarya*-biased) regions from rRNA genes. Although significant seasonal variation occurred in water chemistry characteristics such as salinity, ammonium, dissolved organic carbon, total dissolved nitrogen, and nitrite + nitrate during the same period, overall benthic and water column microbial community structure instead correlated with spatial factors such as latitude and annual rainfall. Despite this, approximately half of the identified third-level clades (i.e. approximately class-level nomenclatures) within these communities exhibited variation in relative sequence abundances between wet and dry seasons. Furthermore, relative abundance changes for approximately three-quarters of these clades correlated with at least one seasonally varying factor. Thus, while microbial communities in the Hawaiian anchialine ecosystem did not show stable composition year-round, overall community structure correlated more strongly with the spatial, rather than temporal, context of any given habitat.

KEY WORDS: Illumina amplicon sequencing · Microbial diversity · Seasonal variation · Sediment · V6 hypervariable region 16S rRNA gene · V9 hypervariable region 18S rRNA gene · Water

INTRODUCTION

The anchialine ecosystem, first defined by Holthuis (1973, p. 3) as 'pools with no surface connection with the sea, containing salt or brackish water, which fluctuates with the tides', includes land-locked bodies of water tidally influenced by a confluence of marine and groundwaters (Bishop et al. 2015). Such habitats are primarily localized to the tropics (Maciolek 1983), lack surface connections to the ocean (Holthuis 1973, Stock et al. 1986), and occur within a variety of basin substrates, including karst caves, cenotes, natural

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wells and springs, fossilized coral reefs, and coastal basalt (i.e. lava) fields (Sket 1996). Given their simultaneous marine and groundwater connections, anchialine habitats can exhibit complex physical and chemical clines in addition to widely varying salinities across the tidal cycle (Stock et al. 1986, Humphreys 1999, Bishop et al. 2004). Although a number of studies have documented great species richness and endemism among anchialine macroorganisms (reviewed in Maciolek 1983, Weese et al. 2013), the inherent challenges of examining microorganisms (e.g. small size, low morphological diversity) have historically resulted in relatively little attention being given to the microbial communities occupying habitats of this ecosystem (Sarbu et al. 1996, Pohlman et al. 1997, Seymour et al. 2007, Garman et al. 2011, Gonzalez et al. 2011, Humphreys et al. 2012, Krstulović et al. 2013, Hoffman et al. 2018).

While spatial factors have been shown to significantly influence microbial community structure of the anchialine ecosystem (e.g. Seymour et al. 2007, Humphreys et al. 2012, Hoffman et al. 2018), less is known regarding how community composition might be impacted by temporal or seasonal fluctuations in environmental variables. Such information is important from an ecosystem perspective since microbes fulfill vital roles such as primary production (Dalton et al. 2013) and the facilitation of nutrient cycling (van der Heijden et al. 2008), and thus any environmental factor influencing the microbial component of an ecosystem can potentially impact higher trophic levels as well. In this context, only 2 studies to date have examined patterns of seasonal variation in microbial community composition from the anchialine ecosystem as well as the water chemistry and environmental factors potentially influencing such dynamics. Specifically, examination of bacterioplankton abundance in anchialine caves in the Yucatan Peninsula (Mexico) found greater bacterioplankton density during the rainy season (Alcocer et al. 1999). This increased density appeared to be driven by population growth due to increased nutrient input in conjunction with transient surface bacteria being washed into the habitat (Alcocer et al. 1999). Similarly, microbial communities from anchialine caves on Mljet Island in the Adriatic Sea were found to exhibit shifts in relative and total bacterial abundance and an influence of bottom-up versus top-down control across a 21-mo sampling period (Krstulović et al. 2013).

Currently, the impact of seasonal fluctuations in environmental variables on the microbial community of the anchialine ecosystem has yet to be explored in the Hawaiian Archipelago (Fig. 1A). The climate in Hawaii is characterized by wet and dry seasons, and this archipelago possesses the greatest concentration of anchialine habitats on the planet. Hawaiian anchialine habitats comprise a wide variety of environmental conditions with respect to basin substrate, temperature, and salinity (Craft et al. 2008, Havird et al. 2013, 2014, Hoffman et al. 2018); the food web within these systems is based on their microbial communities (Bailey-Brock & Brock 1993, Dalton et al. 2013). Notably, a number of anchialine habitats within the Cape Kinau and Kona regions of the islands of Maui and Hawaii, respectively, can possess distinctive, laminated orange cyanobacterial–bacterial crusts (Fig. 1B,C) found nowhere else in the world

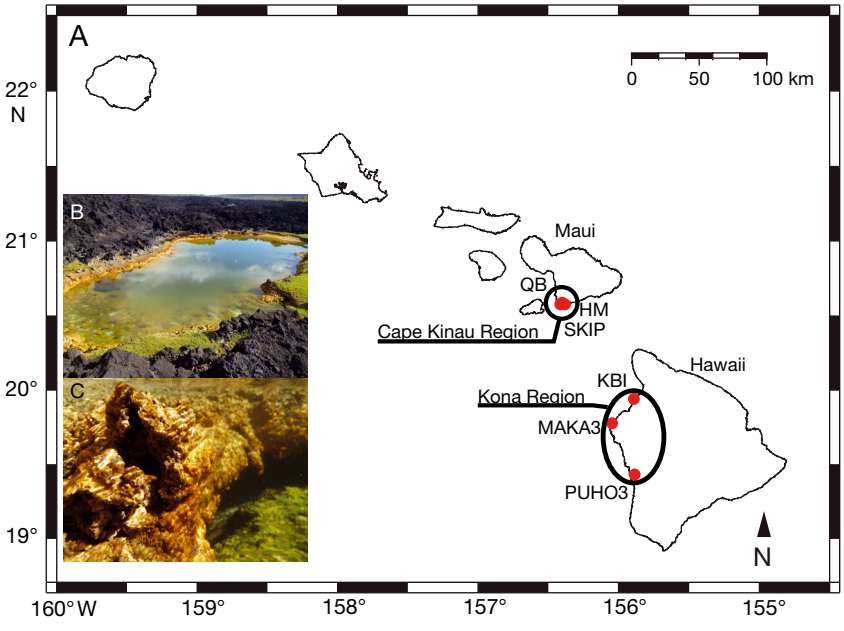

Fig. 1. (A) Map depicting the Hawaiian Islands and sampled anchialine habitats on Maui and Hawaii, with the regions of Cape Kinau and Kona regions indicated with open ellipses. Sites were visited in 2 dry seasons (July 2010 and 2011) and 2 wet seasons (March and December 2011). (B) Example of a Hawaiian anchialine open pool habitat (i.e. site SKIP) with the orange cyanobacterial–bacterial crust community found at Cape Kinau, Maui. (C) Close-up of an orange cyanobacterial–bacterial crust community (i.e. site SKIP) found at Cape Kinau, Maui. See Table 1 for site abbreviations

(Wong 1975, Bailey-Brock & Brock 1993, Hoffman et al. 2018). Phenotypically similar 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 lowering temperatures and increasing nutrient levels (Lacap et al. 2007, Briggs et al. 2013). 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.

Here, we examined the diversity, composition, and distribution among benthic and water-column microbial (i.e. *Bacteria* and micro-*Eukarya*) communities across an 18-mo period from anchialine habitats within the Cape Kinau and Kona regions via high-throughput amplicon sequencing. Previously, we investigated spatial differences among microbial communities from some of these same habitats and demonstrated distinct microbial communities correlating to specific environmental factors on a per-habitat basis (Hoffman et al. 2018). In the Hawaiian Archipelago, however, the year can be broken into 2 broad periods, dry (May through October) and wet (November through April) seasons (Wiegner et al. 2006), with groundwater nutrient levels tracking these differences and being greatest during the wet months (Lau & Mink 2006). Thus, we hypothesized that microbial taxonomic diversity and community structure, as approximated by relative sequence abundance, would exhibit dynamics correlating with known seasonal variation in groundwater nutrient levels and chemistry. Of these, seasonal variation in ammonium, dissolved organic carbon, and salinity were expected to correlate with changes in community structure as these water chemistry characteristics were previously identified as impacting the spatial variation among Hawaiian anchialine microbial communities (Hoffman et al. 2018).

MATERIALS AND METHODS

Sample and environmental data collection

Water column and benthic samples were collected from 6 anchialine habitats, 3 each on Maui and Hawaii, during 4 collection trips (i.e. 2 dry seasons [July] of 2010 and 2011 and 2 wet seasons [March and December] of 2011) over an 18-mo period. 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), while those on Hawaii were Makalawena Beach (MAKA3), Kiawaiki Bay (KBI), and Pu'uhonua O Hōnaunau National Historical Park (PUHO3) (Fig. 1A). Five of the sampled sites possessed the laminated cyanobacterial–bacterial crust, with the exception being PUHO3, which was historically utilized for fish aquaculture (Tango et al. 2012), has a fine sediment as a benthic substrate, and is considered a degraded anchialine habitat. All habitats were open ponds, but varied in whether they were impacted by invasive (or introduced) fish, goats, and humans (Fig. 1, Table 1), with human impact assessed by whether a site has been historically open to public use, including during the sampling periods. Additional environmental variables were acquired from the Hawaii Statewide GIS Program, including annual rainfall, rainfall during the month of sampling, and mean annual solar radiation (Giambelluca et al. 2013, Office of Planning Hawaii Statewide GIS Program 2015). Rainfall 15 mo prior to the month of sampling was also included to account for rainwater input via gravitational movement of groundwater, as previous work found that this duration represented the average transit time of dye injected into inland wastewater reclamation wells traveling out to the ocean (Glenn et al. 2013).

Each set of samples was collected within an 8-d span to minimize potential fine-scale temporal biases. Using disposable sterile sampling spoons, approximately 100 g of benthic material (crust or sediment) from each of 3 sampling locations per site was collected and preserved in RNALater (Thermo Fisher Scientific) until later processing. Additional samples were collected and preserved in 95% ethanol or flash frozen with liquid N_2 in 10% dimethyl sulfoxide (DMSO) or 10% glycerol for archiving in the Hawaiian Anchialine Microbial Repository in conjunction with The Ocean Genome Legacy (https://www.northeastern.edu/ogl/) under accession numbers S23033–S23083. For sampling water column communities, ~1 l of water collected ~5 cm below the surface at each of 2 sampling locations per site was immediately filtered through sterile 0.2 µm Sterivex (Millipore) units and preserved by flooding with cell lysis buffer (Qiagen). For analyses of water chemistry characteristics, two ~0.25 l samples of filtered water from each habitat were stored as recommended at −20°C and transported frozen to the University of Hawaii Hilo Analytical Laboratory, where dissolved organic carbon (DOC), ammonium (NH_4^+), nitrite (NO_2^-) + nitrate (NO_3^-), total dissolved nitrogen

Table 1. Sampled anchialine habitats from the islands of Hawaii and Maui and their environmental characteristics. 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

Habitat characteristics	Site					
	Maui			Hawaii		
	HM	QB	SKIP	KBI	MAKA3	PUHO3
Orange microbial crust	+	+	+	+	+	–
Presence of fish	–	–	–	+	+	–
Presence of goats	+	+	+	–	+	–
Open to public use	–	–	–	+	+	–
Aquifer[a]	Kahikinui	Central	Kahikinui	Hualalai	Hualalai	SW Mauna Loa
Watershed[a]	Kanaio	Ahihi Kinau	Ahihi Kinau	Kiholo	Kiholo	Kauna
Potential warm groundwater[a]	Yes	Yes	Yes	No	Yes	No
Latitude (N)	20.5829	20.5983	20.5970	19.8947	19.7900	19.4210
Longitude (W)	156.4103	156.4386	156.4242	155.9025	156.0291	155.9120
Annual rainfall 2010 (mm)[a,b]	261.1	249.9	253.5	152.9	173.0	439.8
Annual rainfall 2011 (mm)[a,b]	344.0	364.5	358.8	101.5	150.8	491.3
10 yr mean rainfall (mm)[b]	446.0	448.3	452.6	218.4	266.5	587.8
Mean annual solar radiation 2014[a,c] ($W\ m^{-2}$)	216.6	193.5	196.2	226.5	224.8	180.7

[a]Office of Planning Hawaii Statewide GIS Program (2015)
[b]Giambelluca et al. (2013)
[c]2014 data used as approximation, data not available for sampled years

(TDN), orthophosphate (PO_4^{3-}), total dissolved phosphorus (TDP), silica (Si), and salinity (Table 2) were quantified on either a Shimadzu TOC-V + TNM-1 (DOC and TDN) or an AutoAnalyzer (Pulse Instrumentation; NH_4^+, NO_2^- + NO_3^-, PO_4^{3-}, TDP, Si, and salinity).

Sequence data generation

Utilizing the procedures described in Hoffman et al. (2018), DNA was extracted from benthic materials preserved in RNALater using MoBio PowerSoil DNA Isolation Kits (MOBIO) and from lysis-buffer preserved water column filters using Gentra Puregene Yeast/Bacteria Kits (Qiagen). To account for potential spatial heterogeneity within a site, 2 (or 3 in the case of MAKA3 benthic material from July 2010) separate DNA extractions were performed for most samples from at least 2 locations within a site. Amplification of the extracted DNA samples via the PCR utilized 20 ng DNA for each PCR except in cases of low DNA concentration, where the template volume was divided equally between the 2 PCRs. Amplified samples were sequenced as dual-barcoded amplicons on an Illumina HiSeq 2500 platform to obtain paired-end 100 bp reads from 1 of 2 ribosomal RNA (rRNA) genes. The V6 hypervariable region of the 16S-rRNA genes was amplified using the *Bacteria*-specific primers 967–985F and 1078–1061R (Gloor et al. 2010) and the V9 hypervariable region of the 18S-rRNA genes utilized the *Eukarya*-biased primers 1389F and 1510R (Amaral-Zettler et al. 2009). PCR reactions and sequencing runs were performed by the HudsonAlpha Institute for Biotechnology, Genomic Services Laboratory (Huntsville, AL, USA) in duplicate as described in Hoffman et al. (2018). 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–SRX1913629, and SRP077079; BioProject ID number PRJNA325159).

Operational taxonomic unit clustering

PandaSeq v.2.5 (Masella et al. 2012) was used to align forward and reverse sequencing reads, trim

Table 2. Sampled anchialine habitats from the islands of Hawaii and Maui and their seasonal and water chemistry characteristics. See Table 1 for site abbreviations

Site Season	Rainfall (mm)[d,e]	15 mo prior rainfall (mm)[d,e]	Salinity (ppt)	$NO_2^- + NO_3^-$ (µM)	PO_4^{3-} (µM)[a]	Si (µM)	NH_4^+ (µM)[b]	DOC (µM)	TDN (µM)	TDP (µM)[c]
HM										
Jul 2010	14.4	16.6	15.0	48.1	1.65	418	1.41	44.1	49.1	1.53
Mar 2011	17.9	127.7	15.0	38.8	1.17	352	2.52	96.5	47.6	1.30
Jul 2011	17.2	8.30	5.0	32.2	1.16	232	2.89	48.1	36.0	1.17
Dec 2011	22.2	32.1	17.0	43.4	1.24	332	ND	107	59.5	1.23
QB										
Jul 2010	14.5	15.7	26.5	24.6	ND	383	ND	54.2	24.9	ND
Mar 2011	17.0	124.9	21.0	40.2	0.54	341	ND	70.1	44.8	0.71
Jul 2011	18.1	7.32	23.0	22.4	0.14	223	ND	53.1	24.0	ND
Dec 2011	20.5	34.8	21.0	12.5	0.34	236	2.67	138	31.1	ND
SKIP										
Jul 2010	14.8	16.1	24.0	23.9	ND	355	ND	40.1	23.6	ND
Mar 2011	17.4	126.9	15.0	27.9	1.01	398	2.69	45.3	29.2	1.12
Jul 2011	18.3	7.56	12.0	13.7	0.46	204	2.11	53.8	17.4	ND
Dec 2011	20.8	34.9	17.0	38.9	1.29	312	1.16	57.5	43.9	1.09
KBI										
Jul 2010	2.3	6.79	5.0	79.8	1.24	666	1.41	38.3	73.4	1.25
Mar 2011	21.0	68.2	5.0	66.4	1.06	602	1.69	81.6	70.7	1.13
Jul 2011	2.1	10.4	2.0	60.4	1.00	625	1.90	60.8	65.2	0.93
Dec 2011	1.3	3.49	5.0	81.1	1.28	677	1.23	73.1	85.8	1.27
MAKA3										
Jul 2010	7.8	10.2	4.0	46.3	7.24	897	1.16	14.5	42.9	7.50
Mar 2011	2.9	61.4	7.0	75.6	7.89	669	2.06	26.0	78.4	7.95
Jul 2011	1.9	22.1	5.0	54.8	6.49	712	1.72	26.1	59.0	6.39
Dec 2011	8.7	2.85	7.0	70.6	7.57	773	ND	57.1	78.0	7.60
PUHO3										
Jul 2010	34.6	29.0	15.5	41.1	1.88	568	1.73	119	9.9	2.22
Mar 2011	21.4	79.3	13.0	6.9	3.50	461	8.07	195	27.1	3.72
Jul 2011	11.6	42.6	9.0	5.6	1.29	389	3.97	106	15.9	1.54
Dec 2011	8.3	29.4	13.0	7.2	2.62	536	4.55	83.8	19.6	2.62

[a]Not detectable (ND) <0.10; [b]Not detectable (ND) <1.00; [c]Not detectable (ND) <0.050; [d]Office of Planning Hawaii Statewide GIS Program (2015); [e]Giambelluca et al. (2013)

primers, 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 nucleotides in the read using the FASTQ Quality Filter included in the FASTX-Toolkit v.13.2 (FASTQ/A short-reads pre-processing tools, http://hannonlab.cshl.edu/fastx_toolkit/). USEARCH61 (Edgar 2010), a component of the QIIME v.1.8 pipeline (Caporaso et al. 2010b), was then utilized to remove potentially chimeric sequences before submission to UCLUST (Edgar 2010) 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 GreenGenes 13.8 database (DeSantis et al. 2006) as the initial cluster references for the V6 region and the 99% clustered Silva 111 database (Quast et al. 2012) for the V9 region. The 0.005% OTU abundance filter was applied as per recommendations by Bokulich et al. (2013) for improvement of clustering results, and OTUs were lumped, rather than split, using a conservative 95% sequence similarity parameter because previous analyses at 97% sequence similarity did not significantly increase the number of recovered OTUs (Hoffman et al. 2018). Each OTU cluster, as represented by its most abundant sequence, was submitted for taxonomic identification using megaBLAST v.2.2.26 (Altschul et al. 1990) (i.e. sequence identity ≥90%, e-value 1×10^{-6}) and alignment using PYNAST v.1.2.2 (Caporaso et al. 2010a) with default parameters (i.e. minimum length of 75% median input length, mini-

mum identity 75%) (Caporaso et al. 2010a) to the appropriate curated databases discussed above. Those OTUs failing to align using PYNAST were removed from the final tables. Notably, OTUs identified as prokaryotic in nature were not removed from the final V9 OTU table in order to maintain as broad a sampled taxonomic diversity as possible, in accordance with suggestions by Hadziavdic et al. (2014).

Analyses of community composition

Within the R v.3.1.13 statistical environment (R Development Core Team 2008), the package PhyloSeq v.1.10.0 (McMurdie & Holmes 2013) was used to calculate 4 alpha diversity metrics on the final OTU abundance tables: the number of observed OTUs, Chao1 (Chao 1984), Shannon (Shannon et al. 1964), and inverse Simpson (Simpson 1949) diversity indices. Chao1 estimates the total number of taxa present by assuming samples with many rare OTUs and/or taxa likely contain additional undetected taxa. For the Shannon diversity index, higher values reflect greater community diversity by quantifying the uncertainty in predicting to which OTU and/or taxa the next sampled sequence belongs. In contrast, the inverse of Simpson's index reports the richness of a perfectly even community with the same diversity as the observed sample. Rarefaction curves were generated in R for the latter 3 alpha diversity metrics using 10 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 our sampling depth at capturing community diversity. Differences in alpha diversity between samples grouped by whether they originated from benthic material or the water column, hereafter referred to as habitat type, as well as season (i.e. dry versus wet) were tested using one-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test in the R package agricolae v.1.2.3 (De Mendiburu 2015). Differences with $p < 0.05$ were considered significant.

The summarize_taxa_through_plots.py script in the QIIME v.1.8 pipeline (Caporaso et al. 2010b) was used to produce data tables corresponding to taxonomically conservative third-level clades (i.e. class or approximate class-level nomenclatures in the GreenGenes and Silva databases, respectively) that were employed in later analyses. Similarities among microbial communities from whole samples were visualized using non-metric multidimensional scaling (NMDS) ordination with 95% confidence interval ellipses around sample groups in the R package PhyloSeq v.1.10.0 (McMurdie & Holmes 2013). Ordinations were made using the dissimilarity matrices resulting from applying the Bray-Curtis dissimilarity metric and the Jaccard dissimilarity coefficient on the final OTU tables after transformations to even sampling depth. Ecological studies commonly use the Bray-Curtis dissimilarity metric because it is based on the abundance of OTU sequences shared between communities (Bray & Curtis 1957). In contrast, the Jaccard dissimilarity coefficient utilizes only the presence or absence of OTUs to return the proportion of unshared taxa between samples (Jaccard 1908). Transformation of the final OTU tables were not required, as the Jaccard dissimilarity coefficient calculation in R automatically standardizes the data into a presence/absence matrix prior to calculation of the dissimilarity matrix. Ordinations were compared using a Procrustes test with 999 permutations in the R package vegan v.2.3.1 (Oksanen et al. 2015). Phyla-level tables were also generated for samples grouped by site, habitat type (i.e. benthic material versus water column), and season (i.e. wet versus dry) to produce stacked bar plots of the proportion of each phylum present at a sample site. Environmental variables, including site, habitat type, sampling date, and season, with significant explanatory power ($\alpha = 0.05$) of the sample ordinations were identified using the envfit function (999 permutations) and overlaid on the ordinations as vectors scaled by their explanatory power (r) if their $r^2 > 0.5$. Furthermore, the bioenv function in the R package vegan v.2.3.1 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 for all sites by season was evaluated with one-way multivariate analysis of variance (MANOVA) followed by Wilk's lambda post hoc test. Univariate one-way ANOVAs with Tukey's HSD post hoc tests were then performed on water chemistry characteristics to identify which were specifically impacted by season. One-way ANOVAs with Tukey's HSD post hoc tests were also utilized to identify third-level clades whose relative abundance varied by season, and multiple regressions were then used to examine the relationship between clades whose relative abundance varied with season, as well as environmental variables that also varied with season. While the water chemistry characteristics and alpha diversity indices violate the assumptions

of normally distributed residuals and homogeneous variances for ANOVA, use of the nonparametric Welch's ANOVA correction with the Games-Howell post hoc test found no difference in any of the results (data not shown). Thus, the results of the initial ANOVAs are presented here. All R code, QIIME scripts, and other commands employed in this study can be downloaded from www.auburn.edu/~santosr/sequencedatasets.htm.

RESULTS

Sample collection and OTU clustering

Water chemistry characteristics were significantly correlated with season (MANOVA: Wilk's lambda $F_{8,460} = 0.3685$, $p < 0.01$), but not sampling date (multivariate regression: $F_{1,464} = 0.0121$, $p = 0.9124$), for the anchialine habitats examined here. Univariate tests with Tukey's HSD post hoc analysis revealed the wet seasons as having greater ammonium, DOC, $NO_2^- + NO_3^-$, salinity, and TDN ($p < 0.01$) per site. Correspondingly, PO_4^{3-}, TDP, and Si concentrations were not correlated with season.

Biological samples collected from July 2010 (21 and 21), March 2011 (29 and 29), July 2011 (36 and 34), and December 2011 (32 and 32) from 3 anchialine habitats each on Maui and Hawaii were successfully sequenced for both the V6 and V9 regions of the 16S and 18S rRNA genes, respectively. Of the 118 V6 and 116 V9 samples, 33 in both the V6 and V9 samples were from the water column and 85 V6 and 83 V9 samples were from benthic material (Table 3). Overall, the amplicon sequencing produced a total of 51 706 414 demultiplexed bacterial V6 Illumina reads in each paired-end direction, for an average ($\bar{x}$) of 96 828 reads per sequencing replicate sample, hereafter referred to as a sample. For the eukaryotic-biased V9 data, 33 046 764 demultiplexed reads in each paired-end direction were produced ($\bar{x}$ = 61 885 reads per sample).

Following alignment of paired-end reads, quality-filtering, chimera-checking, and abundance filtering of the V6 data, 14 126 948 reads were retained ($\bar{x}$ = 30 057 reads per sample), representing a 72.7% reduction. Similarly, 72.2% of the V9 reads were also removed during processing, resulting in 9 180 794 reads ($\bar{x}$ = 19 701 reads per sample). These stringent filtering parameters were employed to reduce the signal-to-noise ratio as well as potential issues from the relatively short read lengths obtained from the Illumina platform (see below). Lengths of the V6 reads ranged from 63 to 80 bp ($\bar{x}$ = 74 bp), while V9 reads ranged from 65 to 163 bp ($\bar{x}$ = 125 bp). A single V6 OTU was removed due to failure to align in PYNAST, resulting in 1656 OTUs, totaling 12 492 442 sequences ($\bar{x}$ = 26 579 sequences per sample) being retained. In contrast, 15 V9 OTUs failed to align and were removed from the final data set, resulting in 8 450 946 sequences belonging to 1211 OTUs ($\bar{x}$ = 18 135 sequences per sample). Of the 1656 and 1211 OTUs in the final V6 and V9 data sets, respectively, 149 V6 (213 912 sequences, 11.4% of total) and 10 V9 OTUs (13 799 sequences, 0.16% of total) could not be assigned taxonomic identifications using either the GreenGenes 13.8 (DeSantis et al. 2006) or Silva 111 database (Quast et al. 2012). Similarity searches of the unassigned V6 OTUs to NCBI's GenBank repository (Benson et al. 2009, Sayers et al. 2009) using BLASTN v.2.3.0 (Altschul et al. 1990) 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, analogous searches 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 3.3% of the total sequences (range of 0–37.4%, with average relative abundance of 3.8% per sample). No potential chloroplast sequences were recovered from the V9 OTUs.

Analyses of community composition and seasonal factors

Samples originating from the same site, habitat type (e.g. benthic material or water column), or specific sampling date were combined (i.e. treated as members of a single category) for most downstream analyses because they were most similar to each other regardless of which biological sample, DNA extraction, PCR, or sequencing run the read data were generated from (Fig. S1 in the Supplement at www-int-res.com/articles/suppl/a082p087_supp.pdf). Following consolidation, estimates of observed OTU richness were not saturated at depths of 30 000 sequences per sample for either the V6 or V9 data sets (Fig. S2 in the Supplement). In contrast, Chao1, Shannon, and inverse Simpson diversities reached apparent saturation at approxi-

Table 3. Number of biological samples collected from each site that successfully amplified via PCR, technical samples (produced by duplicate PCR reactions from a single biological sample), and the total number of samples sequenced for *Bacteria*-specific V6 rRNA and *Eukarya*-biased V9 rRNA from 2 Illumina HiSeq 2500 runs analyzed in this study. See Table 1 for site abbreviations. Samples with a value of 0 were collected and sent for sequencing but failed to amplify

		July 2010		March 2011		July 2011		December 2011	
		Benthic	Water col.	Benthic	Water col.	Benthic	Water col.	Benthic	Water col.
V6									
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
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
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
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
All sites	Biological samples	16	5	21	8	26	10	22	10
total	Technical samples	32	10	42	16	52	20	43	20
	Sequenced samples	64	20	84	32	104	40	86	40
V9									
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
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
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
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
All sites	Biological samples	16	5	21	8	24	10	22	10
total	Technical samples	31	10	42	16	50	20	44	20
	Sequenced samples	62	20	84	32	100	40	88	40

mately 10 000 sequences per sample (Fig. S2 in the Supplement). Examination of alpha diversity metrics from the V6 and V9 samples grouped by habitat type revealed the benthic material as having significantly greater alpha diversities than the water column samples (Fig. 2A, Table 4). However, when the same V6 and V9 samples were grouped by season, no differences were detected in these same indices (Fig. 2A, Table 4). Furthermore, no linear relationships were observed between the V6

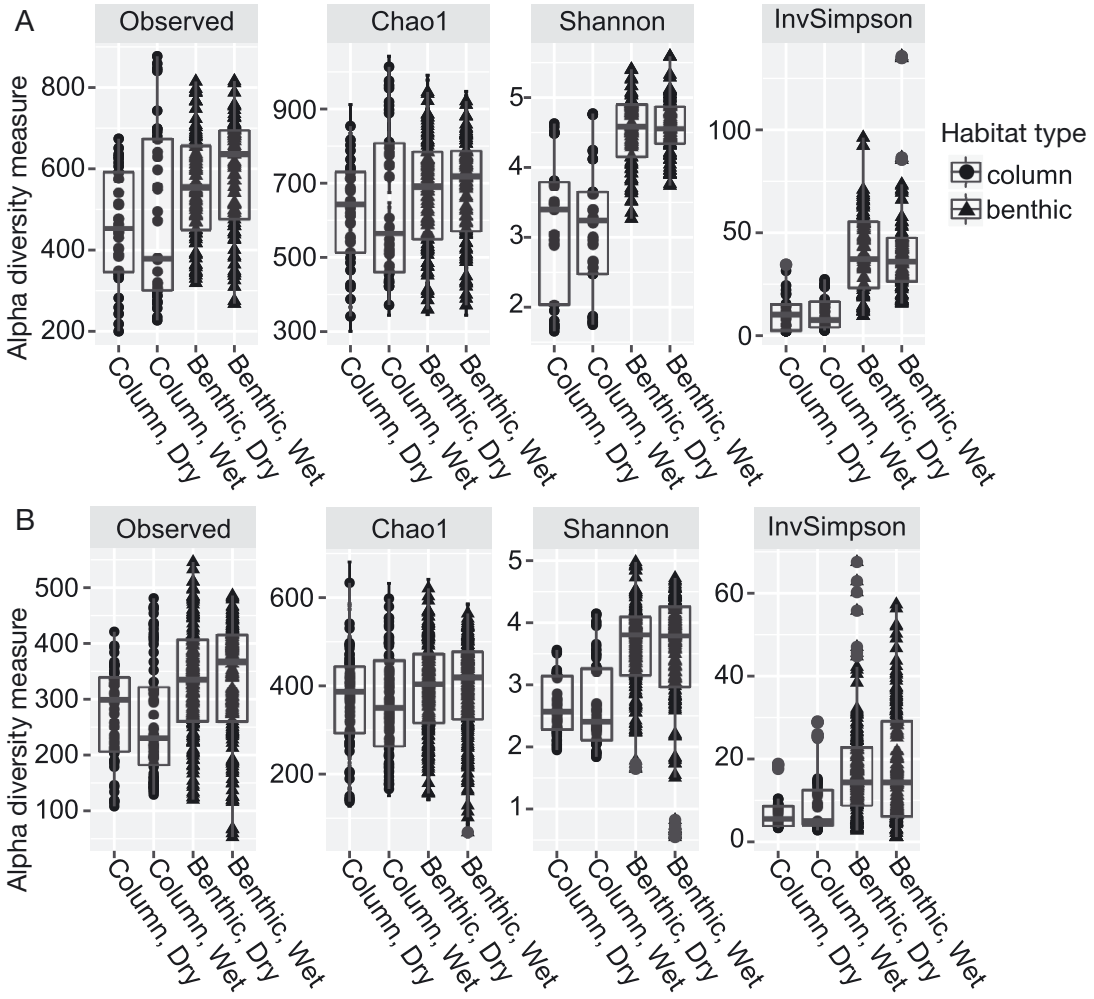

Fig. 2. Diversity estimates as number of observed operational taxonomic units (OTUs), Chao1 diversity, Shannon diversity, and inverse Simpson diversity from benthic material or water column microbial communities and seasons (i.e. wet versus dry) for anchialine habitats on the islands of Maui and Hawaii. Box-and-whisker plots indicate the data median and quartiles for each particular data set. (A) *Bacteria*-specific V6 rRNA; (B) *Eukarya*-biased V9 rRNA

Table 4. Univariate ANOVA of alpha diversity metrics for *Bacteria*-specific V6 and *Eukarya*-biased V9 rRNA data sets grouped either by habitat type or season

Alpha diversity metric	V6 $F_{1,468}$	V6 p	V9 $F_{1,468}$	V9 p
Habitat type				
OTU richness	45.11	<<0.001	37.98	<<0.001
Chao1	233.5	0.008	6.691	0.01
Shannon	435.9	<<0.001	93.8	<<0.001
Inverse Simpson	233.5	<<0.001	58.71	<<0.001
Season				
OTU richness	2.083	0.15	0.203	0.652
Chao1	1.126	0.289	0.239	0.625
Shannon	0.009	0.923	1.962	0.162
Inverse Simpson	0.116	0.734	0.599	0.439

or V9 alpha diversity indices and sampling date (data not shown).

Differences between NMDS ordinations and fitted envfit vectors utilizing the Bray-Curtis dissimilarity metric and the Jaccard dissimilarity coefficient were minimal (V6: Procrustes m^2 = 0.024, p = 0.001; V9: m^2 = 0.034, p = 0.001); hence only the Bray-Curtis ordinations for the V6 (Fig. 3A) and the V9 (Fig. 3B) OTU data are presented here (Jaccard ordinations given as Fig. S3 in the Supplement). Both benthic material and water column samples primarily grouped by island, specifically into a cluster of Maui (i.e. HM, SKIP, and QB) and Hawaii sites (i.e. MAKA3, KBI, and PUHO3) (Fig. 3). Within these island-specific clusters, samples were separated by habitat type (i.e. benthic material versus water col-

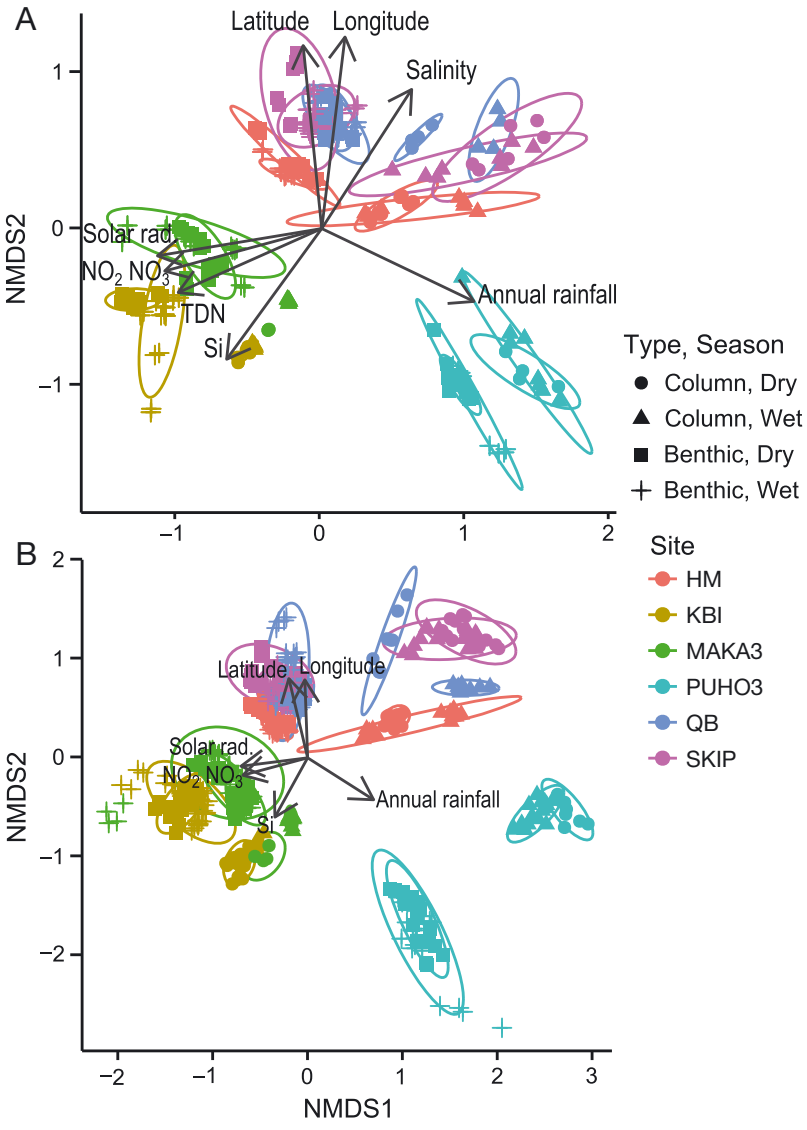

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination using the Bray-Curtis dissimilarity index of samples grouped by benthic material or water column microbial communities within seasons (i.e. wet versus dry) and sites from anchialine habitats on the islands of Maui and Hawaii. Ellipses represent 95% confidence intervals grouping samples by benthic material or water column microbial communities within seasons and anchialine habitats. (A) Samples generated using the *Bacteria*-specific V6 rRNA (stress = 0.1450). (B) Samples generated using the *Eukarya*-biased V9 rRNA (stress = 0.1457). See Table 1 for site abbreviations

umn) that could be further differentiated by specific site (Fig. 3). Notably, the PUHO3 samples were distinctive from the other sites on Hawaii, apparently due to its historically degraded state and lack of the distinctive, laminated orange cyanobacterial–bacterial crust. Season (i.e. wet versus dry) and sampling date did not substantially influence ordinations, with appreciable overlap between samples taken from the same site and habitat type regardless of season or sampling date, with the sole exception being water column samples from QB (Fig. 3).

The bioenv function tested 67 108 863 possible combinations among the categorical and continuous environmental variables as well as site, sample ID, sampling date, season, and habitat type for both data sets. From these, 3 and 4 variables were identified as significant for the V6 (Spearman rank correlation = 0.8345) and V9 data sets (Spearman rank correlation = 0.8116), respectively. Specifically, habitat type, annual rainfall (mm), and $NO_2^- + NO_3^-$ were found as significant for both the bacterial V6 and micro-eukaryotic V9 data, while NH_4^+ was identified as significant only for the V9 data. Using envfit to examine among-individual environmental variables, all categorical variables other than season were identified as significant in explaining the V6 NMDS ordination at $p < 0.05$, with site accounting for the most variation ($r^2 = 0.8157$). In a similar fashion, all categorical variables other than sampling date and season were identified as explanatory of the ordination for the V9 data, with site having the greatest explanatory power ($r^2 = 0.7186$). 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-mo prior rainfall, PO_4^{3-}, and TDP having the least, explanatory power (Table S1 in the Supplement). Along with this, 39 of the 84 third-level bacterial clades (representing approximately class-level nomenclatures) in the V6 data set, 12 of the 30 third-level bacterial clades in the V9 data set, and 18 of the 28 third-level eukaryotic clades in the V9 data set exhibited relative abundances significantly varying with season (Tables 5 & 6). When analyzed in combination, TDN, $NO_2^- + NO_3^-$, DOC, and salinity correlated with both the V6 and V9 clades, while only NH_4^+ correlated with the V6 clades, both those more abundant during wet seasons and those more abundant during dry seasons (Tables 5 & 6).

Table 5. Third-level bacterial clades identified by univariate ANOVA in the *Bacteria*-specific V6 rRNA data set as having greater relative abundances during a specific season, and water chemistry variables that correlated with each particular clade ($p < 0.05$)

Taxa	Abundant season	SS	$F_{1,467}$	Water chemistry variables
Acidobacteria, BPC102	Wet	1.38×10^{-6}	9.894	NH_4^+, salinity
Acidobacteria, OS.K	Wet	1.84×10^{-4}	5.314	DOC, NH_4^+
Acidobacteria, Sva0725	Wet	1.55×10^{-4}	4.977	NH_4^+, $NO_2^- + NO_3^-$
Actinobacteria, *Nitriliruptoria*	Wet	1.91×10^{-6}	7.646	–
Armatimonadetes, 0319.6E2	Wet	4.64×10^{-6}	11.920	TDN, DOC, NH_4^+, salinity, $NO_2^- + NO_3^-$
Bacteroidetes, SB.5	Wet	1.17×10^{-6}	4.189	DOC, NH_4^+
Chlamydiae, *Chlamydiia*	Wet	4.16×10^{-6}	16.050	TDN, DOC, salinity, $NO_2^- + NO_3^-$
Chlorobi, SJA.28	Wet	4.50×10^{-5}	5.060	DOC, $NO_2^- + NO_3^-$
Chloroflexi, *Anaerolineae*	Wet	7.00×10^{-4}	5.561	Salinity
Fibrobacteres, TG3	Wet	7.70×10^{-6}	5.743	Salinity
Gemmatimonadetes	Wet	1.83×10^{-6}	17.111	TDN, NH_4^+, $NO_2^- + NO_3^-$
Gemmatimonadetes, Gemm.1	Wet	5.08×10^{-5}	10.240	DOC, NH_4^+
Gemmatimonadetes, Gemm.2	Wet	2.66×10^{-4}	6.327	TDN, $NO_2^- + NO_3^-$
Gemmatimonadetes, Gemm.4	Wet	1.30×10^{-3}	6.640	NH_4^+
N02, 3BR.5F	Wet	1.10×10^{-5}	4.355	TDN, $NO_2^- + NO_3^-$
Nitrospirae, *Nitrospira*	Wet	7.61×10^{-5}	16.495	DOC
Planctomycetes, C6	Wet	1.72×10^{-5}	6.530	DOC
Proteobacteria, *Gammaproteobacteria*	Wet	6.44×10^{-1}	37.610	–
Thermi, *Deinococci*	Wet	5.34×10^{-4}	15.020	TDN, $NO_2^- + NO_3^-$
Verrucomicrobia, *Pedosphaerae*	Wet	3.91×10^{-3}	7.503	DOC, $NO_2^- + NO_3^-$
Verrucomicrobia, *Spartobacteria*	Wet	2.43×10^{-6}	16.580	TDN, NH_4^+, salinity, $NO_2^- + NO_3^-$
Verrucomicrobia, Verruco.5	Wet	1.72×10^{-4}	14.370	–
WS3, PRR.12	Wet	9.80×10^{-5}	7.125	NH_4^+
WS6, SC72	Wet	1.00×10^{-5}	12.390	TDN, NH_4^+, $NO_2^- + NO_3^-$
Bacteroidetes, *Rhodothermi*	Dry	5.30×10^{-4}	4.372	TDN, salinity, $NO_2^- + NO_3^-$
Bacteroidetes, *Saprospirae*	Dry	2.37×10^{-2}	8.250	DOC, NH_4^+, salinity
Caldithrix, *Caldithrixae*	Dry	4.60×10^{-7}	5.173	DOC, NH_4^+, salinity
Cyanobacteria	Dry	7.70×10^{-3}	9.251	DOC, NH_4^+
Cyanobacteria, Chloroplast	Dry	5.19×10^{-2}	8.997	DOC, NH_4^+, salinity, $NO_2^- + NO_3^-$
Cyanobacteria, *Gloeobacterophycideae*	Dry	9.70×10^{-4}	7.467	DOC
Cyanobacteria, *Synechococcophycideae*	Dry	4.95×10^{-2}	12.560	DOC, $NO_2^- + NO_3^-$
Firmicutes, *Bacilli*	Dry	2.10×10^{-6}	10.620	TDN, DOC, salinity, $NO_2^- + NO_3^-$
Firmicutes, *Clostridia*	Dry	1.03×10^{-3}	7.798	Salinity
GN04, GN15	Dry	3.10×10^{-6}	8.252	NH_4^+, salinity
Planctomycetes, *Phycisphaerae*	Dry	2.21×10^{-4}	4.739	TDN, DOC, NH_4^+, $NO_2^- + NO_3^-$
Planctomycetes, Pla3	Dry	9.60×10^{-7}	4.691	NH_4^+, salinity
Planctomycetes, vadinHA49	Dry	8.23×10^{-7}	40.390	TDN, salinity, $NO_2^- + NO_3^-$
Proteobacteria, *Epsilonproteobacteria*	Dry	1.31×10^{-6}	8.280	Salinity
Verrucomicrobia, *Opitutae*	Dry	5.60×10^{-4}	10.690	DOC, NH_4^+, salinity

DISCUSSION

Hawaiian anchialine microbial communities during wet and dry seasons

Due to their simultaneous subterranean connections to oceanic and groundwater sources, anchialine habitats can potentially exhibit both vertical stratification and water chemistry fluctuations from terrestrial origins (Por 1985). In Hawaii, 2 predictable seasons — wet from November through April and dry from May through October (Wiegner et al. 2006) — are apparent. During wet seasons, significantly higher levels of $NO_2^- + NO_3^-$, NH_4^+, DOC, and TDN were observed in the anchialine habitats sampled here, likely due to an increased influence of groundwater, which has greater nutrient levels than the nearshore seawater surrounding Hawaii (Bienfang 1980). An increase in near-surface salinity was also recorded in wet seasons, which could be induced by disruption of the halocline or movement of higher salinity water towards the surface of the habitats via an increased flow of freshwater, direct rainfall into the surface waters of the habitat, and/or higher levels of wind activity.

In contrast to our initial hypothesis that these orange cyanobacterial–bacterial crust communities from the Hawaiian anchialine ecosystem would ex-

Table 6. Third-level bacterial and eukaryotic clades identified by univariate ANOVA in the *Eukarya*-biased V9 rRNA data set as having greater relative abundances during a specific season, and water chemistry variables that correlated with each particular clade ($p < 0.05$)

Taxa	Abundant season	SS	$F_{1,467}$	Water chemistry variables
Bacteria, *Firmicutes*, *Bacilli*	Wet	3.09×10^{-5}	6.125	–
Bacteria, *Gemmatimonadetes*, BD2.11 terrestrial group	Wet	4.90×10^{-5}	6.633	–
Bacteria, *Gemmatimonadetes*, PAUC43f marine benthic group	Wet	2.35×10^{-6}	4.191	–
Bacteria, *Lentisphaerae*, *Lentisphaeria*	Wet	2.19×10^{-4}	10.948	DOC, $NO_2^- + NO_3^-$
Bacteria, *Nitrospirae*, *Nitrospira*	Wet	8.40×10^{-5}	9.180	–
Bacteria, *Planctomycetes*, OM190	Wet	6.20×10^{-5}	8.637	–
Bacteria, *Planctomycetes*, *Planctomycetacia*	Wet	4.20×10^{-4}	3.872	–
Bacteria, *Proteobacteria*, *Deltaproteobacteria*	Wet	6.80×10^{-4}	4.495	TDN, DOC, $NO_2^- + NO_3^-$
Bacteria, *Proteobacteria*, SPOTSOCT00m83	Wet	4.73×10^{-7}	13.584	TDN, DOC, salinity, $NO_2^- + NO_3^-$
Bacteria, *Verrucomicrobia*, OPB35 soil group	Wet	9.40×10^{-4}	9.863	TDN, DOC, $NO_2^- + NO_3^-$
Bacteria, *Verrucomicrobia*, *Opitutae*	Wet	7.10×10^{-5}	4.992	Salinity
Bacteria, *Verrucomicrobia*, *Spartobacteria*	Wet	4.10×10^{-4}	4.766	–
Eukaryota, Amoebozoa, Conosa	Wet	1.34×10^{-3}	37.169	$NO_2^- + NO_3^-$
Eukaryota, Amoebozoa, Discosea	Wet	2.06×10^{-5}	4.159	–
Eukaryota, Archaeplastida, Chloroplastida	Wet	1.05×10^{-1}	11.850	TDN, DOC, $NO_2^- + NO_3^-$
Eukaryota, Incertae Sedis, Apusomonadidae	Wet	4.69×10^{-7}	8.986	DOC
Eukaryota, SAR, Rhizaria	Wet	4.10×10^{-4}	4.168	TDN, DOC, $NO_2^- + NO_3^-$
Bacteria, *Firmicutes*, *Clostridia*	Dry	4.98×10^{-4}	16.815	DOC
Bacteria, *Proteobacteria*, *Betaproteobacteria*	Dry	6.10×10^{-5}	12.510	–
Eukaryota, Amoebozoa, Lobosa	Dry	1.19×10^{-3}	23.310	Salinity
Eukaryota, Archaeplastida, Glaucophyta	Dry	3.35×10^{-5}	6.657	Salinity
Eukaryota, Archaeplastida, Rhodophyceae	Dry	7.50×10^{-6}	4.375	TDN, DOC, $NO_2^- + NO_3^-$
Eukaryota, Centrohelida, Heterophryidae	Dry	4.58×10^{-5}	10.170	–
Eukaryota, DH147.EKD10.uncultured marine eukaryote	Dry	9.80×10^{-7}	8.992	–
Eukaryota, Excavata, Discoba	Dry	2.79×10^{-4}	25.140	–
Eukaryota, Incertae Sedis, Palpitomonas	Dry	1.38×10^{-6}	10.003	TDN, DOC, $NO_2^- + NO_3^-$
Eukaryota, Kathablepharidae, Roombia	Dry	5.59×10^{-6}	19.478	–
Eukaryota, SA1.3c06. uncultured eukaryote	Dry	6.37×10^{-6}	9.536	–
Eukaryota, SAR, Alveolata	Dry	1.84×10^{-1}	5.515	–
Eukaryota, Zeuk77, uncultured Eimeriidae	Dry	5.40×10^{-6}	4.810	–

hibit dynamics correlating to seasonally varying environmental factors, we found that spatial factors, rather than season or sampling date, correlated with overall community structure with regards to OTU composition and distribution (Fig. 3). Similarly, season was also found to have relatively little correlation with OTU richness or Chao1, Shannon, and inverse Simpson diversities (Fig. 2). Notably, any given sample was more similar to one from the same site and/or habitat type 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), in spite of significant seasonal differences in environmental and water chemistry characteristics (i.e. salinity, NH_4^+, and TDN). Conversely, approximately half of the third-level bacterial and micro-eukaryotic clades exhibited clear variation according to season. Taken together, while seasonal factors did not appear to be strongly correlated with microbial diversity or composition for these crust communities as a whole, they may influence the abundances of particular third-level clades without causing major shifts in the overall community. Along with this, similarity in the Jaccard and Bray-Curtis ordinations suggests any distinctions between samples were likely due to both differences in relative abundance of shared OTUs and different OTU memberships rather than only being abundance based. Indeed, neither the V6 nor the V9 data sets included an OTU that was present in every single sample. Thus, the previously documented distinct and unique nature of individual orange cyanobacterial–bacterial crust communities on the islands of Maui and Hawaii (Hoffman et al. 2018) was apparently maintained despite environmental influences undergoing seasonal variation, as demonstrated here.

One possible explanation for our results is that season and sampling date exerted a greater influence on these Hawaiian anchialine microbial communities than indicated by the analyses conducted here. For example, microbial taxa occupying complex structures such as mats or crusts have been shown to migrate with subsequent patchy distributions (Dillon et al. 2009) that complicate sampling in a temporal fashion. However, seasonal stability has also been observed in numerous other microbial consortia or communities occupying unusual niches, including hypersaline microbial mats (Paerl et al. 2003, Yannarell et al. 2007, Green et al. 2008), cyanobacterial desert soil crusts (Belnap 2003), hot spring microbial mats (Ferris & Ward 1997), and phototrophic microbial/cyanobacterial mats found in a meromictic hypersaline lake (Lindemann et al. 2013, Cole et al. 2014). Likewise, the orange crust communities found in certain Hawaiian anchialine habitats may also demonstrate such stability. In cases where cyanobacterial–bacterial-dominated communities take on a laminated mat nature or form complex structures such as crusts, the formation of microniches can foster greater taxonomic diversity within the community, with subsequent increases in metabolic variation and activity (Stal 1995, Paerl et al. 2000). Furthermore, the broad metabolic diversity of the cyanobacterial component of the community enables persistence under fairly extreme conditions and facilitates formation of mats and crusts by driving productivity, separating oxic from anoxic microniches and creating a transition zone characterized by oxic–anoxic alternations, along with secreting the extracellular polymeric substances that create additional structural cohesion (Stal 1995). As a result, laminated cyanobacterial–bacterial structures allow for greater community diversity and functional redundancy, characteristics that Yannarell et al. (2007) noted as conducive for maintaining compositional integrity of Bahamian hypersaline microbial mats in the face of seasonal hurricane activity (Paerl et al. 2003). Thus, lamination in the orange cyanobacterial–bacterial crust communities found in some Hawaiian anchialine habitats may also increase community diversity and subsequent resistance to seasonal environmental fluctuations.

Another possibility is that these Hawaiian anchialine microbial communities could be resistant to compositional fluctuations and significant alterations in relative abundances if a substantial fraction of community members is not nutrient limited across seasons. For instance, land development in the Hawaiian Archipelago can significantly increase incidents of nitrogen and phosphorus leeching into groundwater, leading to temporally consistent levels well above ambient (Dollar & Atkinson 1992, Wiegner et al. 2006) that could pass through extant anchialine habitats on the 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 to 116% and 22% increases in nitrogen and phosphorus, respectively (Dollar & Atkinson 1992), with some anchialine pools within such developed areas having nutrient levels >70% higher than rivers and estuaries considered heavily polluted (Wiegner et al. 2006). Furthermore, experimental addition of nitrogen and phosphorus to both pristine and anthropogenically impacted anchialine habitats on Hawaii revealed that the benthic microbial community was not nutrient limited and was only impacted by top-down control (Sakihara et al. 2015). Additionally, observed microbial community compositional differences correlating with salinity in these habitats were found to be decoupled from covarying nitrogen or phosphorus levels, suggesting that any bottom-up forces may be complex and/or linked to other nutrients (Sakihara et al. 2015). In contrast, a survey on Hawaii of minimally to heavily impacted anchialine habitats found greater nitrogen and phosphorus levels 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 Hawaiian Islands (Dalton et al. 2013). Notably, most of the salinity and nitrogen levels presented here were more similar to those reported by Sakihara et al. (2015) than those of Dalton et al. (2013), suggesting that microbial communities from anchialine habitats with consistently lower salinities (i.e. such as those surveyed by Dalton et al. 2013) may be more nutrient limited than those from habitats with higher salinity.

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 these anchialine habitats. For example, Hawaiian anchialine pools can experience considerable fluctuations in water chemistry characteristics (specifically pH and turbidity) during the diel cycle, with lower pH and greater turbidity at night versus during the day (Sakihara 2012). 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 characteristics and

thus exhibit minimal shifts due to seasonal nutrient availability or concentrations. Indeed, the greater explanatory power of mean annual rainfall rather than either rainfall 15 mo prior to sampling or rainfall during the sampling month may also be due to the reduced impact of short-term fluctuations; i.e., long-term alterations in environmental conditions may have greater impact on these microbial communities by overcoming community resistance to short-term changes. Consistent with this, 2009–2010 marked a severe drought in the Hawaiian Archipelago due to El Niño that was alleviated somewhat in 2011. Thus, drought conditions, with a decrease in annual rainfall, may have obscured seasonal impacts by applying greater environmental pressure to these microbial communities over extended periods that overcame community resistance to altered conditions.

An exception to the temporal stability exhibited among the microbial communities examined here were water column samples taken from the QB site (Fig. 3). Relative to the wet season, the dry season (July 2010; Table 3) was uniquely dominated (i.e. ~67% versus 1.5% of the total) by sequences belonging to the genus *Cetobacterium* in the *Fusobacteria*, whose members have been identified in mammalian, shorebird, and fish gut microbiomes (Krieg et al. 2010, Larsen et al. 2014, Ryu et al. 2014). Among the habitats included in this study, QB was unusual in that it appeared to function more as a wetland, where the pond basin almost dried out, but remained marsh-like, during low tides. Furthermore, it was the only site inhabited by a population of the Hawaiian stilt *Himantopus mexicanus knudseni* (S. K. Hoffman pers. obs.), a potential source for this particular genus, over the course of this study.

Seasonal water chemistry and relative abundance of taxa

Although seasonality in water chemistry characteristics apparently has little impact on the large-scale diversity and structure of these Hawaiian anchialine microbial communities (see above), approximately one-half of the third-level clades (representing approximately class-level nomenclatures) recovered in our sampling experienced shifts in their relative abundances during different seasons, with about 75% of these correlating with at least one of the water chemistry characteristics that varied in a seasonal fashion (Tables 5 & 6). Of particular note were the cyanobacterial clades being more abundant during dry seasons and correlating with lower DOC, NH_4^+, and $NO_2^- + NO_3^-$ (Table 5). Together with the previous evidence suggesting microbes from these anchialine habitats might not be nutrient limited, the lower nutrient levels and higher irradiances during the dry seasons may favor the oxygenic photosynthetic portion of the microbial community, of which *Cyanobacteria* dominate (Fig. S1 in the Supplement). Further evidence for oxygenic autotrophy being favored during the dry seasons was the concurrent increase in abundance of algal chloroplasts, Rhodophyta, and Glaucophyta (Tables 5 & 6, Fig. S1 in the Supplement). Unsurprisingly, Glaucophyta, exclusively encompassing freshwater microorganisms (Leblond et al. 2010), had increased abundances during the dry seasons and were correlated with reduced salinity. In contrast, the wet seasons appeared to favor microorganisms capable of anoxygenic photosynthesis, as greater abundances of *Acidobacteria*, *Chlorobi*, and *Chloroflexi* were recorded (Tables 5 & 6). Anoxygenic photosynthesizers have been shown to be inhibited by increasing salinity (Sørensen et al. 2004, Yannarell et al. 2006), and although we measured increased salinity during the wet seasons, increased turnover and mixing may have facilitated halocline breakdown and decreased salinities at deeper depths, thus allowing greater activity by members of these groups.

Comparison of the seasonal trends in taxa identified during this study with other work on cyanobacterial–bacterial communities revealed both similarities and differences. For example, *Cyanobacteria* in thermophilic mats of the Philippines and China exhibited greater abundances during the wet seasons (Lacap et al. 2007, Briggs et al. 2013), with both instances correlating with reduced temperatures driven by large rainwater influxes. Although the cyanobacterial clades of our study responded differentially in wet seasons, *Chloroflexi* were more abundant during this period both in the Hawaiian anchialine ecosystem as well as in the Philippine study (Lacap et al. 2007). In this latter case, *Chloroflexi* abundance co-varied with increased phosphate levels, likely due to rainwater influx (Lacap et al. 2007). However, phosphate did not significantly increase during Hawaii's wet seasons, and instead greater *Chloroflexi* abundance was correlated with reduced salinities. In previous studies of Chinese thermophilic mats, *Bacilli* were more abundant during the dry seasons while *Clostridia* were more abundant during the wet seasons (Briggs et al. 2013); here, *Clostridia* were found to be more abundant during Hawaii's dry seasons and correlated with lower DOC and salinity (Tables 5 & 6). Interestingly, the *Bacilli*

recovered in the V6 data set were also found to be more abundant during dry seasons, despite belonging to a different order than those found in Chinese thermophilic mats (Briggs et al. 2013), whereas those in the V9 data set included an OTU from the same order (Briggs et al. 2013) as being more abundant during the wet seasons (Tables 5 & 6).

Of note, several clades identified as differentially abundant in relation to Hawaii's wet and dry seasons were apparently not correlated to any of the water chemistry characteristics that varied with season. Examples of some of the microorganisms identified as more abundant during the wet seasons included members of the *Gemmatimonadetes* and *Spartobacteria*, which may have been washed into these anchialine habitats from other sources. In general, *Gemmatimonadetes* account for approximately 2% of soil bacterial communities (Janssen 2006), while the *Spartobacteria* are considered ubiquitous soil microorganisms (Herlemann et al. 2013), lending credence to their increased contribution to our samples during wet seasons being a result of increased groundwater influence, and thus accounting for their lack of correlation with any of the measured water chemistry characteristics.

CONCLUSIONS

Here, data from high-throughput amplicon sequencing imply that the alternations of wet and dry seasons minimally impact the distinct cyanobacterial–bacterial crust communities unique to the anchialine ecosystem of the Hawaiian Archipelago. Although community composition as a whole appeared to be more heavily influenced by geographic and spatial factors such as island and site, the pronounced wet and dry seasons of the islands did significantly correlate with water chemistry characteristics such as salinity, NH_4^+, DOC, TDN, and $NO_2^- + NO_3^-$. Additionally, shifts in relative abundance for approximately half of the third-level *Bacteria* and micro-*Eukarya* clades were detected, with many of these changes in OTU relative abundance correlated with at least one of the measured and seasonally influenced water chemistry characteristics.

Although this study provides insight into the limited impact of seasonal variation of microbial communities from the Hawaiian anchialine ecosystem, additional work remains to be done. For example, we were constrained to sampling just 2 wet seasons and 2 dry seasons, so much remains unknown concerning the generality and predictability of the observed patterns over longer time periods, particularly because the samplings were performed during and after the El Niño-related dry period in 2009–2010. A second question refers to whether finer-scale temporal variation in microbial community diversity, composition, and distributions exists in this ecosystem. However, anchialine habitats are considered one of Hawaii's most threatened ecosystems due to multiple factors, including invasive organisms such as introduced fish and invertebrate species (Chai et al. 1989, Acly 2003, Capps et al. 2009, Carey et al. 2011, Dalton et al. 2013, Havird et al. 2013, Marrack 2016), development and urbanization (Brock et al. 1987, Hawaii State Data Center 2015), nonpoint source pollution (Dollar & Atkinson 1992, Wiegner et al. 2006), and inundation via projected increases in sea level due to global climate change (Marrack 2016). Given that the abiotic and biotic uniqueness of individual habitats comprising the Hawaiian anchialine ecosystem is only now being recognized (Hoffman et al. 2018), we unfortunately risk losing the opportunity to document the biodiversity contained within them (microbial or otherwise) before they are degraded or destroyed.

Acknowledgements. We thank K. L. Kim and R. A. Kinzie III for generous help and support associated with fieldwork. P. M. Brannock and D. S. Waits provided assistance with sequence processing. P. M. Brannock and M. Newman provided comments and feedback during the writing process. Site access and collections were conducted under the following scientific permits: State of Hawaii Native Invertebrate Research Permit FHM10-232 and MAKA: Kamehameha Schools Permit 4803. The experiments 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). This represents contributions nos. 179 and 82 to the Auburn University (AU) Marine Biology Program and Molette Biology Laboratory for Environmental and Climate Change Studies, respectively. The authors declare that they have no conflict of interest.

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Editorial responsibility: Rutger de Wit, Montpellier, France

Submitted: November 6, 2017; Accepted: July 16, 2018
Proofs received from author(s): September 5, 2018