CHARACTERIZING THE EFFECT OF SUBMARINE GROUNDWATER DISCHARGE ON CORAL REEF PLANKTONIC MICROBIAL COMMUNITIES OF MO‘OREA, FRENCH POLYNESIA

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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For Steffanie Clothier and Charlotte McClintock for being the most supportive family I could ask for and inspiring me to believe in myself and the world. You have fed my curiosity ever since I was a kid leading me to be the scientist I am today.

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I would also like to thank the wonderful graduate students in the Nelson lab including Jessica Bullington, Shayle Matsuda, Wesley Sparagon, and Hendrikje Jorissen for bringing me on to projects, training me on laboratory equipment, and answering all my questions.

ABSTRACT

Submarine groundwater discharge is a natural phenomenon that is common on volcanic islands wherein fresh, nutrient rich water is expelled onto reefs in coastal zones, particularly during low tide. This natural source of nutrients may be helpful or harmful to the coral reef ecosystems that inhabit oligotrophic topical waters. One of the factors influencing this interaction is the microbial assemblage in the water column and the roles these communities play in maintaining coral ecosystem health. The addition of anthropogenic stressors potentially associated with SGD, including eutrophication due to increasing nutrient concentrations and the introduction of microbes associated with wastewater, may tip the scales of SGD influence towards harm. This thesis investigates the changes in microbial communities associated with submarine groundwater discharge in Mo‘orea, French Polynesia. Surveys were designed to resolve microbial dynamics over tidal and diel cycles using synoptic collections of water at the seepage point and at sites throughout the adjacent reef at high and low tide during day and night. Nutrient concentrations were elevated at the seep and greatest during low tide confirming SGD presence and variation due to tidal differences. A group of microbial families associated with SGD were identified, including Burkholderiaceae, Pseudomonadaceae and Arcobacteraceae, all previously associated with freshwater and wastewater habitats. Both wastewater-associated and eutrophication-associated microbes were present at each location with highest abundance at the seep point and lower abundance at the farthest sites demonstrating potential anthropogenic contamination of groundwater at these locations. These results indicate the potential for SGD to both directly and indirectly influence the microbial ecology of coral reefs.

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CHAPTER 1. INTRODUCTION

Coral reefs provide essential services not only to ocean organisms but to humanity. Island nations are particularly dependent on coral reefs for subsistence fishing, economic benefit from tourism, and as a barrier against coastal erosion (Laurans et al. 2013). However, due to anthropogenic actions, coral reefs around the world are degrading faster than they are able to grow (Silbiger 2014). Increased ocean temperature, acidity level, sedimentation, and pollution caused by industrial and residential activities has already eliminated half of the coral reefs since the 1950's (Wetzel 2021). These detrimental activities are not slowing in response to their damaging environmental impacts but instead escalating. This necessitates research into understanding the interactions between human activity and coral reefs as well as the dynamics influencing reef ecosystem's sensitivity to stressors. The following paper will focus on the role of submarine groundwater discharge in structuring reef microbial communities through chronic nutrient loading, changes in seawater chemistry, and potential input of pathogenic microbes.

1.1 Nutrient Enrichment Impact on Reefs

Coral reefs are predominantly found in oligotrophic waters and their productivity, despite the low nutrient density of their habitat, has left researchers questioning the nutrient cycling mechanisms corals possess and how microbes influence this system (Kelly et al. 2018). Coral is a unique organism because, while it is an animal that conducts cellular respiration, there are small algal symbionts known as Symbiodiniaceae that live within the coral's surface tissues that perform photosynthesis (van Oppen & Medina 2020). This results in an extremely efficient and productive symbiosis where the products of one

process can be used as reactants in another: the coral produces carbon dioxide and nitrogen as waste products from cellular respiration that are then utilized by Symbiodiniaceae for carbon fixation through photosynthesis (Morris et al. 2019). The coral can then use that fixed carbon in cellular respiration. This is one reason corals can survive in low nutrient conditions and excess nutrients can disrupt the balance of these ecosystems. Enrichment of nitrogen has been found to shift the relationship between Symbiodiniaceae and coral from symbiotic to parasitic, particularly when occurring without the addition of phosphorus (Morris et al. 2019). These algal symbionts are key in modulating the nitrogen fixation process and maintaining holobiont homeostasis so shifted N:P ratios can impact physiologic responses of coral to environmental stressors.

Coastal eutrophication is the excessive input of nutrients like nitrogen, phosphorus, and silicate that are present in the runoff from industrial, agricultural, and residential activities (Selman 2008). Nutrient loading can create unsuitable environmental conditions for corals. An influx of nitrogen and phosphorus can incite algal blooms that outcompete corals for space and light (Dorgham 2014). This creates a potentially hypoxic environment as the productive microbes use up dissolved oxygen during the decomposition process that follows an algal bloom, lowering the oxygen concentration of the seawater, and promoting coral mortality (Raj et al. 2020). Algal success and interaction can also mean an influx of pathogenic microbes that are fostered by algal exudates and potentially harmful to coral species (Nelson et al. 2013).

Nitrogen loading has been shown to damage coral ecosystems by impacting physiological processes as well. The addition of nitrate and phosphate has decreased net community calcification rates of reef communities and disrupted rates of photosynthesis

and respiration changing pH fluctuation from typical values (Silbiger et al. 2014). A meta-analysis of nutrient studies found that nitrogen enrichment decreased coral calcification rates by 11% (Shantz & Burkepile 2014). Another study found that corals bleached at lower temperature thresholds and bleached more severely in locations where nitrogen pollution was high (Donovan et al. 2020). Sources and impacts of nutrient pollution must be thoroughly studied because adequate net accretion rates to keep up with erosion and sea level rise as well as thermal tolerance are the most important factors of reef resilience in the context of climate change.

1.2 SGD as a Source of Nutrients and Pollution to Reefs

There are many factors that influence how SGD impacts the reef ecosystem. Disturbance can occur through nutrient loading (Couch et al. 2014), alterations of ecosystem metabolism (Silbiger et al. 2020), effects on coral growth (Lubarsky et al. 2018), effects on reef benthic cover (La Valle et al. 2019, 2021), and potentially the influx of wastewater associated microbes (Wiegner et al. 2021). One pathway of nutrient input into reef ecosystems is through submarine groundwater discharge (SGD), a geological phenomenon in which groundwater containing terrigenous materials that have accumulated within porous rocks is expelled into the surrounding coastal ocean and directly onto the reef. When groundwater is contaminated with, for example, agricultural nutrients or untreated sewage, this natural influx of nutrients can become elevated and potentially damaging rather than beneficial to reef ecosystems (Couch et al. 2014). This paper will focus on the detection of groundwater contamination as well as the interaction of submarine groundwater discharge with the microbial plankton communities in a coral reef ecosystem.

Groundwater contamination by wastewater is a common occurrence and typically caused by inaccessible or outdated sewage systems such as leaking pipes, septic tanks, and cesspools that allow untreated waste to reach the surrounding groundwater (Richardson et al. 2015). The most common systems confounded with wastewater contamination on volcanic islands are cesspools, small underground structures that temporarily hold the human waste from individual residences (Mezzacapo et al. 2020). They are typically open-ended cylinders that allow the untreated wastewater to slowly leak out of the tank and into the surrounding soil and, depending on depth of the water table, into the groundwater (McKenzie et al. 2019). The abundant nutrients and pathogens present in wastewater can then be transferred onto the reef through a point of SGD, potentially influencing the ecosystem in detrimental ways. As the water table continues to rise with sea level due to climate change and increases the groundwater's proximity to cesspools, groundwater contamination will be a more common problem exposing the essential nature of this research (McKenzie et al. 2019).

1.3 Microbial Signaling

The homeostasis that microbial communities provide is crucial for maintaining coral reef ecosystem health. Recent research has exposed the essential nature of the microbial community encompassing coral reefs to recycle nutrients (Wegley Kelly et al. 2018) and staving off harmful viruses and pathogens (Pollock et al. 2019). The communities surrounding healthy reefs are composed of highly diverse microbes with abundant autotrophs (Bruce et al. 2012) that assist in maintaining reef metabolism. Disruption from environmental factors could induce changes in the existing microbial

community and disrupt the homeostasis in which corals thrive, selecting for less diverse and more virulent microorganisms (Nelson et al. 2013). Factors associated with SGD including decreased salinity, decreased pH, and increased nutrient concentrations (Garrison et al. 2003) may affect the community of organisms in the reef surrounding an SGD seep, and therefore coral success.

An anthropogenic factor potentially altering microbial communities is groundwater contamination. Certain pathogens that are present in human waste can be used to identify if groundwater had been contaminated with sewage. The main pathogens associated with wastewater contamination are diverse members of the Phylum Gammaproteobacteria, especially the family Enterobacteriaceae (Salmonella spp., Escherichia spp., Shigella spp., Yersinia spp., Klebsiella spp.) as well as genera from additional Gammaproteobacterial families such as, Vibrio cholerae, Aeromonas hydrophila, Legionella pneumophila, Pseudomonas aeruginosa; additional pathogens in wastewater belong to diverse Phyla (e.g. Leptospira spp. Mycobacterium spp.) (Chahal et al. 2016). Eutrophication associated with an influx of nutrients from waste can also indirectly increase the abundance of opportunistic taxonomic orders like Vibrionales, Rhodobacterales, Flavobacteriales, and Cytophagales associated with deterioration in coral health and suppression of coral immune function (Zaneveld et al. 2016). Microbial sampling of the water column at sites of SGD known to be a source of chronic nutrient input can help identify if there is groundwater contamination from human wastewater as well as signal if coral health is threatened through the enrichment of these opportunistic pathogens.

To better understand the tidal and diel dynamics of submarine groundwater discharge, its effect on microbial community structure, and the potential identification of

groundwater contamination through microbial indicators, we investigated the microbial dynamics of SGD in reefs of Mo‘orea, one of the Society Islands of French Polynesia. Tracers of submarine groundwater discharge like radon, silicate, and salinity have been used to identify seepage points near reef ecosystems (e.g., Nelson et al. 2015). These parameters were explored in Mo‘orea and SGD seeps with high levels of elemental isotopes associated with groundwater, low salinity measurements, and high nutrient concentrations were found (Knee et al. 2016) prompting further investigation of SGD locations. In Mo‘orea, a majority of the enriched nitrate concentrations found in the groundwater are anthropogenic in origin and associated with wastewater contamination that enters the groundwater system at low altitudes (Haßler et al. 2018).

Our study involved collecting water at the seepage point of SGD and throughout the reef adjacent to a seep at high and low tide during the day and night. We hypothesize that the seep samples as well as the sites in close proximity to the seep will have increased nutrient concentrations, abundant freshwater microbes with higher microbial diversity that correlate with a drop in salinity and display a gradient of groundwater distribution radiating from the seep. We also hypothesize that this gradient will be the most apparent during low tide when the most SGD is released. Finally, we hypothesize that if groundwater contamination has occurred, pathogens associated with wastewater will be abundant at the seep and follow a similar pattern as the SGD gradient.

CHAPTER 2. METHODS

2.1 Site Selection and Setup

Seepage points of submarine groundwater in Mo‘orea were initially explored through interviews with local experts and through field measurements. In May of 2020, a research team from California State University, Northridge traveled to Mo‘orea where they spoke with local community members living along the coastline of Ha‘apiti who had knowledge of SGD seep locations. Salinity measurements were performed using a YSI conductivity sensor to confirm the location of freshwater seepage at two sites, here called Varari (-17.540, -149.899) and Cabral (-17.516, -149.912) (figure 1). The owners of the land adjacent to these seeps graciously allowed access to these sites as sampling locations. Observations of current patterns were used to guide selection of 20 fixed sampling sites at each location to ensure that the gradient of SGD dispersion throughout the reef was captured. The current at Varari has a dominant flow direction perpendicular to the coast moving northwest so a site was placed upstream of the flow and the remaining sites were distributed through the reef radiating outwards from the seepage point. The Cabral location has no dominant flow pattern so sampling sites were disseminated at varying proximities from the seep. At each site, a stainless-steel rod was secured into the reef where the sensors and sampling devices would later be attached.

Figure 1. Satellite Image of Sampling Locations Varari (left) and Cabral (right) with the SGD seep shown in red and the spatial distribution of sampling sites shown in blue. The northwest current at Varari is the reason for the placement of a site upstream of the seep and the remaining sites are dispersed downstream. With no dominant flow at Cabral, the sites were placed throughout the reef radiating out from the seep.

2.2 Autosampler Deployment and Sampling

In 2021, a team returned to Mo‘orea for this SGD project composed of microbiologists from the University of Hawai‘i at Mānoa, ecologists from California State University, Northridge, and geologists from California State University, Long Beach. Water collections at each site were primarily conducted using two types of autosampler. At all reef sites water was collected four times over a 24 hour period using a sub-surface automated dual water sampler (SAS) (Enochs et al. 2020). The design was developed by NOAA and the University of Miami, recreated through 3D printing, and assembled by technicians at California State University, Northridge. The SASs are designed to collect two water samples simultaneously with programmable pumps that are activated at specific

times and push a set volume of water into a collection bag. This allowed for easier sampling throughout tidal cycles during the day and the night. The seep samples were collected at higher frequencies (every 3-4 hours over a multi-day period) using a 12-channel autonomous multiport water sampler created by Dr. David Mucciarone at Stanford University (Mucciarone et al. 2021). This machine uses a peristaltic pump run by an Arduino computer that can be programmed to collect specific volumes of water at a set time with the advantage of multiple ports, so it does not need to be reprogrammed as often as the SAS autosamplers. The autosamplers were programmed with a flushing protocol to flush the manifold and ensure all collected water originated from the time of the sampling event. The device was placed as close to the seep point as possible so that the inflow valve would be immersed during high and low tide with a weight belt to keep it secured in place.

Autosamplers collected two samples at each sampling event timepoint. One collection bag was made of Tedlar, preloaded with mercury chloride ($HgCl_2$), and used for chemical measurements of pH, salinity, and total alkalinity. The second bag had a more complicated forked inflow system in which water moved first into a 5 mL syringe preloaded with 0.5% paraformaldehyde to preserve samples for plankton counts. A syringe blocker was placed on each syringe stopping the sample at 5mL and a one-way valve was attached to the luer to ensure there was no backwash of chemicals into the rest of the samples. When the syringe was full, water would then flow through the other side of the forked inflow tube and into a 0.2 μm polyethersulfone filter (Millipore Sterivex) for microbial analysis and the remaining filtered seawater was collected in a Mylar bag that was acid and methanol leached, then deionized water rinsed for nutrient and dissolved organic matter concentration measurements.

Tidal schemes were used to determine the sampling schedule so that the signal of high and low tide during the day and night could be captured throughout the reef samples (table 1) and samples were taken at the seep every three hours to acquire higher data resolution (table 2). Before the scheduled sampling time, a field team snorkeled throughout the sampling locations attaching SAS autosamplers already fitted with collection bags to all twenty sites using zip ties, the SASs were activated using a magnet, and programmed with a remote to collect 500mL of seawater. The team then retrieved the full bags as soon as possible after the collection finished and attached a new set. Samples were recovered within a maximum of 6 hours after collection and then immediately driven back to UC Berkeley's Gump Station where laboratory processing took place.

Table 1. SAS Water Sampling Schedule to capture tidal schemes during the day and night. Sampling began at the Varari location on August 5th, 2021, and continued until August 8th, 2021. The sampling at Cabral immediately followed beginning on August 9th, 2021, and finishing that same day.

Location	Tidal Scheme	Diurnal Scheme	Date & Time
Varari	High	Night	8/5/2021 00:00
Varari	High	Day	8/5/2021 11:57
Varari	Low	Day	8/8/2021 07:30
Varari	Low	Night	8/8/2021 18:30
Cabral	High	Night	8/9/2021 01:00
Cabral	Low	Day	8/9/2021 07:00
Cabral	High	Day	8/9/2021 13:00
Cabral	Low	Night	8/9/2021 19:00

Table 2. 12-Channel Autosampler Seep Water Sampling Schedule following the tidal schedule. Sampling began at the Varari location on August 4th, 2021, and continued to August 8th, 2021 but was disrupted by a high wave event where the machine had to be pulled out of the water on August 7th for a day. The sampling at Cabral immediately followed beginning on August 9th, 2021, and finishing that same day.

Location	Tidal Scheme	Diurnal Scheme	Date & Time
Varari	Low	Night	8/4/2021 18:45
Varari	High	Night	8/4/2021 23:51
Varari	Mid	Night	8/5/2021 02:51
Varari	Low	Day	8/5/2021 06:45
Varari	Mid	Day	8/5/2021 08:51
Varari	High	Day	8/5/2021 11:57
Varari	Mid	Day	8/6/2021 08:30
Varari	Low	Night	8/6/2021 19:00
Varari	Low	Day	8/8/2021 07:30
Varari	Low	Night	8/8/2021 18:30
Cabral	Low	Night	8/8/2021 19:00
Cabral	Mid	Night	8/8/2021 22:00
Cabral	High	Night	8/9/2021 01:00
Cabral	Mid	Night	8/9/2021 04:00
Cabral	Low	Day	8/9/2021 07:00
Cabral	High	Day	8/9/2021 13:00
Cabral	Mid	Day	8/9/2021 10:00
Cabral	Mid	Day	8/9/2021 16:10
Cabral	Low	Night	8/9/2021 19:00
Cabral	Mid	Night	8/9/2021 22:20
Cabral	High	Night	8/10/2021 01:20
Cabral	Mid	Night	8/10/2021 04:20
Cabral	Low	Day	8/10/2021 07:00

2.3 Laboratory Analysis

The Tedlar bags for chemical analysis were processed as soon as possible and pH, salinity, and total alkalinity values were recorded. The Mylar bags were first disassembled, and the syringe samples were transferred into labeled 2mL cryogenic tubes and placed in a -40 °C freezer. The Sterivex filters were detached and drained by repeatedly injecting air through the filter using a clean 10 mL syringe and then stored in a -40 °C freezer. A portion of the remaining filtered seawater collected was transferred into sterile 50mL Falcon conical centrifuge tubes after three rinses with the sample and kept in a -40 °C freezer for nutrient concentration measurements. The remaining seawater was transferred into 20mL glass amber vials that were previously acid washed and combusted and rinsed with sample three times and placed in a 4 °C refrigerator for fDOM analysis.

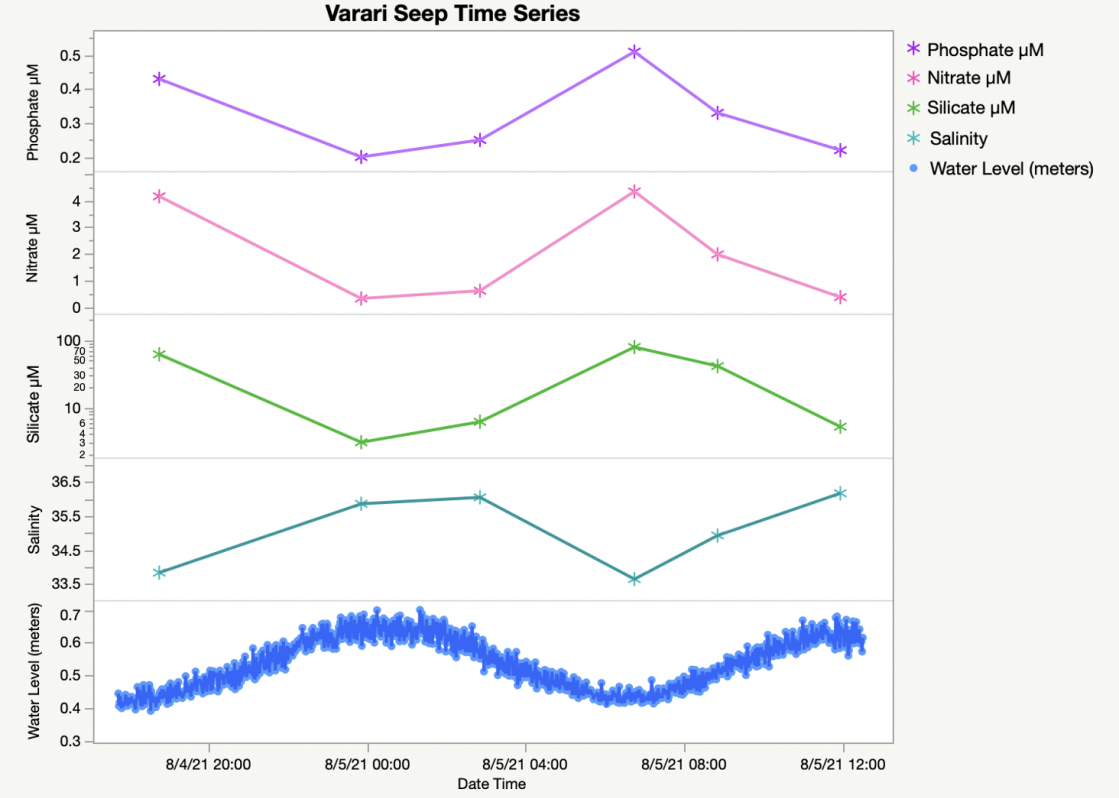
After completion of work in Mo‘orea, water samples were brought back to Hawai‘i for less than 1 month of storage (-80, -80, -20 and 4 °C, respectively) before being analyzed in laboratory facilities at the University of Hawai‘i at Mānoa. Nutrient samples were processed by the SOEST Laboratory for Analytical Biogeochemistry for dissolved inorganic nutrient concentrations. Flow cytometry samples were run on a Cytoflex Flow Cytometer stained with 7 µL of 40 µL mL^{-1} Hoechst 34580 (1 µg mL^{-1}, final concentration) following Nelson et al. (2015). Sterivex filters were cracked and removed using a sterilized blade and placed into PowerBead tubes where they were then submitted to the Microbial Genomics and Analytical Laboratory for 16S amplicon-based metagenomic library sequencing. This process included DNA extraction using a Qiagen MagAttract PowerSoil DNA kit followed by PCR targeting the v4 hypervariable region of the 16S gene and normalization for multiplex sequencing library preparation following standard Earth

Microbiome Project protocols and primers (Caporaso et al. 2012, Walters et al. 2016, Thompson et al. 2018). DNA concentrations were checked using a Qubit dsDNA HS Assay Kit and then sequenced. Fluorescent dissolved organic matter analysis proceeded on an Aqualog fluorometer following the procedures of Nelson et al. (2015).

CHAPTER 3. RESULTS

3.1 Evidence of SGD Presence and Tidal/Diel Variability

The samples collected at the seep had elevated concentrations of silicate, nitrate, and phosphate paired with lower salinity values. These values varied throughout tidal sequences with salinity dropping as the tide fell coupled with elevated nutrient concentrations at the seep during low tide (figure 2). Silicate and salinity were selected as indicators of groundwater because of their strong response to tidal variation. Silicate concentrations covaried with nitrate ($R^2 = 0.82$, $p < 0.0001$) and phosphate ($R^2 = 0.97$, $p < 0.0001$) concentrations and were well above average at the seep (marked by an asterisk) when compared to the reef samples (figure 3) and changed with tidal variation expressing that the seep was a major source of nutrients.



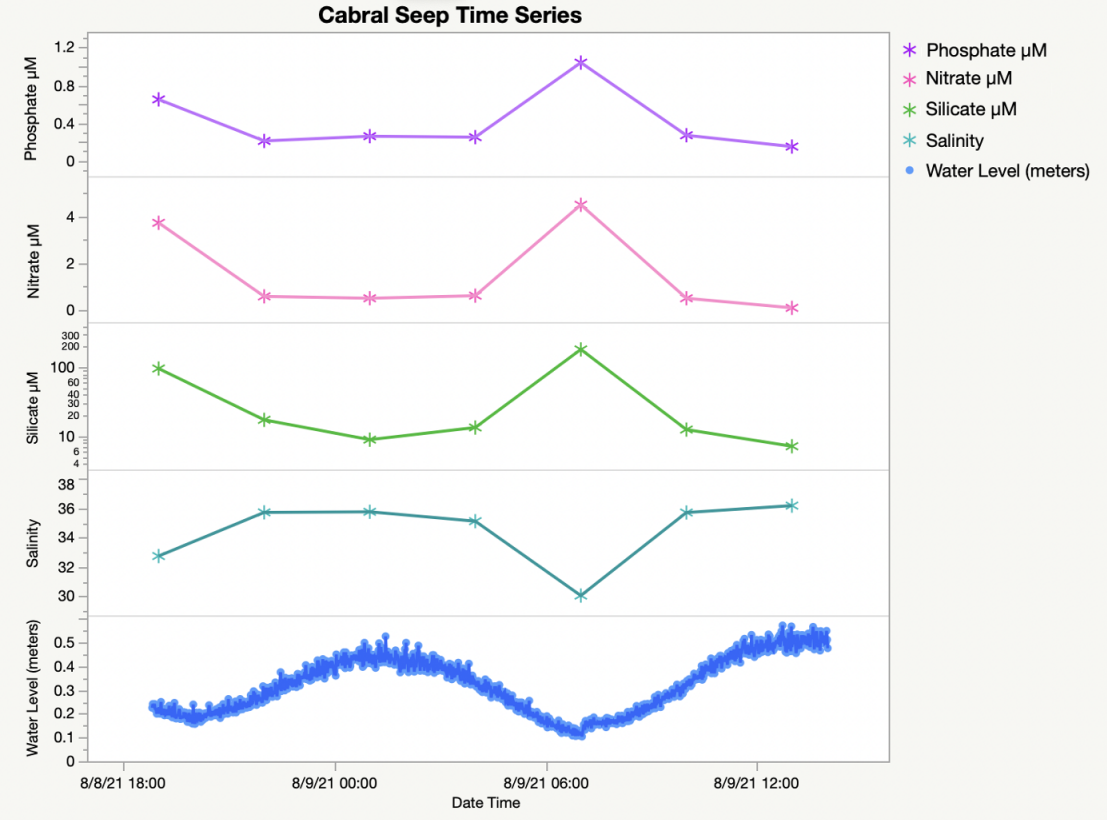


Figure 2. Time Series of water level, salinity, silicate, nitrate, and phosphate at the seep point at Varari (top) and Cabral (bottom) displaying the influence of tidal cycles on SGD discharge. Water level was found by placing instruments at the seepage point to measure the distance from the seafloor to the surface. When the water level was lower, nutrient concentrations including silicate, nitrate, and phosphate increased while salinity decreased.

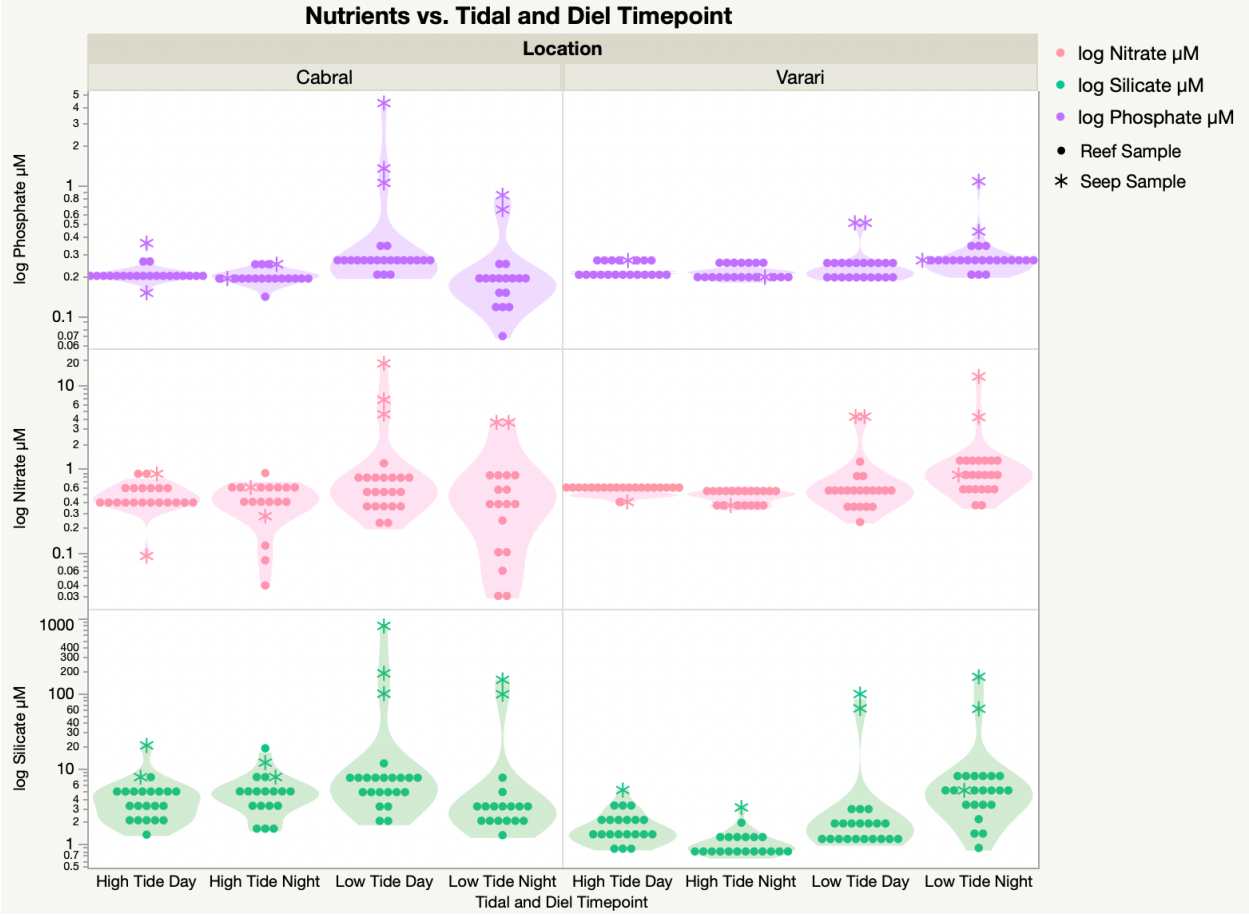


Figure 3. Violin Plot of log Nutrient Concentrations at each location across all timepoint with Cabral (left) and Varari (right). The seep samples are marked with an asterisk.

3.2 Differentiation of Microbial Communities Between Sampling Locations and Tidal Cycles

A multidimensional scaling plot displays the grouping of microbial communities by sampling location (figure 4). Statitstical analysis using the adonis function in R showed that the two sampling locations had differing microbial communities ($R^2 = 0.35$, $p < 0.001$); therefore, further analysis was performed investigating the sites separately. The communities appear to be influenced by the tidal schemes displayed in a multidimensional scaling plot (figure 5) in which low tide during the night at Cabral was the most different

and low tide during the day at Varari was the most different. Day and night samples seemed to separate out at Cabral regardless of tide while there was more overlap of microbial communities between day and night at Varari (figure 6).

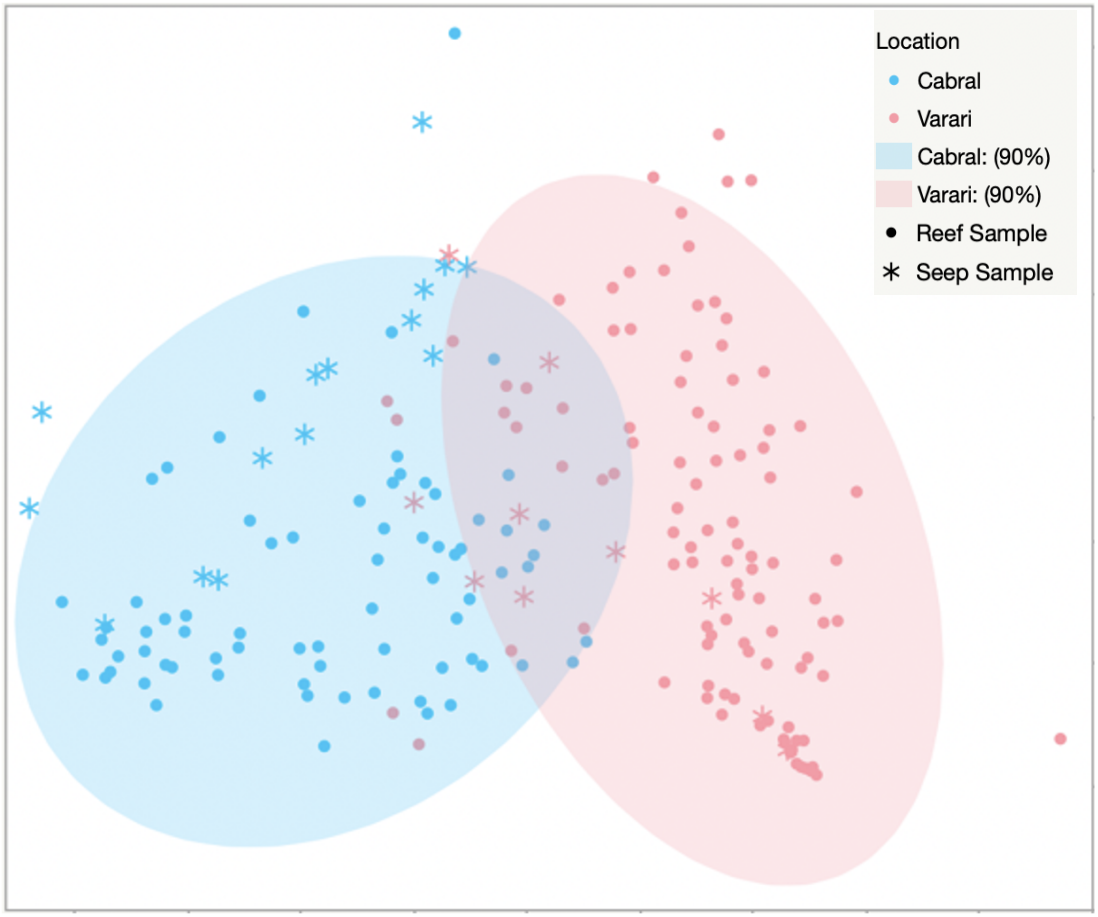


Figure 4. Multidimensional scaling plot showing the differentiation of microbial communities at each sampling location with ellipses displaying 90% confidence using microbial ASV data with the seep data points represented with an asterisk.

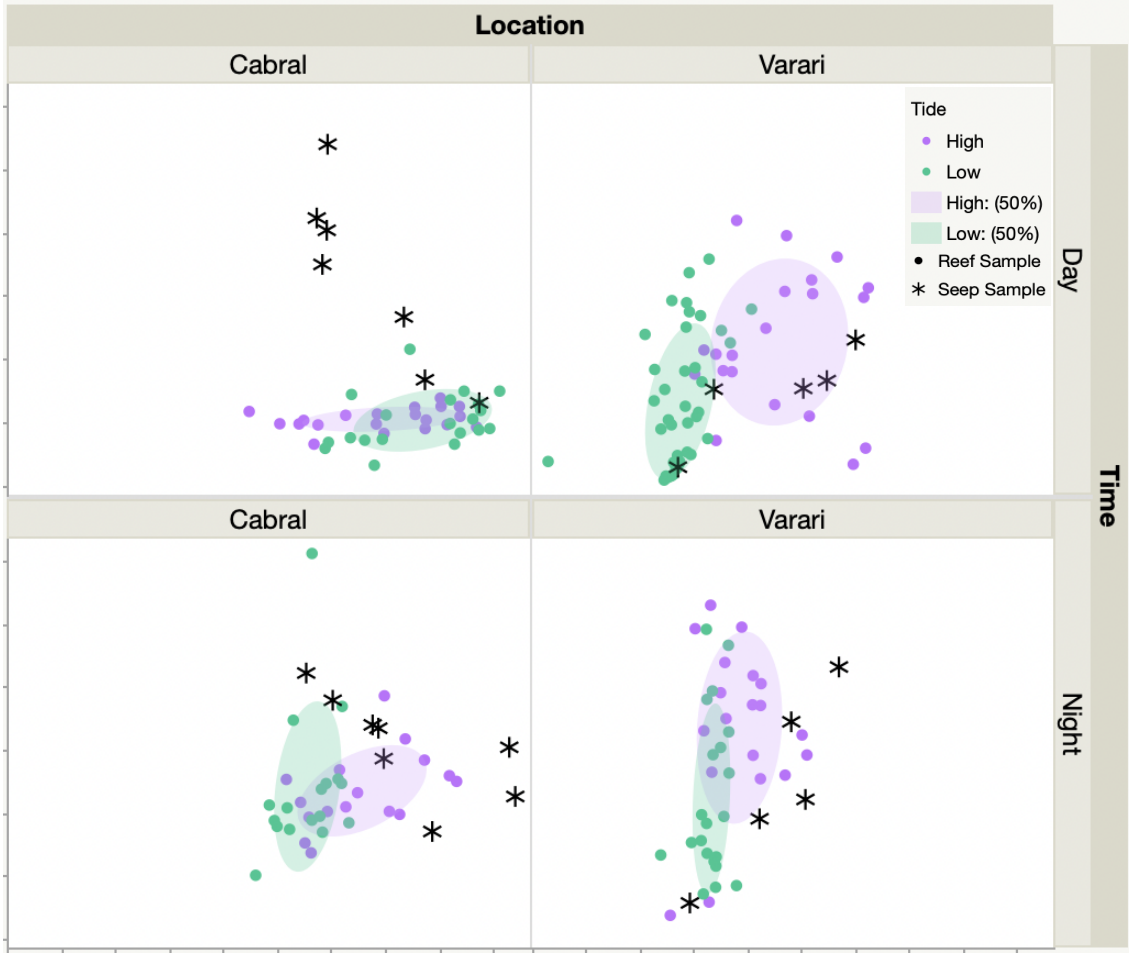


Figure 5. Multidimensional scaling plot showing the differentiation of microbial communities at high and low tide broken out by location and time with ellipses displaying 50% confidence using microbial ASV data.

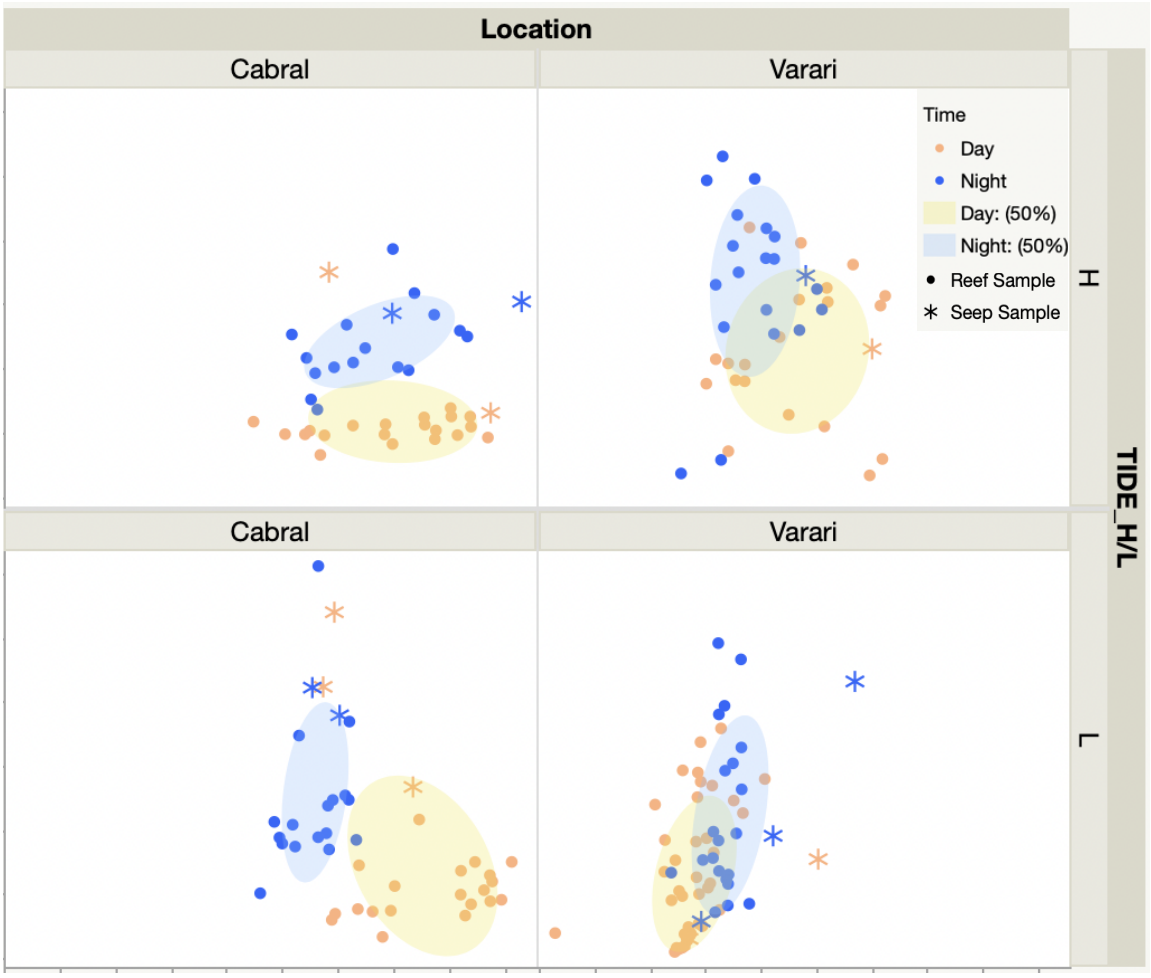


Figure 6. Multidimensional scaling plot showing the differentiation of microbial communities during the day and night broken out by location and tide with ellipses displaying 50% confidence using microbial ASV data.

3.3 Spatial Distribution of Freshwater Microbes Displaying SGD Gradient

To explore SGD influence on microbial communities, hierarchical clustering using Ward's minimum variance method was performed (figures 7 & 8) after angular transformation. At Cabral, a cluster of seep-associated microbes that were enriched in seep samples were identified (table 3), largely comprising taxa widely recovered from

freshwater and groundwater habitats. Three families of Bacteria - Burkholderiaceae, Pseudomonadaceae, and Arcobacteraceae - were relatively abundant in the seeps (maxima greater than 10%) significantly correlated with Silicate ($p < 0.05$) and significantly enriched in the Seep samples ($p < 0.05$) (figure 9). Correlations between the summed relative abundances of these seep-associated microbes and silicate concentrations indicating groundwater discharge confirmed that this microbe module was associated with SGD output (figure 11; $p < 0.0001$) at both Varari and Cabral. The summed abundance of the freshwater module overlaid on the coordinates of a multidimensional scaling plot performed for each location emphasized that the abundance of the taxa in this seep-associated module had a significant influence on the overall beta diversity displayed in the ordination (figure 11).

To understand the distribution of SGD nutrients throughout the reefscape, a contour plot of silicate concentrations at Cabral and Varari was produced (figure 12). Silicate concentrations appeared to be affected by tide with high concentrations at Varari during low tide compared to high tide with smaller changed occurring at Cabral. The samples collected closer to the seep have higher silicate concentrations and seemed to follow the current patterns. Cabral has no dominating current while Varari has a current moving northwest that is apparent in the silicate bloom. The summed abundances of the freshwater microbe cluster were then plotted along with the latitude and longitude coordinates of the site to display the spatial distribution of these families at each sampling time point across tidal and diel schemes (figure 13).

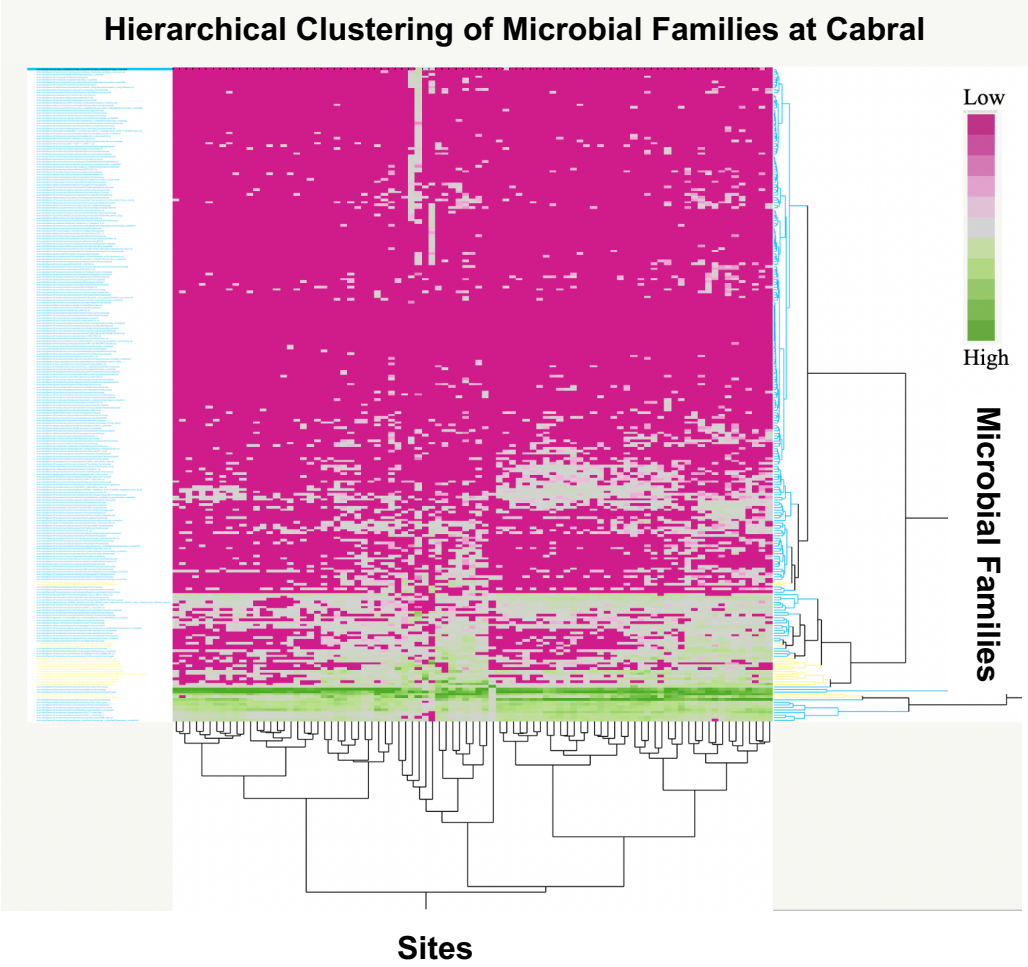


Figure 7. Hierarchical clustering of microbial abundances at Cabral that were summed by family and transformed using arcsinsqrt. Green represents high abundance while pink represents low abundance. The freshwater module appears in yellow with higher abundances in the seep samples than the reef samples and the remaining families appear in blue.

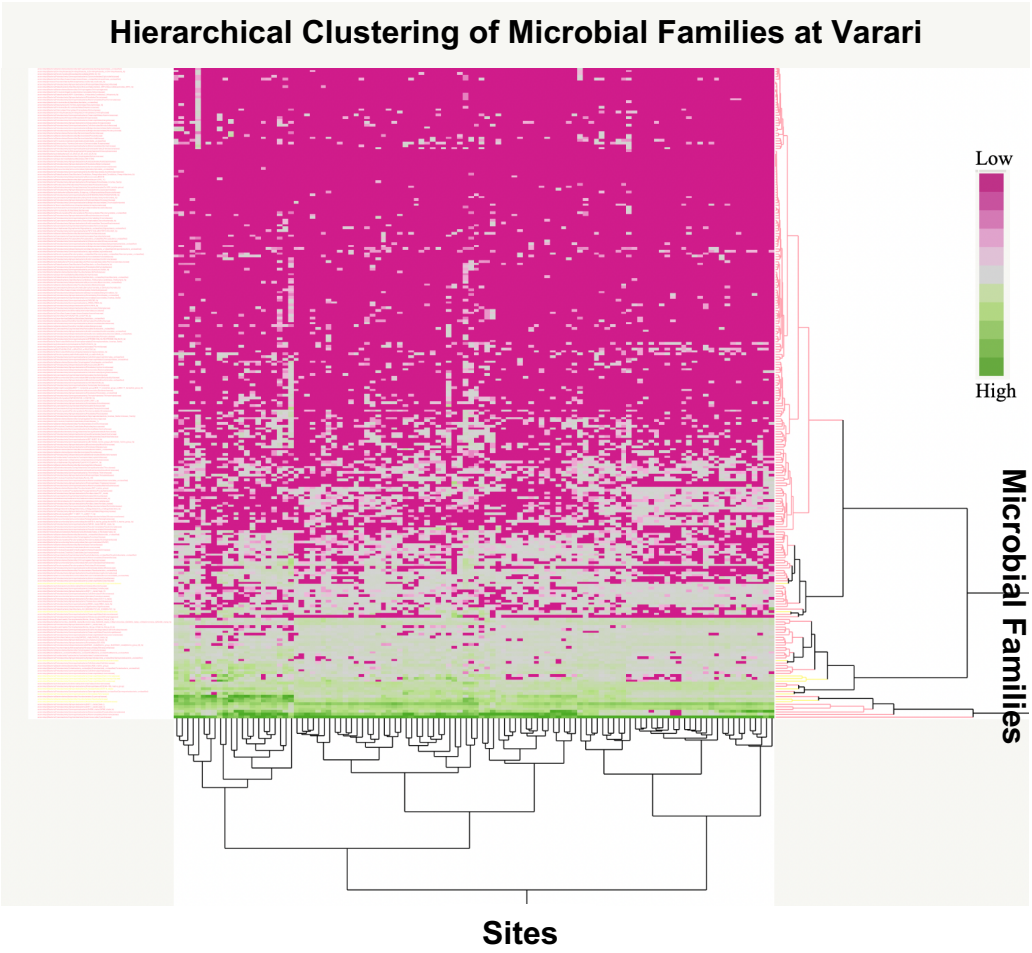


Figure 8. Hierarchical clustering of microbial abundances at Varari that were summed by family and transformed using arcsinsqrt. Green represents high abundance while pink represents low abundance. The freshwater module appears in yellow with higher abundances in the seep samples than the reef samples and the remaining families appear in red.

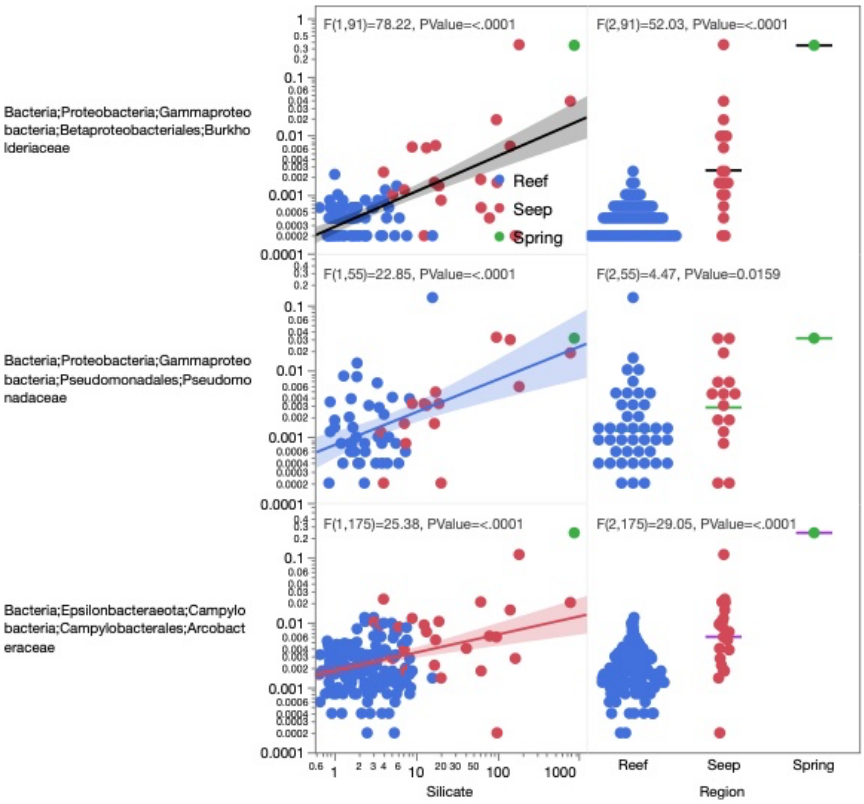


Figure 9. Correlation of the three most abundant families in the seep-associated microbial module and their distribution among reef, seep, and spring samples.

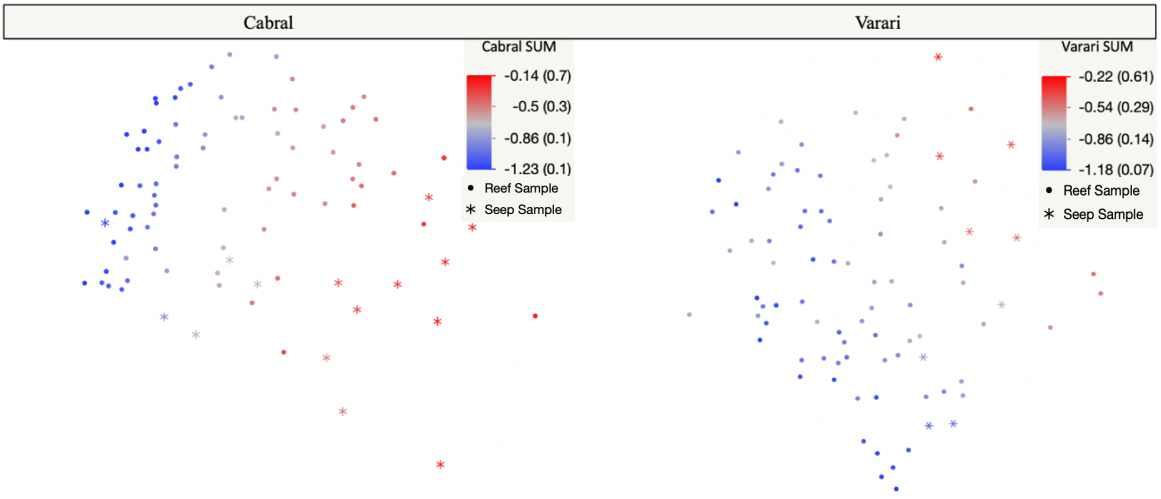


Figure 10. Multidimensional scaling plot overlaying the freshwater module's abundance using microbial data from Cabral (left) and Varari (right) that was summed by family and transformed using arcsinsqrt colored by the summed abundance of the freshwater microbe module where blue is low abundance and red is high abundance.

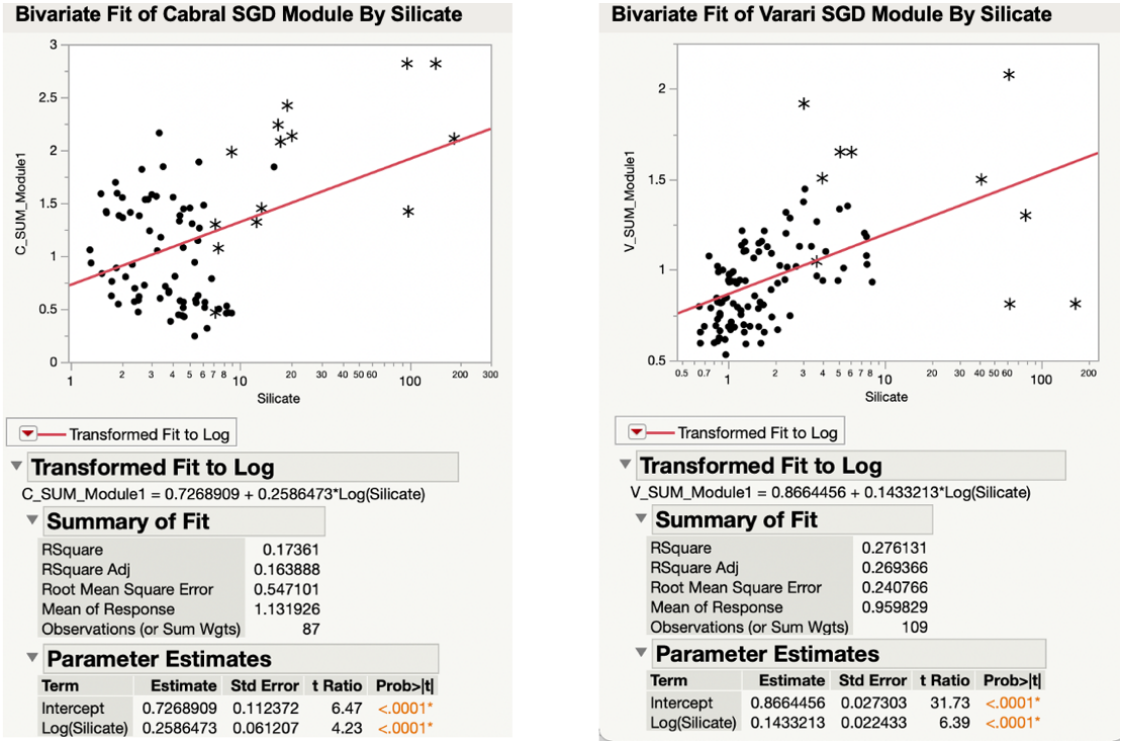


Figure 11. Correlation and summary statistics of summed abundance of the freshwater microbe module vs. log silicate concentration. Higher silicate concentrations are associated with higher freshwater microbe abundance and are elevated in the seep samples.

Table 3. SGD Associated Bacteria identified in figure 7 & 8 as a freshwater microbe module at each sampling location.

Location	Class	Order	Family
Varari	Bacteroidia	Flavobacteriales	Flavobacteriaceae
Varari	Campylobacteria	Campylobacterales	Arcobacteraceae
Varari	Alphaproteobacteria	Puniceispirillales	SAR116_clade
Varari	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae
Varari	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae
Varari	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae
Varari	Gammaproteobacteria	Oceanospirillales	Marinomonadaceae
Varari	Gammaproteobacteria	Oceanospirillales	Nitrincolaceae
Varari	Gammaproteobacteria	Oceanospirillales	Saccharospirillaceae
Varari	Gammaproteobacteria	Vibrionales	Vibrionaceae
Cabral	Bacteroidia	Flavobacteriales	Flavobacteriaceae
Cabral	Campylobacteria	Campylobacterales	Arcobacteraceae
Cabral	Alphaproteobacteria	Caulobacterales	Hyphomonadaceae
Cabral	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae
Cabral	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae
Cabral	Gammaproteobacteria	Alteromonadales	Alteromonadaceae
Cabral	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae
Cabral	Gammaproteobacteria	Arenicellales	Arenicellaceae
Cabral	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae
Cabral	Gammaproteobacteria	Cellvibrionales	Spongiibacteraceae
Cabral	Gammaproteobacteria	Gammaproteobacteria_unclassified	Gammaproteobacteria_unclassified
Cabral	Gammaproteobacteria	Nitrosococcales	Methylophagaceae
Cabral	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae
Cabral	Gammaproteobacteria	Oceanospirillales	Marinomonadaceae
Cabral	Gammaproteobacteria	Oceanospirillales	Nitrincolaceae
Cabral	Gammaproteobacteria	Oceanospirillales	Saccharospirillaceae
Cabral	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae

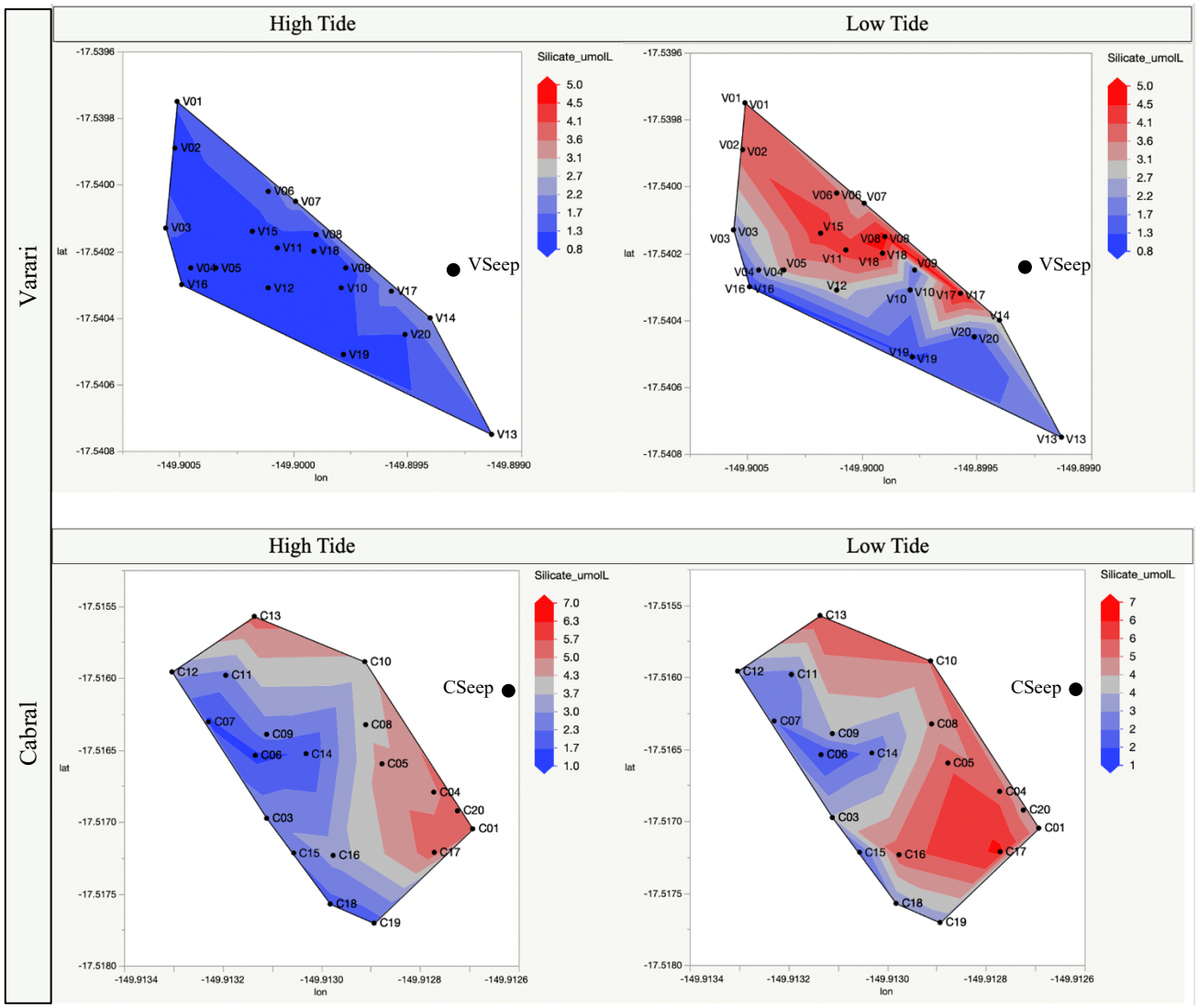


Figure 12. Contour plot of silicate concentrations excluding the seep point at Varari (top) and Cabral (bottom) displaying a nutrient bloom with higher concentrations closest to the SGD seep point distributing downstream with the direction of the current at Varari and spreading across the reef at Cabral where there is little current. Silicate concentrations at the seep skewed the contours because they were much higher so seep samples were excluded in the figure and a point where the seep would be located was added.

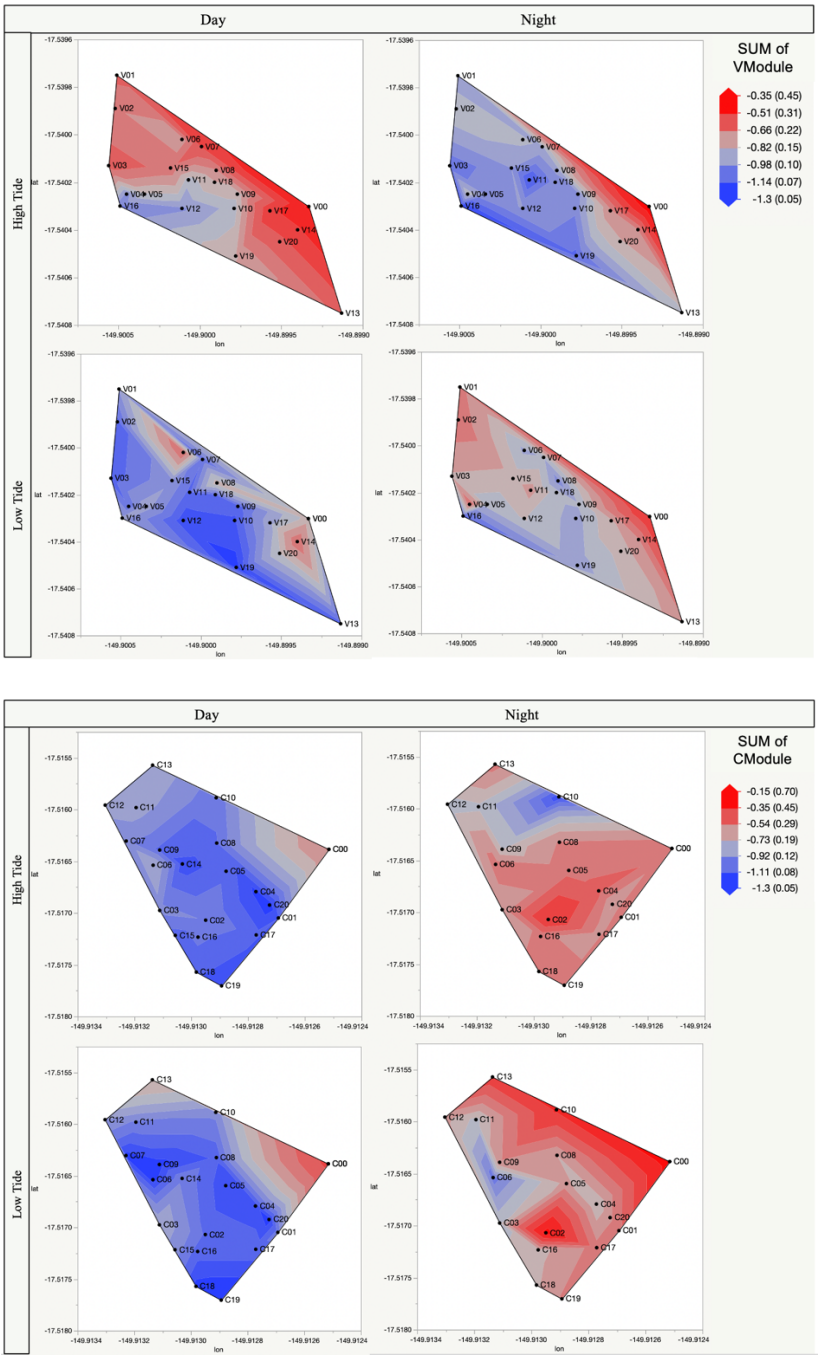


Figure 13. Contour plot displaying the spatial gradient of the abundance of the freshwater microbe module abundance at Varari (top) and Cabral (bottom). The abundance of freshwater microbes is greatest at the seep point during high tide at Varari and low tide at Cabral.

3.4 Detection of Microbes Associated with Wastewater Pollution

The potential pathogens associated with wastewater contamination identified by Chahal et al. 2016 were searched for in the ASV datasheet and their relative abundances were aggregated by site if present (table 4; figure 14). Abundance of these microbes was highest at the seepage point reaching a relative abundance of 1.4% at Cabral and 1.6% at Varari.

Table 4. Microbes Associated with Wastewater Contamination detected in microbial samples.

Class	Order	Family	Genus
Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia-Shigella
Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonadaceae_unclassified
Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
Leptospirae	Leptospirales	Leptospiraceae	Leptospiraceae_unclassified
Gammaproteobacteria	Legionellales	Legionellaceae	Legionella
Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas
Gammaproteobacteria	Legionellales	Legionellaceae	uncultured
Actinobacteria	Corynebacteriales	Mycobacteriaceae	Mycobacterium

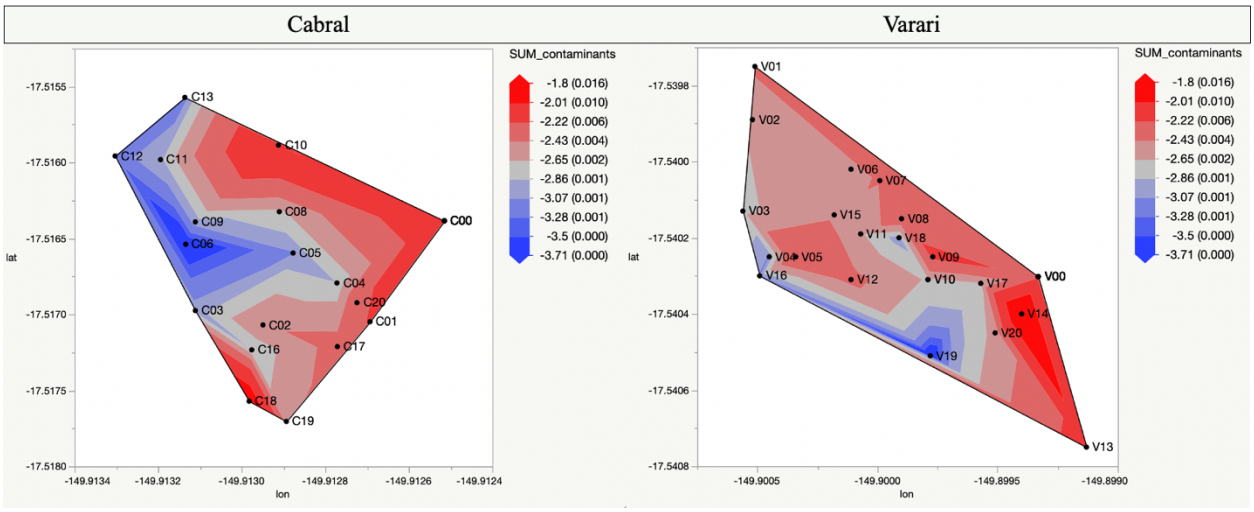


Figure 14. Contour plot showing spatial distribution of the summed log abundance of wastewater associated microbes (table 4) at Cabral (left) and Varari (right) during low tide at night.

3.5 Alpha Diversity Metrics

A boxplot of sobs richness, shannon index, and shannon evenness shows the changes in diversity across tidal and diel schemes. A Tukey test of these alpha diversity metrics at Cabral showed a significant difference between night and day at low tide, between high and low tide during the night, and between high day and low night for sobs richness, shannon index score, and shannon evenness ($p > 0.02$). A Tukey test of samples at the Varari location showed no significant differences in sobs richness across time or day. There were changes in the evenness of the communities with the shannon index score and shannon evenness showing a significant difference between day and night across tidal schemes ($p > 0.05$) as well as between high tide and low tide across diel schemes ($p > 0.05$).

Cabral Varari

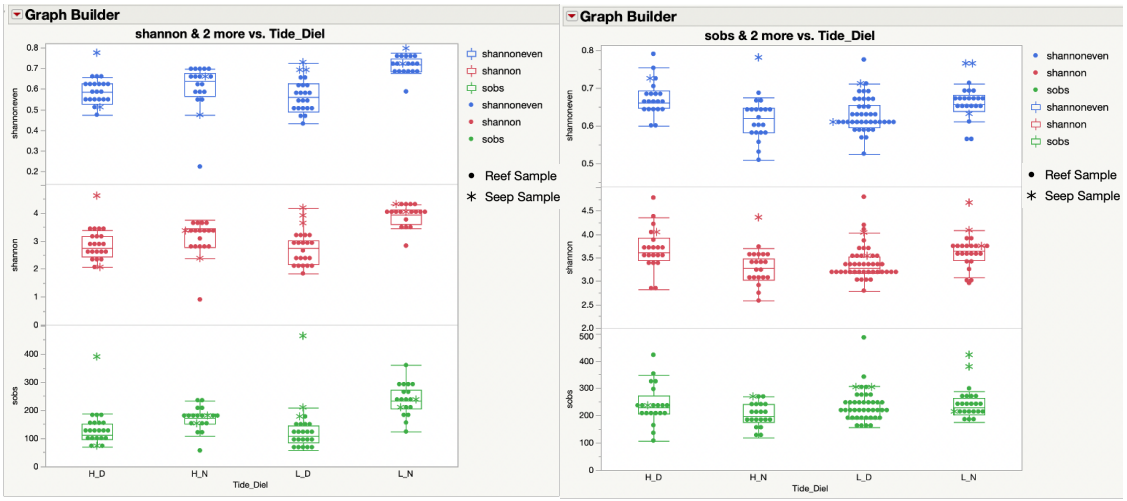


Figure 15. Boxplot of alpha diversity metrics at each combined diel and tidal scheme of Cabral (left) and Varari (right) with the seep samples represented as an asterisk. The sobs score describes the richness of the population, the shannon index score describes the evenness of the population.

CHAPTER 4. DISCUSSION

The data showed variability of environmental parameters like silicate, nitrate, phosphate, and salinity that corresponded to water level signaling tidal influence on SGD discharge. Consistent with previous findings (Moore 2010), there was variability of these metrics between high and low tide with elevated nutrient concentrations during low tides paired with lower salinity levels signaling more SGD influence at those times. Additionally, at both of the two sampling locations, there were key differences between samples taken at the point of SGD seepage and the rest of the sites distributed throughout the adjacent reef. Nutrient concentrations including silicate, nitrate and phosphate were higher while salinity was lower at the seep than the reef samples allowing for these parameters to be selected as indicators of groundwater throughout the reefscape.

An ordination of the microbial data displayed the significant difference of microbial communities at Varari and Cabral. The exact reason for this is unknown as the locations are not particularly far away from each other and have similar reefscapes. Further analysis separating out the ordination by location, time, and tide showed that microbial assemblages differed by high and low tidal schemes as well as during the day and night. The difference in assemblage by tide could be because of the SGD's increased flow during the low tide, selecting for freshwater associated microbes. Diel patterns of change in communities have been observed in previous work and thought to be a method of energetic provisioning of reef systems (Wegley Kelly et al. 2019).

Hierarchical clustering of the microbial abundances run separately for Varari, and Cabral exposed a module of microbes that were more abundant in the seep samples than the reef samples. The sum of the abundance of this module overlaid on the ordination shows

a gradient of abundance moving from left to right with the seep samples clustering closer to the right side of the ordination where the module's summed abundance was greatest. A graph of the modules summed abundance versus logged silicate concentrations showed a correlation in which samples with higher silicate concentrations had higher abundances of the microbes included in the module. This provides evidence that the list of microbes identified in this module are SGD-associated. Almost all of the microbes in the module were of the phylum Proteobacteria specifically of the classes Gammaproteobacteria and Alphaproteobacteria, and some of the most abundant families in the module (figure 9) have been previously associated with groundwater and freshwater habitats on tropical islands (Kirs et al. 2020).

Other families found enriched in the seep-associated cluster have been previously associated with algal exudates and algal-dominated reef systems. The algal exudates of the invasive species *Turbinaria ornata,* common in Mo‘orea and observed at the sampling locations, have been found to select for Gammaproteobacteria families such as *Vibrionaceae* and *Pseudoalteromonadaceae* that have higher virulence factors (Nelson et al. 2013). These families were present in the freshwater module indicating that SGD input from the seep could be fostering algae species like *Turbinaria* that proliferate in high nutrient environments causing the bacterioplankton population to be dominated by opportunistic pathogens that associate with their exudates.

The spatial distribution of the freshwater module showed highest abundance at the seepage point of SGD at both Cabral and Varari. At Cabral, these freshwater microbes were most abundant during low tide at night while at Varari, the tidal pattern was less clear. This could be due to the consistent water flow due to the current at Varari that is not present at

Cabral. Despite this heterogeneity we resolved three abundant bacterial families across the reef ecosystem that exhibited distributional patterns across sites indicating they were sourced from SGD (figure 9). The family *Burkholderiaceae* is a well-known freshwater-associated clade of the *Betaproteobacteriales,* (Linz et al. 2019, Newton et al.). The family Pseudomonadaceae is widespread in environmental samples but primarily associated with freshwater and terrestrial habitats and includes several human pathogens in the Pseudomonas genus. The family *Arcobacteraceae* is associated with wastewater ecosystems and is a compelling indicator of wastewater-associated contamination of the SGD in these ecosystems. Bacteria in the family *Arcobacteraceae* were identified in the freshwater module at both Varari and Cabral, a family found to be highly prevalent in sewage as well as groundwater (Venâncio et al. 2022) prompting further investigation into potential wastewater contamination at these sites. All three exhibited relative abundances of less than 1% in the reef samples but rose above 10% in the SGD samples, suggesting they are at least somewhat persistent in the reef.

Microbes associated with wastewater contamination identified by Chahal et al. 2016 were present in the microbial samples at both Varari and Cabral and were majorly dominated by Gammaproteobacteria. While the abundance was relatively low, the spatial distribution at Varari and Cabral displayed higher concentrations at the seep than the reef samples with a gradient moving away from the seep location. This provides evidence that wastewater pollution from anthropogenic activities may be entering the groundwater at these locations and discharging onto the reef at the seepage point consistent with previous investigations of SGD (Valiela et al. 1990). This means that the elevated nutrient

concentrations found at the seepage point might not only be from environmental factors but from an influx of nitrogen and phosphorus found in residential waste.

Samples taken at Cabral during low tide at night appeared to have a slightly higher richness and evenness of microbial assemblage with the high tide day samples having the second highest alpha diversity. At Varari, samples taken during low tide at night and high tide during the day appeared to have slightly higher richness and evenness of microbial communities. Previous studies of planktonic microbial communities above a reef have found microbial richness to be higher during the day than the night (Weber & Apprill 2020). A majority of the seep samples had higher alpha diversity metrics (richness and evenness) compared to the reef samples at both locations which may be due to the mixing of freshwater and saltwater as well as the influx of nutrients at that location.

CHAPTER 5. CONCLUSION

As expressed in my results, seepage points of submarine groundwater discharge were found at the two locations studied in this research and the SGD did influence the microbial community present across the reef environment. The SGD was characterized by low salinity as well as high phosphate, nitrate, and silicate. Tidal cycling impacted the amount of submarine groundwater that was discharged with increased flow at low tide shown by the decrease in salinity and the increase in nutrient concentrations at that time. These elevated nutrients were distributed throughout the reef and followed the current pattern prevailing at each location. A cluster of families that were more abundant at the seepage point than the reef samples could be identified and included bacteria that are commonly found in freshwater and wastewater habitats. When the summed abundance of this cluster was overlayed on a multiple dimensional scaling plot of microbial community data, it exposed a gradient of SGD influence over the reefscape. Finally, wastewater associated microbes were identified in low relative abundances at both location and appeared to be coming from the seepage point indicating that a portion of the elevated nutrient concentrations and influx of wastewater bacteria could be anthropogenic in origin.

Further research is necessary to determine how this microbial community shift may affect the reef ecosystem. Analysis into the community composition of marine organisms at each site as well as their physiological changes would be beneficial to assess if this influx of nutrients is doing any harm to the local reef system. The percent coverage of coral versus algae at each sampling location could shed light on how low salinity SGD with excess nutrient load is affecting a potential phase shift of these ecosystems. Measurements of coral growth at these sites would be profitable to compare with previous studies on nutrient

enrichment's effect on calcification and bioerosion rates. If further research confirms that elevated nutrient concentrations are from wastewater through fecal indicator bacteria and source tracking techniques, and that it is damaging the reef ecosystem, then the necessary steps can be made to update wastewater systems.

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