A COMPARATIVE STUDY OF THE BACTERIAL COMMUNITIES IN CALIFORNIA VERNAL POOLS

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Dana Lynn Carper

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A COMPARATIVE STUDY OF THE BACTERIAL COMMUNITIES IN CALIFORNIA VERNAL POOLS

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Department of Biological Sciences

Abstract

of

A COMPARATIVE STUDY OF THE BACTERIAL COMMUNITIES IN CALIFORNIA VERNAL POOLS

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Dana Lynn Carper

Microbes usually provide important functions as the basal trophic level of ecosystems. Their high reproduction and dispersal capabilities can result in their ubiquity. The “everything is everywhere” hypothesis of bacterial biogeography has been challenged recently by an indication that abiotic factors determine bacterial communities. Vernal pools are insulated environments, which can function similar to islands in regard to the movement of organisms. An alternative hypothesis is that vernal pools in close proximity will have increased movement of organisms from one to the other, while pools that are further away would have less flow of organisms resulting in closer pools supporting similar bacterial communities. California vernal pool communities (primarily plants) have been researched at the local and landscape level but little to no characterization of the microbial community has been done. For this environment it is hypothesized that there will be a difference in the bacterial communities in different

California vernal pool ecosystems (CVPE) and that the difference will be driven by location or abiotic factors.

Six vernal pools from three different sites were used to evaluate whether location or abiotic factors drive bacterial community structure. Two vernal pools were sampled from each of these three locations; Mather Field Air Force Base, Travis Air Force Base and Beale Air Force Base. Turbidity, pH, phosphates and conductivity were used as abiotic factors. All of the bacterial analysis was carried out using community analysis techniques including; community level physiological profiles, terminal restriction fragment length polymorphism and next generation sequencing. Bacterial cells in each sample were enumerated using fluorescent staining and flow cytometry. Biolog EcoPlates™ were used to assess differences in metabolism for the communities. Terminal restriction fragment length polymorphism (t-RFLP) and next generation sequencing (NGS) were used to evaluate bacterial community diversity. Water samples contained between $4x10^7$ and $6x10^7$ cells per ml of water. Clustering analysis using the Biolog EcoPlates™ data showed two distinct groups: one contained Travis2, and both Beale pools and the other containing Travis1 and both Mather pools. Principal component analysis (PCA) was conducted showing the same grouping. Axis 1 showed a moderate negative correlation with phosphates ($r = -0.473$, $r^2 = 0.223$, tau = -0.200) and axis two showed strong negative correlations with phosphates ($r= -0.760$, $r^2 = 0.577$, tau= -0.333) and pH (r = -0795, $r^2= 0.632$, tau= -0.733).

t-RFLP showed differences dependent on the enzyme used, with all enzymes showing low values of the Bray-Curtis index of similarity. The highest value produced

was from using the restriction enzyme *RsaI* between Travis1 and Travis2 (0.37). NGS showed six main classes of bacteria found in each pool, although relative abundance of each differed between pools: Spartobacteria, Verrucomicrobiae, Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, and Sphingobacteria. All are common freshwater bacteria, which generally function in degradation of organic matter. The Bray-Curtis Index of similarity was higher using NGS data, with the highest value being 0.89 between Travis1 and Beale2. A PCA was conducted using NGS data also showing correlations with pH, phosphates and turbidity. UniFrac Unweighted Pair Group Method with Arithmetic Mean (UPGMA), resulted in two strongly supported groups one containing the Travis pools and the other containing the Beale and Mather pools. This indicates that an evolutionary significant branch was found in the Travis pools, which was not located in the rest of the pools.

All of this data together supports the idea that there are differences in the bacterial communities in CVPE and that the data supports that abiotic factors are driving those differences. Further research will be needed to understand any biogeographical patterns, since only a few pool exhibited biogeographical influences.

_____, Committee Chair
Dr. Enid T. Gonzalez-Orta

_____
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INTRODUCTION

Microbial ecology is the study of microbes and their roles and interactions in ecosystems. Examining the bacterial composition of an environment is fundamental to understanding the functioning of ecosystems and the services (i.e. recreation, healthy environments) that the ecosystem can provide people. Bacteria are primarily decomposers, and therefore are crucial for nutrient cycling and can act as the basal trophic level in many food webs. While many studies have focused on how local environmental factors influence density and diversity within communities (Cho and Tiedje 2000, Gutknecht *et al.* 2006, Fierer *et al.* 2007, Chong *et al.* 2009), less information is available about the regional or biogeographical distributions of communities.

Beijerinck (1913), put forth the hypothesis that "everything is everywhere", postulating that since bacteria have such high dispersal rates and high reproductive rates, that all bacteria are essentially everywhere. The "everything is everywhere" hypothesis has been at the center of the microbial biogeography debate, and this hypothesis argues that microbes have no discernable biogeographical patterns, while others suggested that microbes have biogeographical patterns that had not yet been elucidated (Shade *et al.* 2013).

Bacteria are transported easily by both biotic and abiotic mechanisms. Griffin *et al*. (2001), documented the travel of microbes in African Dust clouds across the Atlantic

ocean to North America that included bacterial species such as, *Bacillus* sp, *Sphingomonas* sp, *Coccodinium* sp and *Microbacterium* sp. The bacteria survived the transport time of about a week, indicating the resilience of bacteria to other factors such as radiation, lack of nutrients, and desiccation (Reche *et al.* 2009). Bacteria are also easily transported by biotic vectors, such as birds, insects and large mammals (Schlichting Jr. and Sides 1969, Foissner 2006, Van der Gucht *et al.* 2007, Psaroulaki *et al.* 2010). Humans also participate in microbial dispersal; this has been especially detrimental with the transport of the plant pathogen *Pseudomonas syringae,* which causes disease in many crop plants (i.e. brown spot disease in snap beans) (Bashan 1986, Hirano and Upper 2000). This mechanism is often passive but tires, boots, and human activities can aide in dispersion, unintentionally enhancing distribution of these microorganisms (Foissner 2006, Wilkinson 2011, Perrigo *et al.* 2012).

Baas Becking (1934), amended the Beijerinck hypothesis with "everything is everywhere but the environment selects." This suggests bacteria can disperse to all environments, but that environmental conditions cause the differences in the bacterial composition. Carbon type and availability is one factor in the environment that has been shown to contribute to bacterial community diversity, with only certain bacteria using specific carbon sources. This gives support to the idea of niche-diversification as an explanation for the patterns seen in bacterial communities (Jones *et al.* 2009). Niche-diversification results from organisms specializing on resources in a way that reduces competitive interactions with other species. For example, Shade *et al.* (2008) examined bacterial communities in stratified versus polymictic lakes, and determined that bacterial

composition and diversity increased with stratification. Other environmental factors such as pH, dissolved oxygen and other local factors (e.g. depth of aquatic habitats and carbon availability) influence bacterial diversity (Langenheder *et al.* 2005, Judd *et al.* 2006, Fierer *et al.* 2007, Van der Gucht *et al.* 2007, Carrino-Kyker and Swanson 2008, Lindstrōm and Langenheder 2012).

While some studies have attributed environmental factors to influencing diversity others have pointed to geographic distance. Xiong *et al.* (2012), examined diversity in bacterial communities in lakes, and noted communities that were more geographically distinct had more dissimilar communities and 12% of the variation within samples was dependent on geographic distance. Reche *et al.* (2005), applied the theory of island biogeography to microbial communities within lakes. Microbial diversity increased with lake size. Although distance did not increase differences in community assemblages (MacArthur and Wilson 1967). Other research indicates a combination of environmental and geographical influences shaping the bacterial diversity (Yannarell and Triplett 2005, Bissett *et al.* 2010). The presence of bacterial generalists contributes to the difficulty in determining the influences of geography and environmental factors. Generalist bacteria are able to survive in the majority of environments, and some scientists believe that these are the majority of microbes within aquatic environments (Langenheder *et al.* 2005). Sommaruga and Casamayor (2009) examined bacterial communities in high altitude lakes, to determine the influence of environmental factors on microbial community assemblages. Their study found certain bacteria were generalists while other bacteria were specialized to specific areas. The specialized bacteria were constrained

geographically due to the local environmental factors that limited wide spread distribution.

Hughes Martiny *et al*. (2006) reviewed the literature on microbial biogeography, and determined that bacteria follow both taxon-area relationships and distance-decay relationships, both indicative of a biogeographical pattern. They also noted that historical environmental conditions could influence the bacterial communities. Under the taxon-area relationship, the number of species found in an area is related to the size of the area in which they live, whereas, the distance-decay relationship hypothesizes that as distance between organisms increases, the similarity between two organisms will decrease (Nekola and White 1999, Horner-Devine *et al.* 2004).

There are many ways to determine the bacterial composition in an environment. Cultivation-dependent studies were the most common studies performed to determine community composition, but these techniques had some limitations. Fægri *et al*. (1977), found that soil bacteria densities using fluorescent microscopy were increased 100-1000 fold as compared to agar plate counts. This led researchers to conclude that only a fraction, ~1% of bacteria can be isolated and cultured within the laboratory (McArthur 2006). This problem is due to the community relationships between bacteria within an environment and the lack of understanding of the nutrients that are needed for bacteria to thrive in laboratory conditions. Torsvik *et al*. (1990), cultured bacteria from soil isolating 200 distinct colonies, and isolated total DNA from the sample, in order to determine if the cultured bacteria were a good representation of all the bacteria living in the soil sample. The authors found that the amount of DNA extracted from the sample was a 170

times greater than could be produced through cultivation-based studies, suggesting that culturable bacteria are not a good representation of the community as a whole. Collectively these findings have led to the development of new methods, which have utilized DNA characterization of the communities and other physiological tests that do not require culturing (i.e. culture-independent techniques).

One culture-independent technique is based on differences in DNA sequence of the 16S ribosomal RNA (rRNA) gene. The 16S rRNA codes for the small subunit of the ribosome in bacteria and is generally used for bacterial classification. The 16s rRNA acts as an evolutionary clock, in that changes in the sequence can be cataloged to create lineages. The 16s rRNA gene is usually used due to its function and secondary structure providing functional constraints, which makes aligning and examining sequences of distinct organisms easier because the secondary structure can not change. This functional constraint leads to regions of DNA that are highly conserved (i.e. those involved with the function of the RNA) and some that are highly variable (Woese and Fox 1977, Woese 1987, Weisburg *et al.* 1991, Case *et al.* 2007). In order to ascertain the identity of bacteria from a complex sample using 16s rRNA sequences, one must extract the total DNA from the sample, or metagenomic DNA, followed by amplification of the 16s rRNA gene using traditional polymerase chain reaction (PCR). Traditional Sanger sequencing requires resultant 16s rRNA PCR products to be cloned into a suitable *E.coli* vector, therefore creating a library of 16s rRNA genes from various bacteria cloned into individual plasmids. These plasmids must then be re-isolated before being individually sequenced (Sanger and Coulson 1975). Next generation sequencing technologies, such as

the Illumina® MiSeq® system, allow for analysis of complex samples with out the need to create 16s rRNA gene plasmid libraries. The Illumina® MiSeq® system amplifies short regions of the 16s rRNA gene, usually up to 450 base pairs. The regions chosen for amplification of the 16s rRNA gene are hyper-variable regions in the DNA, which allow for identification of bacteria within the sample down to species level. Although only a small section of DNA is amplified, this method has been shown to be accurate in characterizing the communities in complex environmental samples (Liu *et al.* 2007, Caporaso *et al.* 2011, Caporaso *et al.* 2012a).

Another culture independent technique molecular fingerprinting analysis, such as terminal restriction fragment length polymorphism (t-RFLP), has allowed microbiologists to examine microbial communities and eliminate some of the biases of culture-based studies. Again, this technique requires amplification of 16s rRNA genes from metagenomic DNA samples. Once amplified, the resultant PCR product is digested with a restriction enzyme, such as *HhaI* to generate a restriction pattern. The restriction pattern generated and the intensity of the fragments give rise to the number of phylotypes and their relative abundance present in a sample, respectively. This method is usually used to study complex microbial communities and monitor populations within the communities (McArthur 2006).

Although next generation sequencing and t-RFLP, address the question of abundance and diversity of bacteria within a sample, the question of the function of these bacteria is still unknown. Physiological testing on the community is required to understand the function. Metabolic testing, such as BIOLOG™ plates, utilizes dyes (e.g.,

tetrazolium chloride), which are linked with varying nutrient sources (e.g. sugars, amino acids, etc.). When bacteria in the community digest the substrate the dye changes color, the tetrazolium chloride is reduced changing the solution from clear to purple, allowing visualization and quantification of community metabolism, using a spectrophotometer (McArthur 2006). The combination of diversity analyses using culture-independent techniques such as next generation sequencing and t-RFLP analysis with physiological methods, such as the Biolog EcoPlates™ (i.e. microbial ecology analyses) allows researchers to address questions in sub-fields, such as microbial biogeography. Most of these studies and techniques have taken place and been used to study lake and stream environments but little research has applied these ideas to the bacterial communities found in vernal pools.

Vernal pools are temporary ponds that form over a layer of hardpan and clay soil that is depressed relative to the surrounding habitat, resulting in a perched water table (Holland and Jain 1981). The Mediterranean climate creates the seasonality of the pools; due to its long dry summers and wet winters; and it is the seasonality, which creates the distinct phases that restricts the types of organisms that can live in this habitat year around. Vernal pools are characterized by three phases: the aquatic phase, the flowering phase, and the dry phase. The aquatic phase occurs during late fall to early winter, when rainfall inundates the pools; this phase supports a complete aquatic ecosystem. The flowering phase occurs in spring as the pools begin to dry out. During this phase a terrestrial ecosystem emerges characterized by many endemic flowering plants. The third phase is the dry phase, in which most organisms enter a state of dormancy (Bliss and

Zedler 1998). The uniqueness of the environment supports organisms that are not found in other ecosystems, such as the Tiger salamander (*Ambystoma californiense*), profuseflower mesa mint (*Pogogyne floribunda)*, and vernal pool fairy shrimp (*Branchinecta lynchi*) (Keeler-Wolf *et al.* 1998).

Organisms, both eukaryotic and prokaryotic, that reside in these pools year-round need a dormancy strategy to survive through the desiccation period (Stevenson 1978, Schwartz and Jenkins 2000); however not all reside year-round, some species can disperse into and out of these ecosystems (Wiggins *et al.* 1980). Many species of plants and animals that inhabit the vernal pools are listed as endangered or threatened (Federal Register 2003). Protection of these endangered species is leading the interest in conservation of these pools, research into studying the formation and functioning of these pools has been allowed to monitor and determine the best conservation strategies for these pools. In total, this ecosystem offers unique understanding in ecosystem functioning but information has been lost due to destruction (Leidy and White 1996). Many vernal pools have been lost due to their conversion to agricultural and/or urban areas. Those that are left are under the increased risk of eutrophication (Sharpley *et al.* 2003).

Characterization of multicellular eukaryotic organisms is somewhat well established (Holland and Jain 1981, King *et al.* 1996, Bliss and Zedler 1998, Kneitel and Lessin 2010) but little information is known about the bacterial communities. Carrino-Kyker and Swanson (2008) examined the bacterial communities of vernal pools in Ohio, based on the temporal and spatial locations. They found evidence to support the hypothesis that most of the bacteria (*Alphaproteobacteria* and *Betaproteobacteria)* in

these communities function as decomposers in the food web. Understanding the bacterial community structure is imperative to the understanding of the relationships between the biota and the environment, using this information to assess the effect of environmental stresses such as human influences and for management (Torsvik *et al.* 1996). The first step in understanding the role that bacteria play in a community is to characterize the bacterial community within these pools. To identify community structure two fundamental questions must be answered: (1) which bacteria are in the community? and (2) what is the role they play in community structure (McArthur 2006)?

The aim of this study is (1) to examine the microbial ecology and microbial distribution among vernal pools by characterizing the bacterial community composition, and (2) evaluate the collective bacterial metabolic profiles in different California vernal pools. This study will elucidate if there is a significant difference in the bacterial composition among the different California vernal pools, followed by the determination if the community is influenced by abiotic factors, that can affect which microbes thrive, or if location influences community.

Hypotheses and Objectives

The two hypotheses for this study are: (1) there is a difference in the bacterial community compositions in different California vernal pools and (2) the difference will be the result of either location or abiotic factors.

To accomplish this water samples will be collected from six different vernal pools at three locations. Community level physiological profiles will be used to assess

metabolic differences using clustering analysis and principal component analysis. Metagenomic DNA will be extracted; community structure and composition will be assessed using terminal restriction fragment length polymorphism and next generation sequencing techniques. Analysis of the DNA based techniques (i.e. t-RFLP and next generation sequencing) using principal component analysis and Bray-Curtis index of similarity will determine any differences between the vernal pool communities.

METHODS

Water Collection

Six vernal pools were sampled: two from Travis Air Force Base, two from Mather Field Air Force Base and two from Beale Air Force Base. Two replicates of each pool were sampled for accuracy. Pools were named based on their location, Beale1and Beale2 located at Beale Air Force Base, Mather1 and Mather2 located at Mather Field Air Force Base and Travis1 and Travis2 located at Travis Air Force Base. Using a sterile glass vial, 500 mL of water was collected from the pools between the months of April 2012 and May 2012. Measurements of turbidity, conductivity, pH, phosphates and dissolved oxygen were taken for each pool to evaluate the abiotic factors that could influence the community. An Oakton pH/CON 300 meter was used to measure pH and conductivity, an Oakton pH/DO 300 meter to measure dissolved oxygen. A LaMotte 2020i turbidity meter was used to quantify turbidity after water was filtered through a 500μm mesh to remove coarse particulates. Phosphates were measured using the Hach DREL/2800 Complete Water Quality Lab (Hach, Loveland CO). Dissolved oxygen was not obtained for the following pools: Beale1 and Beale2 and was subsequently not used for the rest of the analysis. Data for pH for Travis1 and Travis2 was not obtained at the time of sampling and previously recorded pH were used for analysis.

Enumeration of Bacterial Populations from Vernal Pools

Bacteria samples were enumerated using the Bacterial Counting Kit for Flow Cytometry (Invitrogen, Grand Island NY) and the Guava easycyte 6HT flow cytometer (Millipore, Billerica, MA). Each sample was run twice and each run consisted of three enumerations. Bacterial population counts were used to determine appropriate dilutions needed to conduct community-level physiological profiling.

Community Level Physiological Profiles

Water collected from each of the six vernal pools was used to conduct community-level physiological profiles. Individual wells of 96-well Biolog EcoPlates™ (Biolog, Hayward CA) were inoculated with 125 μl of vernal pool water (Garland 1996, Mackenzie *et al.* 2011). Plates were incubated at 30°C in the dark in cardboard boxes with water soaked cloth to prevent evaporation. The absorbance of each well was read at a wavelength of 590nm using a Promega GloMax-Multi plus (i.e. microplate reader) (Madison, WI) (Islam *et al.* 2011, Zhang *et al.* 2011). Absorbance was recorded at 20, 24, 30, 36, and 48h post-inoculation to account for the bacterial lag phase, and then data was recorded every 24 hours until 168 hours (Zhang *et al.* 2011).

Statistical Analysis of Community Level Physiological Profiles

A one-way ANOVA was run on the bacterial counts to assess statistical differences in the numbers of bacteria between the pools. The data obtained from the Biolog EcoPlate™ experiments were processed by subtracting the absorbance value

obtained for the blank well from absorbance values obtained for each well containing substrate. Time point for the analysis was chosen based on Stefanowicz (2006), which determined that the greatest amount of variation was shown when the average well color was 75% of the highest average well color. A graph of substrate utilization by pool was used to visually compare differences in community metabolic profiles. A graph of categorized carbon sources was also used to determine differences between pools (Islam *et al.* 2011). Both graphs were created using GraphPad Prism version 6.0b for MacOSX (GraphPad Software, La Jolla California USA, www.graphpad.com). A dendrogram containing both metabolic data and abiotic data was created using nearest Neighbor-Joining method (Cover and Hart 1967). Principal component analysis (PCA) using the categorized carbon source data was used to compare the relative similarity of the profiles and to determine which variable was causing the most variation within the samples (Garland 1997, San Miguel *et al.* 2007, Albrecht *et al.* 2010). PCA was calculated using PC-ORD v. 6 for Windows 7 (MJM software design, Gleneden Oregon USA, http://home.centurytel.net/~mjm/index.htm).

Bacterial DNA Extractions from Water

Total community DNA (i.e metagenomic DNA) was extracted following the protocol for the Metagenomic DNA Extraction Kit for Water (Epicentre Biotechnologies, Madison WI). Two DNA extractions were performed per vernal pool water sample collected (4 total per pool). DNA extractions were confirmed using gel electrophoresis on

a 1% (w/v) agarose gel + 1X TBE stained with GelRed™ (Phenix Research Products, Candler, NC). DNA was stored at -20°C until needed.

Terminal Restriction Fragment Length Polymorphism

Six metagenomic DNA samples, one for each pool, were shipped on ice overnight to the Microbiome Core Facility at the University of North Carolina School of Medicine (Chapel Hill, NC) and their t-RFLP methods were as follows. Metagenomic DNA was amplified using 16s rRNA universal primers. The E8F forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') was fluorescently labeled with 6-Carboxyfluorescein (6-FAM) (Life technologies™, Grand Island, NY) and was paired with the 1492R reverse primer (5'-CGGTTACCTTGTTACGACTT-3') for amplification via polymerase chain reaction (PCR) (Liu *et al.* 1997, Schüette *et al.* 2008). Four independent PCRs were performed for each sample. Amplification was performed using the HotStar HiFidelity DNA Polymerase Kit (Qiagen, Hilden, Germany). The final PCR volume was 50 μl and was composed of the following components: 500 nM each primer E8F and 1492R, 100 ng of metagenomic DNA, and buffer and enzyme per the manufacturer's instructions. PCR was conducted on the Veriti thermal cycler (Life Technologies, Grand Island, NY) using the following cycling parameters: an initial denaturation at 95°C for 5 minutes, followed by 25 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes with a final extension at 72°C for 10 minutes. A 1% (w/v) agarose gel was run with 5 μl of the four-pooled PCR reactions to verify amplification. PCR products were cleaned up using the QIAquick PCR purification kit (Qiagen, Hilden,

Germany). DNA concentration was measured by absorbance at 260nm, 280nm and 230nm to analyze PCR quality and for standardizing concentrations.

Three restriction enzymes were used to digest the PCR samples according to the respective manufacturer's instructions. The enzymes used were *HhaI*, *RsaI* (Fermentas) and *Hpy188III* (New England Biolabs, Ipswich, MA). In each digest 400ng of the PCR product was used. Digests were cleaned up using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sample fragment analysis was performed on a 3130XL Genetic Analyzer (Life Technologies, Grand Island, NY) using the POP-7 polymer. Sample and molecular size standards (GeneScan ROX 1000, Life Technologies, Grand Island, NY) were used according to the manufacturer's instructions. Tables and text files of peaks were created using the GeneMapper software (Life technologies, Grand Island, NY) using the chromatogram (fsa) files from the run on the 3130XL Genetic Analyzer.

Statistical Analysis of Terminal Restriction Fragment Length Polymorphism

Peaks < 30bp and >950bp were discarded to remove chance of measuring peaks from primers and non-digested DNA, respectively. Shannon's diversity index and Shannon's evenness was calculated for each profile to assess species richness and evenness using peak area as an assessment of abundance (Blackwood *et al.* 2007). Shannon's diversity index is a calculation that takes into account the amount of diversity as well as the abundance of each individual taxonomic classification. Evenness values range from 0 to 1, with 1 representing a community where each taxonomic classification has an even number of individuals, i.e no species is dominating the sample. Differences

between treatments were assessed using the Bray-Curtis Index of Similarity using presence/absence data for each site (Bray and Curtis 1957, Carson *et al.* 2010). The Bray-Curtis Index of Similarity was calculated for each enzyme. This calculation is a pairwise comparison based on the number of taxonomic significant units that are found in both locations divided by the total number of taxonomic units in each location; this gives an index of numbers ranging from 0 to 1, with 1 being the communities share all the same species and 0 being they have no species in common.

Next Generation Sequencing

Twelve metagenomic DNA samples, two for each pool, were shipped overnight on ice to uBiome (San Francisco, CA) their sequencing protocol is as follows. Amplicon sequencing libraries were prepared by PCR. Primers to amplify the 16s rRNA gene variable region 4 (V4) were designed by Caporaso et al. (2012b) (5'-TAGTGCCAGCMGCCGCGGTAA-3’ forward and 5'-ACGGACTACVSGGGTATCTAAT-3’ reverse). The primers contained a proprietary sequence on their 5’ end that allows dual-index barcoding and sequencing on the Illumina® platform; this assigns each read to a specific DNA sample. Eight PCR cycles were used with these primers, which reduce primer bias in the samples. The amplified band at ~450bp was gel extracted for each sample and the amplicons were then pooled. The pooled samples were further amplified using the Solexa primers (5'-AATGATACGGCGACCACCGA-3’ and 5'-CAAGCAGAAGACGGCATACGA-3’). The pooled library was then sequenced on the Illumina® MiSeq® machine using the

paired end 150bp cycling protocol following all manufacturer instructions. Sequences were debarcoded on the MiSeq machine. FASTA files were renamed and combined into one file containing all sequences for all pools (custom perl script see A1).

Statistical Analysis of Next Generation Sequencing

Raw sequencing reads were processed through the Quantitative Insights into Microbial Ecology program (QIIME) (Caporaso *et al.* 2012a). Raw sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/) (For accession numbers see C1). Reads were assigned to taxonomic status and the counts of sequences per taxonomic unit were determined. Sequences determined to be unclassified, that could only be identified to the kingdom *Bacteria* and sequences belonging to other domains were removed from analysis (Custom perl script see B1). Shannon's diversity index and Shannon's evenness were calculated for each sample. Shannon's diversity index was also calculated through QIIME using an alpha rarefaction of 500 sequences per sample. Bray-Curtis index of similarity was calculated for each sample using class as the classification level and discarding any sequences that could only be classified to kingdom or phylum. Using QIIME, UniFrac Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to create a tree based on evolutionary relatedness of the samples (Lozupone and Knight 2005). PCA using only the major class data was conducted, allowing for observance of the effects the pools conditions have on the variation in the communities (Zhang *et al.* 2008).

RESULTS

Abiotic Data Analysis

A clustering analysis using Bray-Curtis and nearest neighbor methods was created using the abiotic data for each pool (Table 1) (Figure 1). Beale1 and Mather1 were most similar at an abiotic level, followed by Beale2 and Mather 2, respectively. Travis2 was the most distantly related indicating that the abiotic factors for this pool was the most distinct of all six pools.

Community Level Physiological Profiles Analysis

Flow cytometry indicated that each pool differed significantly (ANOVA: $F_{(5,30)}=15.54$, $p<0.0001$), in the number of bacterial cells present but all have on the same order of magnitude, 10^7 bacterial cells/mL of water (Figure 2). The water samples were within suggested concentration for the inoculation of the Biolog EcoPlates™ so although those values differed no alterations of water samples was used. All of these counts were potentially overestimations since the stain did not distinguish between living and dead cells.

Biolog EcoPlates™ replicate values were combined and then each substrate was separated into categorized carbon sources. Six distinct types of carbon sources were used; polymers, carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds. Mather1, Mather2 and Travis1, displayed similar metabolic patterns to each other, while

Table 1. Abiotic measurements from each of the vernal pools sampled

Vernal pool	Dissolved oxygen (mg/L)	pH	Conductivity (μS)	Turbidity (NTU)	Phosphates (mg/L)
Mather 1	4.80	6.19	284.0	5.67	0.12
Mather 2	6.90	6.23	427.0	7.03	0.36
Travis 1	11.95	7.35*	71.5	10.03	0.66
Travis 2	11.37	7.45*	1676.0	0.68	0.13
Beale 1	---	6.09	290.0	0.74	0.25
Beale 2	---	6.46	276.0	13.70	0.06

* Indicates pH obtained from earlier samplings

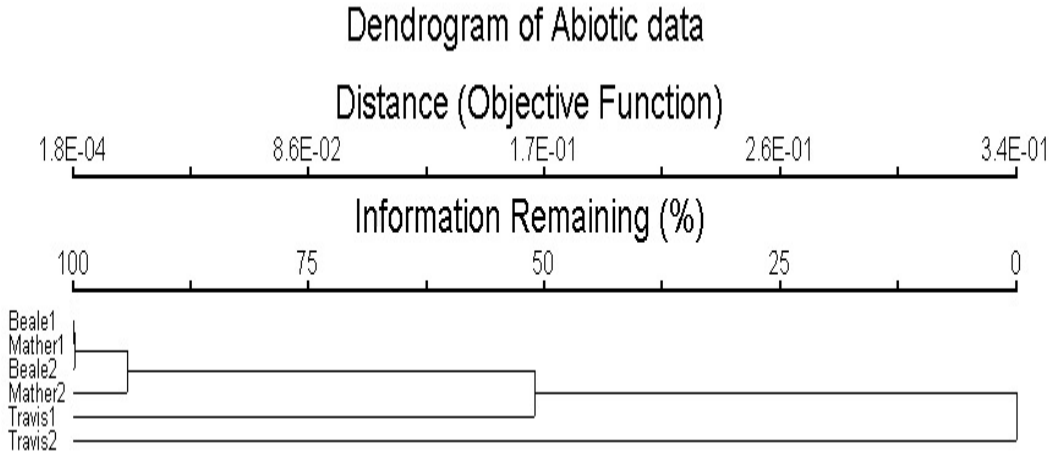

Figure 1. Dendrogram of abiotic data created using Bray-Curtis and nearest neighbor methods. The pools clustered closest share the most similar abiotic characters.

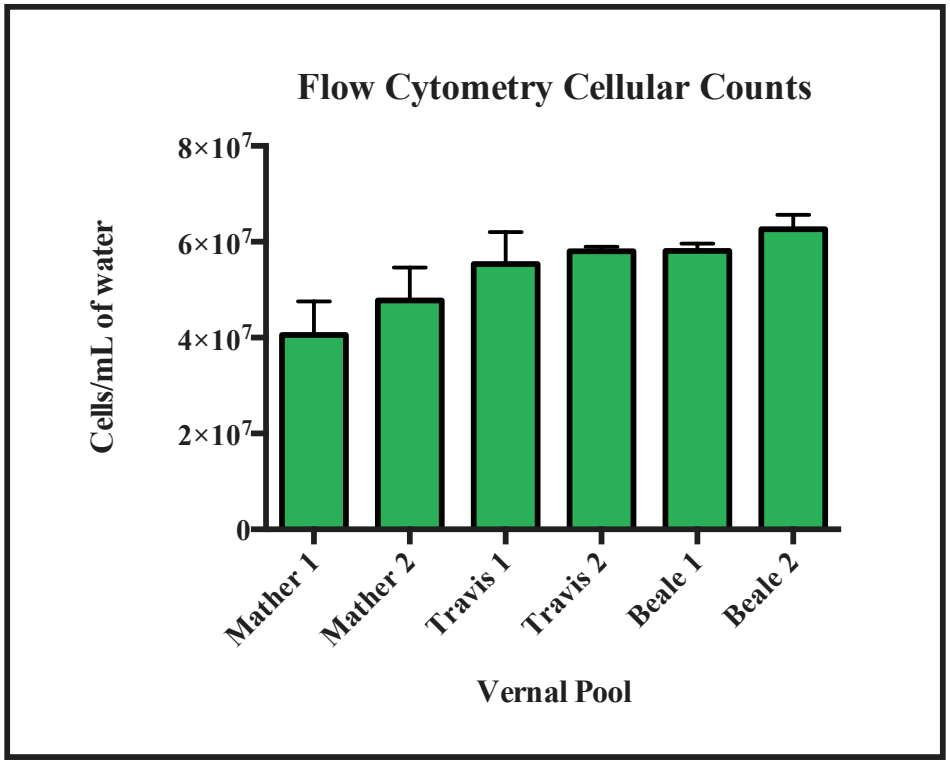

Figure 2. Mean bacterial densities (± standard deviation) found in each of the vernal pools. Bacterial populations were determined via flow cytometry.

Travis2, Beale1 and Beale2 indicated similar patterns (Figure 3). Travis2, Beale1, and Beale2 metabolized most categories at the same rate; phenolic compounds were used the least by Mather1, Mather2 and Travis1. These groupings suggests that similar metabolic substrates are available within those sites, and select for similar bacteria or bacteria with similar metabolic capabilities. Principal component analysis (Figure 4), Mather1, Mather2, and Travis1, all form a group towards axis 2, while Beale1, Beale2 and Travis2 form a tight grouping away from axis 2. Axis 1 and axis 2 accounted for 97.98% of all the variation seen. The randomization test revealed that axis 1 was significant ($p<0.001$), with the real eigenvalue being greater then the predicted value. Axis 1 was negatively correlated with phosphates ($r = -0.473$, $r^2 = 0.223$, tau = -0.200). Axis 2 was also negatively correlated with phosphates ($r= -0.760$, $r^2 = 0.577$, tau= -0.333) and pH (r = -0795, $r^2= 0.632$, tau= -0.733).

Terminal Restriction Fragment Length Polymorphism Analysis

Each enzyme was treated individually and peaks could not be compared across enzymes because the same peak created by two different enzymes would represent two different taxonomic groups. Each enzyme produced different number of peaks. *HPY188III* produced the lowest amount of terminal restriction fragments (TRF's) (3 for Mather2 pool) (Table 2). *RsaI* produced the greatest number of TRF's (46 for Travis1). This variation in the number of TRF's is a result of the fidelity of the enzyme on those samples and not a reflection of the bacterial numbers within each sample (Engebretson and Moyer 2003). Shannon's diversity index was greatest with the enzyme that produced

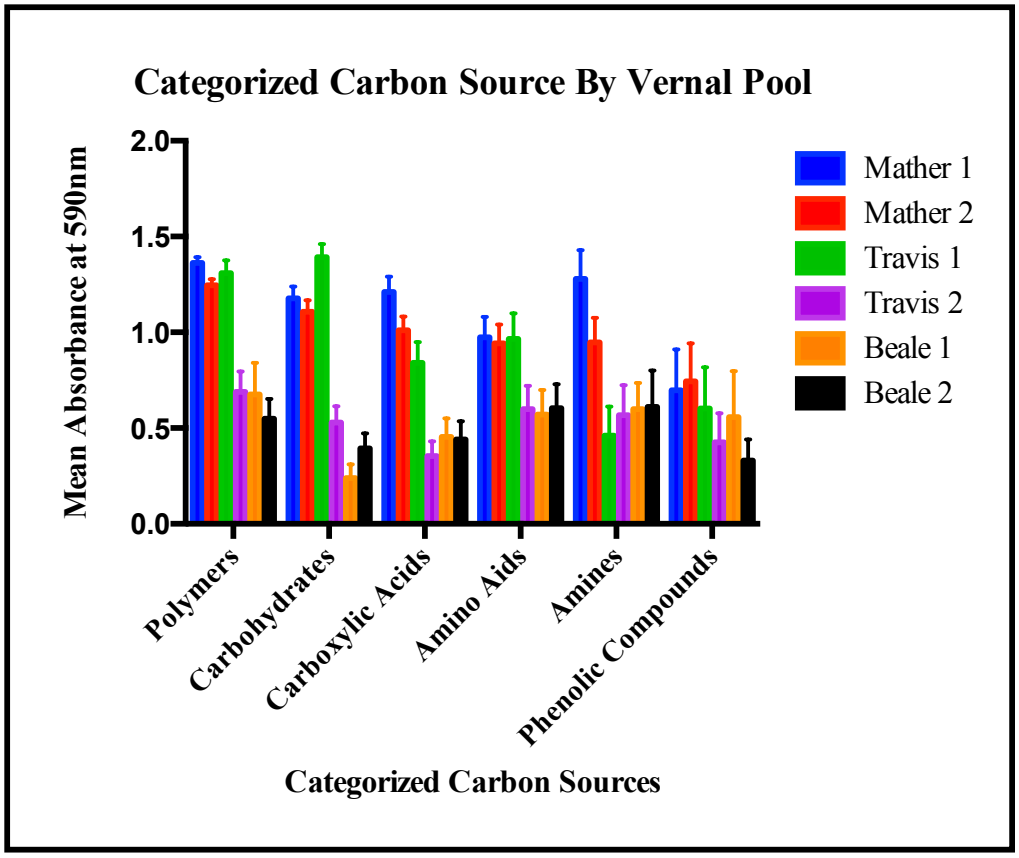

Figure 3. Metabolic profiles of bacteria found in vernal pools. BIOLOG Ecoplate™ results, substrates were divided into six types of substrates, the bars represent the combined average (± standard error) for all substrates within a category.

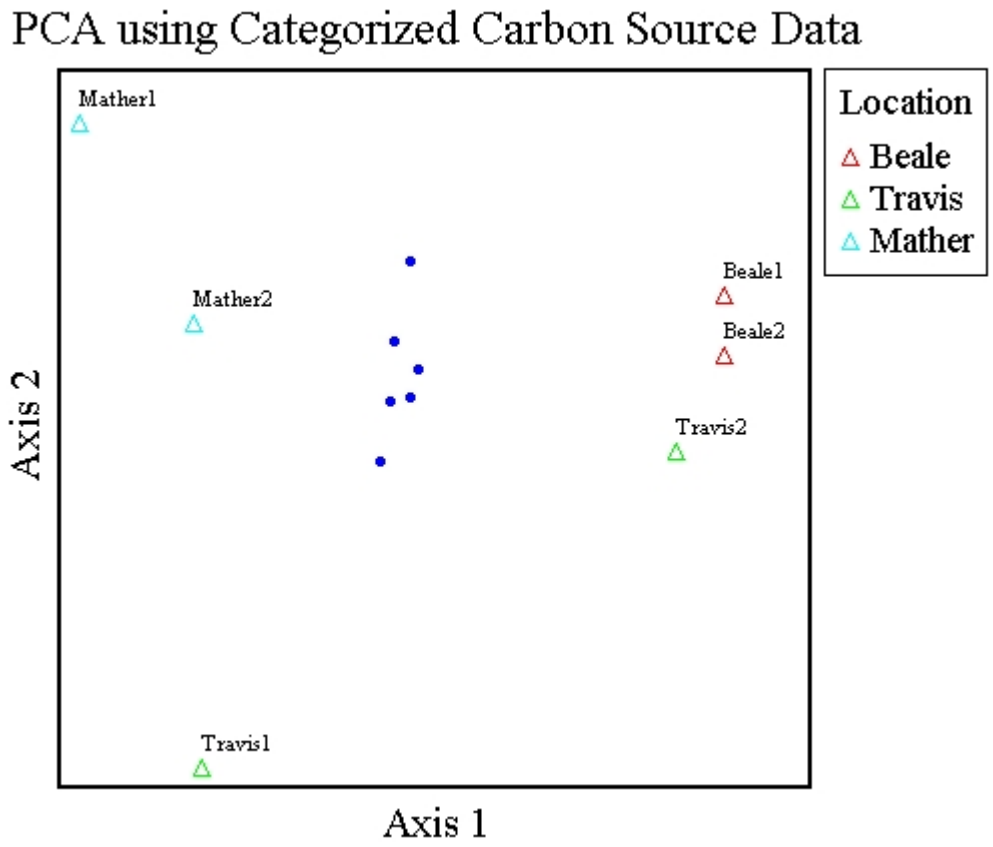

Figure 4. Principal component analysis using categorized carbon sources based on BIOLOG® data. Axis 1 and Axis 2 accounted for 97.975% of the variation (84.759% and 13.216% respectively). Based on the randomization test (# of seed =100, # of runs=9999), Axis 1 was the only significant ($p<0.001$) axis with the real eigenvalue being greater then the randomized eigenvalue. The blue dots represent each categorized carbon source across all pools.

Table 2. Shannon's diversity and evenness indices for each pool for each restriction enzyme

Vernal pool	Restriction Enzyme	# of TRF's	*H'*	*J'*
Mather 1	*HhaI*	8	1.88	0.97
Mather 2	*HhaI*	8	2.03	0.90
Travis 1	*HhaI*	15	2.60	0.96
Travis 2	*HhaI*	20	2.84	0.95
Beale 1	*HhaI*	7	1.79	0.92
Beale 2	*HhaI*	21	2.88	0.94
Mather 1	*HPY188III*	4	1.33	0.96
Mather 2	*HPY188III*	3	0.91	0.83
Travis 1	*HPY188III*	19	2.81	0.95
Travis 2	*HPY188III*	6	1.72	0.96
Beale 1	*HPY188III*	5	1.46	0.91
Beale 2	*HPY188III*	5	1.65	0.92
Mather 1	*RsaI*	13	2.45	0.95
Mather 2	*RsaI*	37	3.49	0.97
Travis 1	*RsaI*	46	3.70	0.97
Travis 2	*RsaI*	35	3.44	0.97
Beale 1	*RsaI*	10	2.08	0.90
Beale 2	*RsaI*	36	3.44	0.96

the greatest number of TRF's per pool, *H'*=3.70 for Travis1 with the *RsaI* enzyme (Table 2). The lowest diversity value was from Mather2, *H'*=0.91 for the *HPY188III* enzyme. Shannon's evenness did not fluctuate as diversity did with the changing number of TRF's. Evenness ranged from *J'*=0.83-0.97, across all pools and enzymes (Table 2).

The Bray-Curtis measurements ranged from 0 to 0.37 across the enzymes and the pools (Table 3). The *RsaI* enzyme between Travis1 and Travis2 pools produced the highest similarity of 0.37. *HPY188III* produced the lowest similarity values for the majority of the pools; this could be an artifact of the small number of TRF's and is not reflective of the community.

Next Generation Sequencing Analysis

The forward sequence run on the Illumina® MiSeq® of the v4 region of the 16s rRNA gene was analyzed. After processing the samples and removing unidentified sequences and those with low quality reads the following number of sequences were analyzed from each pool; Beale1=2669, Beale2=975, Travis1=2545, Travis2=1084, Mather1=729, and Mather2=2457. Taxonomic classification led to the identifications of major classes within each sample. The major classes of bacteria found were Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Sphingobacteria, Verrucomicrobiae and Spartobacteria. These classes were considered dominant with 2% or more in relative abundance in an individual sample. Relative abundance of each bacterial class differed per pool (Figure 5). The major classes that differed between the samples were Betaproteobacteria that made up between 17.61% and 57.80% of the

Table 3. Bray-Curtis Index of Similarity using TRF's from each restriction enzyme

	Mather 1	Mather 2	Travis 1	Travis 2	Beale1
Mather 1					
Mather 2	0.12				
Travis 1	0.10	0.24			
Travis 2	0.13	0.28	0.37		
Beale 1	0.00	0.04	0.14	0.00	
Beale 2	0.08	0.22	0.29	0.28	0.17
Mather 1					
Mather 2	0.25				
Travis 1	0.09	0.26			
Travis 2	0.07	0.07	0.11		
Beale 1	0.00	0.00	0.00	0.07	
Beale 2	0.07	0.07	0.11	0.15	0.00
Mather 1					
Mather 2	0.00				
Travis 1	0.17	0.00			
Travis 2	0.00	0.00	0.24		
Beale 1	0.00	0.00	0.17	0.00	
Beale 2	0.20	0.00	0.16	0.00	0.00

Color of the square indicates fragments used from specific enzyme; white=*RsaI*, yellow=*HhaI*, grey=*HPY188III*

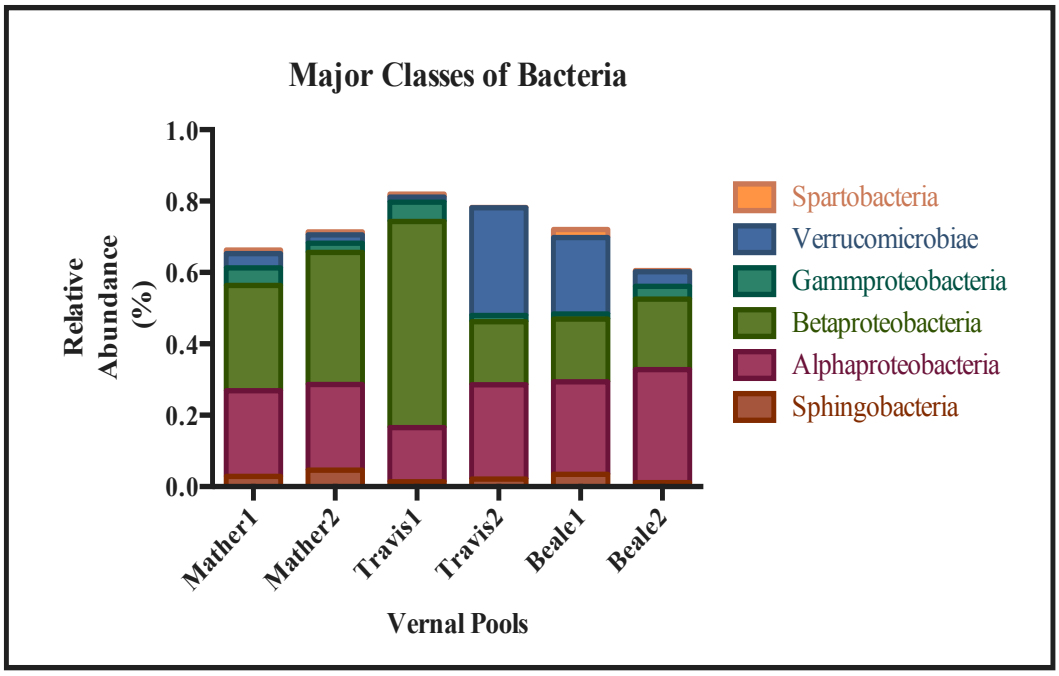

Figure 5. Graph of relative abundance of major classes of bacteria per vernal pool sample. Taxonomic assignment was carried out using QIIME using the Ribosomal Database project with a confidence of 60%. The remaining values out of 100% were comprised of bacterial classes representing less then 2% and a large amount that could only be classified to phylum. Betaproteobacteria and Verrucomicrobiae differ in abundance the most between vernal pool samples.

samples, the most being isolated from Travis1 and the least being from Beale1, and Verrucomicrobia*e* that made up between 1.45% and 30.17% of the samples, the most being isolated from Travis2 and the least being from Travis1.

Within the class of Alphaproteobacteria the main groups were from three distinct orders Rhizobiales, Rhodobacterales and Rickettsiales. Only within the order Rhodbacterales could a genus be identified, that genus being *Rhodobacter*. Gammaproteobacteria was dominated by the genus *Pseudomonas.* The class Sphingobacteria contained only members of the order Sphingobacteriales with the most abundant genus being *Emticicia*. The class Spartobacteria, contains only one order Chthoniobacterales and one family Chthoniobacteraceae. One of the classes that contributed to the most variation in the samples was the Betaproteobacteria, which contained members of the family Comamonadaceace, the dominant genera, were *Rhodoferax, Janthinobacterium,* and *Polynucleobacter*. The other major class that contributed to the most variation within the samples was the Verrucomicrobiae, which contained members of the genus *Prosthecobacter*.

Using class-level identifications Shannon's diversity and evenness indices and, Shannon's index were calculated using a rarefaction of 500 sequences per sample through QIIME. The highest diversity calculated was Travis1 (H'=1.17) (Table 4). Evenness was extremely low across the classes, which indicates that certain classes are dominating within the pools. The diversity calculations were significantly higher when using the rarefaction through QIIME because of the use of a lower taxonomic level than class in the calculation. The greatest amount of diversity was seen in Beale2 (H'=8.40), Travis1

Table 4. Shannon's diversity and evenness indices for sequencing data

Vernal Pools	*H'* *	*H'*	*J'*
Mather 1	7.97	0.47	0.15
Mather 2	7.54	0.59	0.17
Travis 1	5.28	1.17	0.40
Travis 2	6.86	0.57	0.20
Beale 1	7.19	0.47	0.14
Beale 2	8.40	0.43	0.15

* Indicates values based on average from 10 rarefaction samplings of 500 sequences from each pool

that showed the highest diversity at the class level, showed the lowest diversity at a lower taxonomic level (*H'*=5.28). This could be the result of the pool having the most classes but few orders, families, and genera within those orders. The Bray-Curtis index of similarity showed a high degree of similarity for all of the pools (Table 5). All of the values were greater than 0.50, indicating that all of the pools shared over half of the classes the same. The highest similarity was between Travis1 and Beale 2 (0.89). The lowest value was between Mather2 and Travis1 with a value of 0.59, which still showed that they had similar classes within the pools.

A principal component analysis was conducted using data from the major classes of bacteria. Axis1 and axis2 accounted for 98.85% of the variation in the samples (84.42% and 17.43% respectively) (Figure 6). Based on the randomization test, no axis was significant. The Pearson and Kendall correlations for the first two axes resulted in a strong positive correlation between axis 1 and phosphates (Table 6). Axis 2 was correlated with all of the variables measured, but the strongest included a negative correlation with pH and a positive correlation with turbidity.

A final analysis was carried out using UniFrac with Unweighted Pair Group Method with Arithmetic Mean. This analysis clusters groups together that are evolutionarily similar; support values at the nodes indicate the level of support for those clusters. The tree shows that Mather1 and Mather2 clustered together and Travis1 and Travis2 clustered together, while Beale1 was more closely related to Mather1 and Mather2. Beale2 was sister to the Mather and Beale1 group (Figure 7). The node values for the Mather and Beale group were lower than 80, which meant there was not strong

Table 5. Bray-Curtis Index of Similarity calculated based on sequencing data

	Mather 1	Mather 2	Travis 1	Travis 2	Beale1
Mather 1					
Mather 2	0.77				
Travis1	0.71	0.59			
Travis2	0.68	0.60	0.74		
Beale1	0.82	0.83	0.76	0.73	
Beale2	0.71	0.63	0.89	0.74	0.76

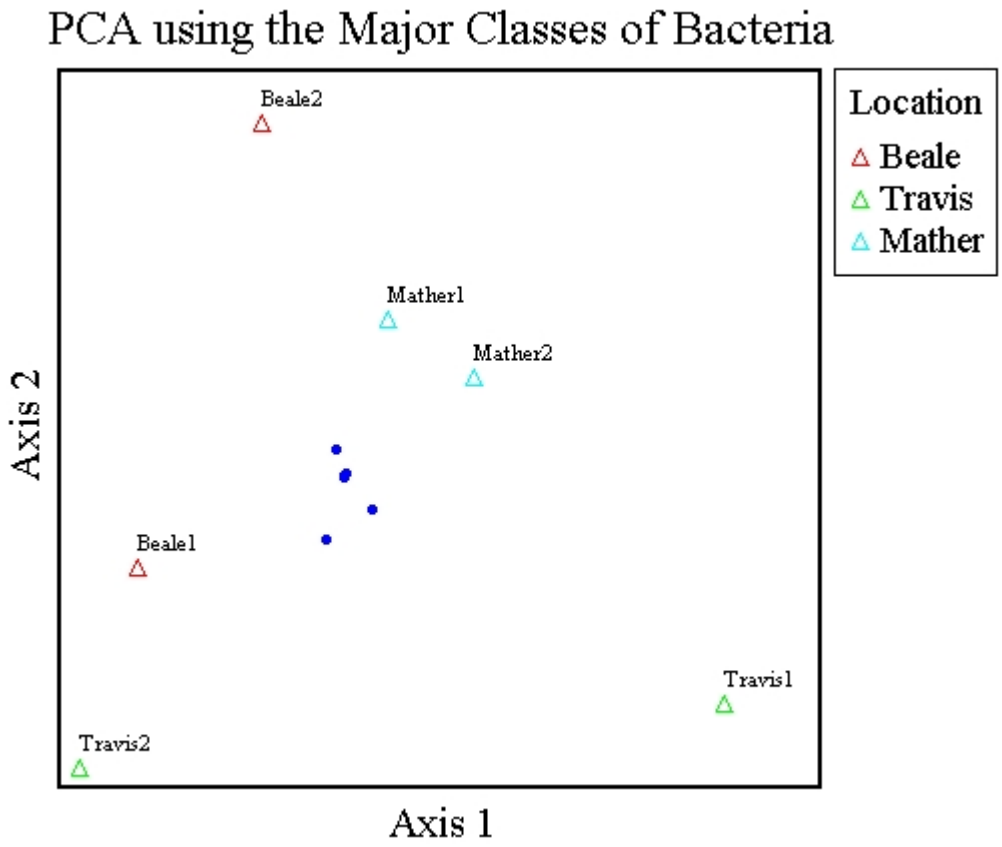

Figure 6. Principal component analysis using the major classes of bacteria from the sequencing data. Axis1 and Axis2 accounted for 98.845% of the variation within the data (81.417% and 17.428% respectively). Based on randomization no axis was significant, the lowest p-value (0.0955) was for Axis1.

Table 6. Pearson and Kendall correlations for PCA of major classes of bacteria

Axis	1			2		
	r	r-sq	tau	r	r-sq	tau
Conductivity	-0.620	0.384	-0.467	-0.480	0.231	-0.333
Phosphates	0.803	0.645	0.467	-0.492	0.242	-0.467
Turbidity	0.574	0.330	0.600	0.613	0.376	0.467
pH	0.153	0.023	0.067	-0.693	0.481	-0.333

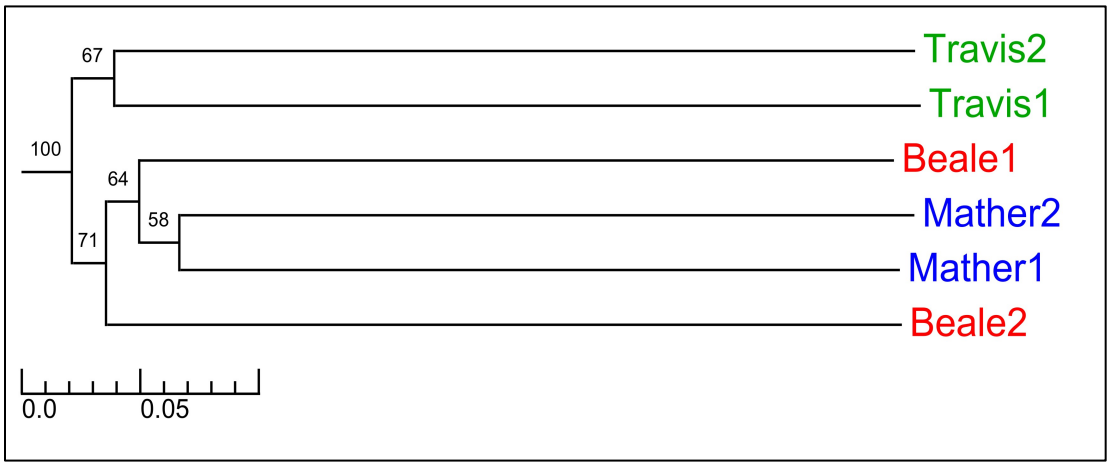

Figure 7. UniFrac UPGMA clustering of phylogenetic data. Clusters areas with similar evolutionary diversity, node values indicate support for the evolutionary separation between the areas. Low support indicates that bacteria within those pools have not acquired adaptions for that specific environment.

support for those clusters. Low values at the nodes, supports the idea that bacteria living within the pools in the cluster (Beale and Mather pools), have not adapted so that they can only reside within one pool, such as the Mather1 pool. There was strong support for the divergence between the Mather and Beale pools and the two Travis pools (node =100). This would mean that the bacteria within the Travis pools have adapted to live within the pools on Travis Air Force Base.

DISCUSSION

The main objectives of any microbial ecology study are to determine what microbes are present and their function are these organisms performing in the environment. To this end, a metagenomic and metabolic study was conducted to determine the identity and function of the bacterial community found in the California vernal pool ecosystem (CVPE). A metabolic study was performed on water samples collected from six different CVPE from three locations (two pools were sampled from each location): Beale Air Force Base, Mather Air Force Base and Travis Air Force Base. The metabolism profiles produced two groups; one containing both Beale pools and one Travis pool (Travis2), while the other contained both Mather pools and the other Travis pool (Travis1). This suggests that bacterial metabolism can be explained by biogeography (location). However, since the Travis pools have substantially different metabolic patterns it appears that some other factor may be influencing the metabolic profiles.

Both Beale pools and Travis2, had a bacterial composition with the inability to use the nutrient D-Xylose, whereas the other pools had high utilization; studies have pointed to the decrease in use of this substrate as an indication of nutrient poor or cold fresh water communities (Boteva *et al.* 2009, Mackenzie *et al.* 2011). Bacterial communities associated with organic aggregates (i.e. detritus floating in water) demonstrated different metabolic patterns as compared to water-associated (i.e. free living) communities (Simon *et al.* 2002). Water-associated communities have extremely high utilization of certain metabolic substrates, while aggregate associated communities

have overall lower utilization of specific substrates but a wider range of utilized substrates. The CVPE followed closely the water-associated community except for the Beale and Travis2 grouping, in which the metabolism of four substrates (α-Cyclodextrin, Tween 40, D-Cellobiose and D-Galacturonic acid) was more similar to aggregate communities (Lyons and Dobs 2012). With the previous similarity to nutrient-poor communities the Beale and Travis2 pools may have bacteria that are aggregate associated due to the limited amount of nutrients available. The principal component analysis (PCA) results indicated phosphates and pH were driving the metabolic diversity in the vernal pools.

Both the terminal restriction fragment length polymorphism (t-RFLP) and the next generation sequencing techniques (NGS) found different patterns of bacterial diversity. T-RFLP produced much lower values than the NGS data in both Bray-Curtis index of Similarity and the Shannon's diversity indices, this is more than likely an artifact of the method's sensitivity and not a reflection of the community. The restriction enzymes used in t-RFLP, cut at specific restriction sites, groups that are evolutionarily similar could have similar locations of those restriction sites. It is impossible to resolve the multiple taxonomic groups per fragment, resulting in a lower diversity. The evenness was much higher in the t-RFLP data then in the NGS data; this could be the result of the taxonomic level used (Class level for the next generation sequencing data) to calculate the evenness. However, this is not unexpected as evenness is traditionally low in aquatic communities when compared to soil microbial communities (Zwart *et al.* 2002).

The major classes identified were the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Sphingobacteria, Spartobacteria and Verrucomicrobiae. All of these classes were found in all of the pools; relative abundance differed between the pools. All of these classes are commonly found in freshwater studies (Zwart *et al.* 2002, Tamaki *et al.* 2005, Van der Gucht *et al.* 2005, Zul *et al.* 2007); the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria were also found in significant densities in constructed Ohio vernal pools (Carrino-Kyker and Swanson 2008). These bacteria are considered generalists due to their multiple metabolic capabilities along with many respiratory capabilities; they are common in most water and soil communities and function as organic material decomposers and rely on their metabolic plasticity to survive on changing resources (Carrino-Kyker and Swanson 2008, Carrino-Kyker *et al.* 2013). The class Alphaproteobacteria contains most of the phototrophic genera, bacteria that are able to photosynthesize to synthesize their own nutrients (Bergey's manual of systematic bacteriology 2005b, Hahn *et al.* 2011). The Gammproteobacteria have members that exhibit a wide range of aerobicity and temperature adaptations, the bacteria in the vernal pools were mainly identified as belonging to the genus *Pseudomonas* (Bergey's manual of systematic bacteriology 2005a, Williams *et al.* 2010). *Pseudomonas* species are found in both soil and aquatic habitats and can exist as both free-living and pathogenic organisms (Bashan 1986, Cho and Tiedje 2000, Hirano and Upper 2000, Bisson *et al.* 2007).

Another common class of bacteria found within the vernal pools was the Sphingobacteria, which was mainly comprised of the genus *Emticicia.* Sphingobacteria

are recently discovered aquatic bacteria, which contribute to nutrient cycling. They also function in the degradation of complex biopolymers including chitin, which is the main component of fungi cell walls, arthropod exoskeletons, plant cellulose, an important structural component in plant cell walls (Bergey's manual of systematic bacteriology 2010). Spartobacteria was another class that was found within the California vernal pools, another recently identified bacterial class. A recent study indicated that these bacteria have a close relationship with filamentous algae and have a heterotrophic metabolism able to breakdown many substrates including cellulose and chitin (Herlemann *et al.* 2013).

The main two classes of bacteria that differed among the vernal pools were the Verrucomicrobiae and the Betaproteobacteria. The Verrucomicrobiae is a group of bacteria that has only been recently identified and very little is known about their function within ecosystems. This group of bacteria is found in freshwater habitats, but is most abundant within soils, especially in habitat rich in phosphorus (Hedlund *et al.* 1997, Lindstrōm *et al.* 2004, Herlemann *et al.* 2013). Orders belonging to Betaproteobacteria are commonly associated with freshwater communities and are known generalist bacteria. They have multiple respiratory strategies and metabolic capabilities that result in adaptations to changing environments and/or plasticity (Bergey's manual of systematic bacteriology 2005b, Salcher *et al.* 2008, Hahn *et al.* 2011). This group's density is influenced by pH, temperature, salinity and water retention times (Lindstrōm *et al.* 2005, Salcher *et al.* 2008). Of those variables only pH was measured in this study and did not correlate with the abundance of the Betaproteobacteria.

Betaproteobacteria and Verrucomicrobiae are common freshwater classes; although in general Betaproteobacteria are usually found in high abundance in samples while the Verrucomicrobiae are relatively rare (Lindstrōm *et al.* 2005, Newton *et al.* 2006, Zul *et al.* 2007, Martinez-Garcia *et al.* 2012) and they seemed to be negatively correlated. Thus far there has been no documented correlation between Betaproteobacteria and Verrucomicrobiae abundances, although this data could imply possible competition between the groups. Biological interactions (i.e. competition) are another factor that could contribute to the differences in abundances. Competition in bacteria has been well documented, *Pseudomonas aeruginosa*, excretes an antibiotic that decimates *Brevibacillus borstenlensis* in soil biofilms (Hibbing *et al.* 2010). Further, other studies have documented the effects of bacterivorous organisms on the structuring of bacterial communities. Specific types of bacteria (e.g. Alphaproteobacteria and members of the *Cytophaga-Flavobacterium* group) are found in higher abundances when bacterivorous organisms are within the community (Pernthaler *et al.* 2004, Nishimura and Nagata 2007). However, it is unclear whether such an interaction would favor Verrucomicrobiae and was not examined in this study.

PCA of the main abiotic factors (pH, conductivity, turbidity and phosphates) showed variation of the CVPE water could be attributed to pH, phosphates and turbidity. These factors explained the metabolic variation as well. Given that phosphates played a strong role in structuring the vernal pool communities, changes in phosphorus levels could cause a distinct shift in the communities. Phosphorus is a key nutrient element, and is a common limiting factor for many organisms (including plants) in most ecosystems

(Elser *et al.* 2007). However, increases in phosphorus can cause detrimental effects to the ecosystem. Eutrophication is a process in which nutrients, more specifically phosphorus and nitrogen, is excessively added into an ecosystem (Likens *et al.* 1971). This is mainly due to human activities such as farming and wastewater runoff (Sharpley *et al.* 2003, Schindler 2006). Eutrophication has been shown to create major shifts in ecosystems; this can be caused by the increase in the nitrogen-fixing cyanobacteria. The blooms of cyanobacteria can be toxic to ecosystems by creating a hypoxic state and disrupting the food webs of the community (Conley *et al.* 2009). In vernal pools, eutrophication is characterized by algal mat growth and significant decrease in eukaryotic diversity (Smith *et al.* 1999, Kneitel and Lessin 2010). In one study of four shallow eutrophic lakes, an increase in phosphorus and especially nitrogen showed a decrease in Betaproteobacteria, a group seen in some of the vernal pools. However, Verrucomicrobiae did not seem to replace them as seen in the CVPE (Van der Gucht *et al.* 2005). CVPE are under increased risk of eutrophication due to expanding agricultural practices (Sharpley *et al.* 2003), so monitoring of pools has increased in importance. The bacterial composition could play an important role that has yet to be determined.

Bacterial communities are increasingly used as bio-indicators of ecosystem health in wetlands (Merkley *et al.* 2004). Bacteria have not commonly been used as indicators due to techniques being too expensive for common use, but this has changed with the advent of newer technologies. Bacteria function as the bottom of food webs and are directly affected by changes in the environment. Bacteria, due to their high reproductive rates, have the potential to quickly respond to changing environmental conditions (Sims

et al. 2013). Since aquatic bacterial communities have been documented to shift with season, a temporal study would be needed to assess changes in the communities over a longer time period (Shi *et al.* 2013). Further data is needed on the microbial community in California vernal pools before they can be used as bio-indicators. However, with the data from this study it would seem that a metabolic study looking for the utilization of the D-Xylose substrate would indicate whether the CVPE are a nutrient poor environment.

CONCLUSIONS

The results of this study showed that there is a difference in the community composition of bacteria among California vernal pools, and that the differences are based on abiotic factors, although location had some influence. Further research would be needed to identify the abiotic factors that are directly influencing the community composition and more pools would be required to get a greater view of the bacteria present. Further research will also be needed to fully elucidate if the bacteria in California vernal pools have any biogeographical patterns. Since only a few pools exhibited biogeographical patterns, a sampling approach that includes larger spatial scales may be needed. Although this study did not fully elucidate the roles of bacterial communities in California vernal pools it provides the foundation for future work within this system.

APPENDIX A

Custom FASTA rename Perl script

```
#!/usr/bin/perl
use warnings;
$sample_num = 0;

print "Enter source file name: ";
chomp($source_name = <STDIN>);

print "Enter dest file name: ";
chomp($out_name = <STDIN>);

print "Enter sample name: ";
chomp($sample_name = <STDIN>);

open (INFILE, $source_name);
open (OUTFILE, ">", $out_name);
while (<INFILE>){

		if (/\A>/){
				$sample_num += 1;
				$str = ">$sample_name" . "_" . "$sample_num\n";
	print OUTFILE $str;
		} else {
				print OUTFILE $_;
		}

}
close (INFILE);
close (OUTFILE);
```

Figure A1. Custom FASTA rename Perl script. Takes the first line of the original FASTA file and allows for renaming with the name of the vernal pool and a sequential number.

APPENDIX B

Custom Perl script to remove sequences

```
open (OTU, $source_name);
print "Processing $source_name.....";
while (<OTU>){
		chomp;
		next if $_ =~ /^\#/;
		($otu_number, $classification) = (split /\t/)[0,7];

		if ($classification =~ /Unclassified/ || $classification =~ /Archaea/ ||
$classification !~ /p/)
		 {
				push @otunums, $otu_number;
		 }
}
print "Done\n";
print scalar @otunums, " OTUs identified for removal\n";
close (OTU);



open otu_fasta, "dana_allpools_otus.txt";
print "Processing dana_allpools_otus.txt.....\n";
@otuFasta = <otu_fasta>;
foreach (@otunums){
		@fasta = split("\t", $otuFasta[$_]);
		push (@remove, (splice @fasta, 1));
		if ((@remove%10000) == 0){
				print scalar @remove, " fasta sequences found for
removal\n";
		}
```

Figure B1. Custom Perl script to remove sequences. The script removes unclassified sequences, sequences identified only to Kingdom Bacteria and sequences belonging to Kingdom Archaea and puts the remained of the sequences into new FASTA file.

```
@fasta = ();
}
print "Done processing dana_allpools_otus.txt...." . scalar @remove . "fasta
sequences found for removal\n";
close (otu_fasta);
open FASTA, "dana_allpools.fasta";
print "Processing dana_allpools.fasta.....\n";

@fasta_seq = <FASTA>;
$num_remove = 0;
#$track = 0;
chomp(@remove);
foreach $nums (@remove){
                $index = 0;
                foreach $search (@fasta_seq) {
                        if ($search =~ />$nums\s/) {
                                splice(@fasta_seq, $index, 2);
                                $num_remove++;
                                if (($num_remove%1000) == 0){
                                        $percent =
($num_remove/@remove)*100;
                                        print "$num_remove
sequences removed $percent% complete\n";
                                }
                                #print "$num_remove\n";
                                last;
                        }
                        $index++;

   }
   #$track++;
   #print "$nums Removal list:$track   Removed:$num_remove\n";

}
```

Figure B1. cont.

```
#$index = 0;
#chomp(@remove);
#foreach $nums (@remove){
#		for ($i = 0; $i <= @remove-2; $i += 2){
#			if ($fasta_seq[$i] =~ />$nums\s/) {
#				splice(@fasta_seq, $i, 2);
#				$num_remove++;
#				#print "$num_remove\n";
#				last;
#			}
#		}
#		$index++;
#		print "$nums Removal list:$index   Removed:$num_remove\n";
#}

print "Done processing dana_allpools.fasta\n";
close (FASTA);

print "Opening output.fasta\n";
open (OUTFILE, ">", "output.fasta");

print "Writing fasta sequences to output.fasta\n";
print OUTFILE @fasta_seq;
close (OUTFILE);
print "Done\n";
```

Figure B1. cont.

APPENDIX C

NCBI Sequence Read Archive accession numbers for samples

Table C1. NCBI Sequence Read Archive accession numbers for samples

BioProject Accession: SRP027582			
Vernal Pool Sample	BioSample Accession	Experiment Accession	Run Accession
Beale1	SRS463031	SRX323347	SRR942735
Beale2	SRS463943	SRX327573	SRR942745
Mather1	SRS463946	SRX327576	SRR942748
Mather2	SRS463947	SRX327577	SRR942749
Travis1	SRS463944	SRX327574	SRR942746
Travis2	SRS463945	SRX327575	SRR942747

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