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Microbial biogeography in Mediterranean Vernal Pools of western North America

A dissertation submitted in partial satisfaction of the requirements

for the degree Doctor of Philosophy

in

Environmental Systems

By

Jorge A. Mandussí Montiel Molina

2021

Committee in charge:

Jon Keeley

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University of California, Merced

2021

This work is dedicated to the past, the present and the future generations…

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ACKNOWLEDGEMENTS

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I apply theories stated in classic ecology for larger organisms but not conclusive for microbial organisms. I study the distribution of taxa across landscapes and, in parallel I study the interspecific interactions between microorganisms -endophytes- and plants. Currently, I work with threatened ephemeral wetlands in Baja California Mexico and the USA. For my work, I use a wide range of molecular tools such as PCR and sequencing, and bioinformatics.

Research experience
Herbarium Assistant, 2009-2010. IB-UNAM National Herbarium of Mexico, Fungal collection.
Project: Fungal diversity of Chamela, Jalisco.
Supervisor: M.Sc. Celia Elvira Aguirre Acosta

Researcher assistant Microbiology Laboratory, 2010-2011. CICESE.
Project: Distribution of Coccidioides spp. at Cañon de los Laureles, Tijuana.
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Consultant and biodiversity explorer, 2011-2013. Terra Peninsular A.C.
Project: Biodiversity in Valle Tranquilo Natural Reserve
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Project: Nocturnal Rodents distribution in Reserva Natural Valle Tranquilo
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Project: California native plants microbial endophytes
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Education
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2013. M. Sc. Conservation Biology, CICESE, Baja California, México.
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Thesis & Publications
Montiel-Molina J (2010). Biodiversidad de ascomicetes de la estación de biología Chamela. Informe servicio social. **Thesis.** Universidad Autonoma Metropolitana, Xochimilco. Mexico. 31pp.
Montiel-Molina J (2013). Distribución de flora rara y endémica de charcas vernales en relación a las propiedades fisicoquímicas del suelo. **Thesis.** Centro de Investigacion Cientifica y Educacion Superior de Ensenada**.** México. 68 pp.
Sexton JP, **J Montiel-Molina** , J Shay, M Stephens, and R Slatyer (2017). Evolution of ecological niche breadth. *Annual Review of Ecology, Evolution, and Systematics* 48:1, 183-206.
Montiel-Molina J, JP Sexton, M Beman, C Frank (2019). Visualizing diversity and distribution patterns for

microbial communities in vernal pools; *in:* **Vernal pool landscapes**: Past, Present and Future. *eds.* Schlising, Guilliams & Castro. California State University, Chico. ISBN 978-0-9761774-7-0

Montiel-Molina J, C Frank, JP Sexton & M Beman, (*in revision*) Archaeal and bacterial diversity and distribution patterns in Mediterranean vernal pools of Mexico and the western USA. ***Microbial Ecology***

Shay J, **J Montiel-Molina**, L Pennington, D Towes, B Hendrikson, JP Sexton (*in revision*) Rules of plant species ranges: applications for conservation strategies. ***Frontiers in Ecology and Evolution***

Honours and awards

2005-2009. SEP-UAM Pronabes Scholarship award
2010-2012. CONACyT Scholarship award for excellence graduate program
2012 Scholarship award CICESE's Life Science masters program.
2012-2013. JIJI Foundation small Conservation Grant. Vegetative characterization on Vernal Pools of Baja California.
2013-2014. JIJI Foundation Research Grant. Research on nocturnal rodents of Reserva Natural Valle Tranquilo
2015-2020. UC MEXUS - CONACYT PhD studies fellowship award
2016 BLUM center-MESAT Fellowship award
2016 ES Summer Fellowship award
2017 ES Summer Fellowship award
2018 SNS bobcat fellowship award spring
2018 SNS bobcat fellowship award fall
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2019 SNS bobcat fellowship award spring
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2019 CEP Upward Bound Fellowship award
2020 Grad student travel award
2021 UCMEXUS COVID relief fellowship

Teaching experience

- Evolution BIO 141. 2015.
- Introduction to molecular cell biology BIO 002. 2016.
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- Introduction to Earth system science. ESS 001. 2017.
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Workshops and trainings

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2020 Microbial community analysis, Jackson Laboratory, Connecticut, USA.

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Oral presentations

2018. Microbes in vernal pools. Aqualliance conference, Vernal pool landscapes: Past, Present and Future, Aqualliance and the Vernal pool recovery plan implementation group.
2020. Islas en el tiempo y el espacio, las charcas vernales de Baja California. Seminario de licenciatura en biología de la Universidad Autónoma Metropolitana, Xochimilco.
2020. In between worlds the amphibious life in mediterranean vernal pools. 46th Annual Southern California Botanists Symposium Living on the Edge - Plants in Extreme Environments.
2020. Archaea and bacteria distributions in Californian ephemeral wetlands. MELiSSA conference: Current and future ways for closed life support systems. MELiSSA Fundation/European Space Agency.

Poster presentations
2020. Bacterial and Archaeal Diversity, and Community Assembly in Water and Sediments of Ephemeral Aquatic Ecosystems: Mediterranean Vernal Pools. ASM Microbe Online ePoster presentation.
2018. Montiel, J. Los hongos endófitos alivian el estrés de plantas en charcas vernales? XIII Congreso, Asociación Mexicana para el Estudio de los Hongos. Jalapa, Veracruz, México.
2018. Montiel, J. Do fungal endophytes alleviate plant stress in vernal pools? 8th Symbiosis Workshop, Sierra Nevada Research Station, Wawona, Yosemite National Park, CA.
2015. Toews D., J. Montiel, J. Sexton, C. Frank, M. Stephens, M. Beman. The new frontiers in Vernal Pool research. 41 Symposium of the SCB, Back to the flora: A journey through southern California, Claremont College, CA.
2015. Montiel J. A. Physico-chemical soil properties and its relation with vernal pool rare and endemic flora. CNPS Conservation Conference. Celebrating 50 years of Progress and Promise, San Jose CA.
2012. Montiel J. A. Influence of physico-chemical soil properties on vernal pool rare and endemic flora from Baja California mediterranean region. X Simposio de Botánica en Baja California, México.
2010. Baptista R., Catalán-Dibene J., Romero-Olivares A.L., Montiel J.A., Fungal biodiversity in Baja California:Impact in human health and social affairs. Congreso Anual de Microbiología, CICESE. Baja California, México.

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2017 **Co-Director & Instructor**. MESAT PROGRAM. Migrant Education Merced County Office of Education and UC Merced Grad Division. https://www.youtube.com/watch?v=uIOYZ0RpzzY&feature=youtu.be
2018 **Co-Director & Instructor**. SUMMER UPWARD BOUNDARY PROGRAM. Saty Science workshops 12K minorities, Center for Education Partnerships UCM.
2018 **Co-Director & Instructor.** SUMMER UPWARD BOUNDARY MATH PROGRAM. Stay Math workshops 12K minorities, Center for Education Partnerships UCM.
2018 **Co-Director & Instructor.** FALL UPWARD BOUNDARY MATH PROGRAM. Stay Science workshops, 4 Venir & Center for Education Partnerships UCM.
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California Native Plant Society (San Diego & Baja California Chapters) Member

DISSERTATION ABSTRACT

Microbial biogeography in Mediterranean Vernal Pools of western North America

Jorge Armando 'Mandussí' Montiel Molina

University of California, Merced

Environmental Systems

2021

Microbial organisms play multiple important roles in all ecosystems, yet their diversity and distributions in differentl natural environments still need to be assessed. This doctoral thesis focuses on the study of macroecological distribution patterns and environmental constraints acting over different microbial groups inhabiting ephemeral ecosystems —vernal pools. Vernal pools are temporal wetlands, with contrasting desiccation periods and water availability periods; fundamentally they are well-delimited ecosystems, embedded in completely terrestrial habitats and populated by specialized organisms. 16s Hightroughput-sequencing was used for the analysis of Environmental DNA (eDNA) collected from different substrates: soil, water and plant tissues; to investigate the microbial communities living in these endangered ecosystems.

The initial chapter of this manuscript highlights the paradigms underlying modern microbial ecology, and how microbiology in medical science incorporated theoretical backgrounds from classical ecology. The niche theory and neutral theory are central frameworks to describe the distributions expected in microorganisms, and these theories can be extrapolated to study vernal pool microbial communities of the California mediterranean ecosystems. Compendia of taxa recorded in other regions with wetlands similar to vernal pools show similarities among vernal pools from different continents. Vernal pools occur around the world in other Mediterranean regions and since they act as isolated islands or archipelagos makes it possible to test hypotheses such as the "Latitudinal Diversity Gradient," "Community Isolation by Distance," and "Community Succession." These principles are often applied to larger organisms but are still debatable for microorganisms. This chapter concludes with some suggested questions focusing on ecological principles applied to bacteria, archaea and fungi, free living and in association with larger organisms as symbionts.

The second chapter addresses the distribution patterns of Archaea and Bacteria in vernal pools. Beta diversity analyses showed distinct microbial communities, one from soils and another from water. Soil and wet soil sample community composition indicated

poor environmental selection regardless of anoxic conditions during the water saturation periods in soils. With two defined communities –aquatic and soil communities–, spatial distribution patterns were analyzed across the California Mediterranean region. A latitudinal transect of 1200 km, including sites with vernal pools from Baja California Mexico and California USA, was analyzed with a variety of diversity metrics and correlated against latitude and spatial distances from site to site; only aquatic communities were sensitive to spatial effects. A latitudinal diversity gradient was detected within aquatic microbial communities, but this gradient was inverted relative to expectations: at higher latitudes microbial communities were more diverse and with higher species evenness in comparison with lower latitudes. Precipitation, which decreases from north to south, is a potential driver behind the observed gradient. Additionally, the effect of distance between sites with regards to species composition ("distance decay") was examined. Closer vernal pools had lower dissimilarity in comparison with distant vernal pools. To understand the drivers behind the distance-decay pattern, beta diversity metrics were correlated with precipitation and temperature (environmental filtering) and geographical distances from site to site (dispersal capacity). Environmental variation (precipitation) explained about 15% of the community isolation whereas dispersal capabilities of the microorganisms represented by the distance between sites explained 6% of community isolation.

The third chapter focuses on the microbial endophytes of the California native plant species *Eryngium castrense* Jep. (apiaceae). Referred to as an amphibious plant, *E. castrense* has the ability to perform a metamorphosis according to the environmental conditions in vernal pools. In this study, diversity and community composition was examined in a transect spanning 5 km at the UC Merced Vernal Pools and Grassland Reserve, to address differences in community assembly in different plant tissues. We amplify and sequence the 16s rRNA region of bacterial DNA extracted from the roots and the shoots of 95 specimens of *E. castrance*. In parallel two different morphologies of the plant species, aquatic and terrestrial, were examined. Finally, microbial endophytic abundance and diversity was contrasted with an anthropogenic gradient. Our results demonstrate that host plant habitats and environmental factors correlate with species assemble, with significant differences between shoots and roots. More interestingly the analysis of the communities showed three communities associated to different morphologies regarding aquatic and terrestrial stage (isoetoide or weedy morphology) and plant compartments. Our results also suggest gradual increment in the abundance of some bacterial taxa given by the anthropogenic influence.

Chapter 1. Visualizing the Microbial diversity and community composition in vernal pools

Abstract

In this review, we propose research focused on the study of microbial groups (archaea, bacteria, fungi, protista) within vernal pools, which are well-delimited ecosystems but can vary in many environmental characteristics. In order to understand the diversity and community composition of microorganisms within vernal pools, we suggest research based on the following questions: 1) Do microbial communities vary by distance? 2) How do microbial communities vary across the Californian Mediterranean region? 3) How much of the variance in communities is explained by biogeographic scale? The distribution of vernal pools across the Californian Mediterranean region provides a suitable geographical extent to characterize biogeographical patterns such as distance decay and/or a latitudinal diversity gradient. Finally, since vernal pools tend to become terrestrial habitats after inundation, we explore these questions: 4) How do aquatic vernal pool communities compare with post-aquatic or terrestrial vernal pool communities? 5) Is any existing overlap indicative of taxa exchange? Our methods comprise the analysis of eDNA using high-throughput sequencing and the estimation of different diversity metrics. Vernal pools are understudied in terms of microorganisms, yet this natural component may be important for ecological equilibrium and resilience at local and global scales.

Introduction

Unlike plants and animals, microorganisms are not commonly the subject of discussion in vernal pools studies, yet microorganisms are likely essential components of the ecosystem. Despite their size, microorganisms are behind a massive number of biological and biogeochemical processes—including nutrient cycling via decomposition, carbon storage via photosynthesis and fermentation, and several other metabolic-ecosystemic processes occurring under diverse habitat conditions (Hättenschwiler and Vitousek, 2000; Morgavi et al., 2010; Weitz and Wilhelm, 2012; Jacoby et al., 2017). In parallel, microbial organisms can live associated with larger organisms as symbionts, having profound effects over the host's life cycle, and as consequence, influencing ecosystem processes (Kerney et al., 2011; Pita et al., 2018). After almost a hundred years of recognition of vernal pools as a unique type of habitat, very little research has been conducted on vernal pool microorganisms [see https://www.vernalpools.org/literature.htm]. Therefore, the microbial world of vernal pools remains a frontier to explore (Fig 1). Microbiology refers to the study of those small organisms – *micro*organisms – that cannot be observed with the naked eye. This includes groups of the smallest existing organisms within the five kingdoms: amoebas, zygomycetes, tardigrades, chytridiomycetes, and several bacteria taxa. Because of their physical properties, vernal pools are appropriate systems to test ecological theories

pertaining to microorganismal diversity and distribution. Vernal pools can be seen as water islands that extend across the western edge of the North American continent. They are complete ecosystems in which microorganisms could move among the soil matrix, water column, and biosphere (e.g., inside plant tissues). Microorganismal diversity and their spatial and temporal distribution across the landscape are aspects studied by microbial ecologists and the concepts extrapolated from the study of larger organisms could inform microbial distribution patterns. In this paper we review potential existing diversity of microorganisms inhabiting vernal pools, based on similar ecosystems, and concepts in ecology that could apply to understand microbial distribution patterns. We begin with a brief summary of the origins of microbiology and microbial ecology.
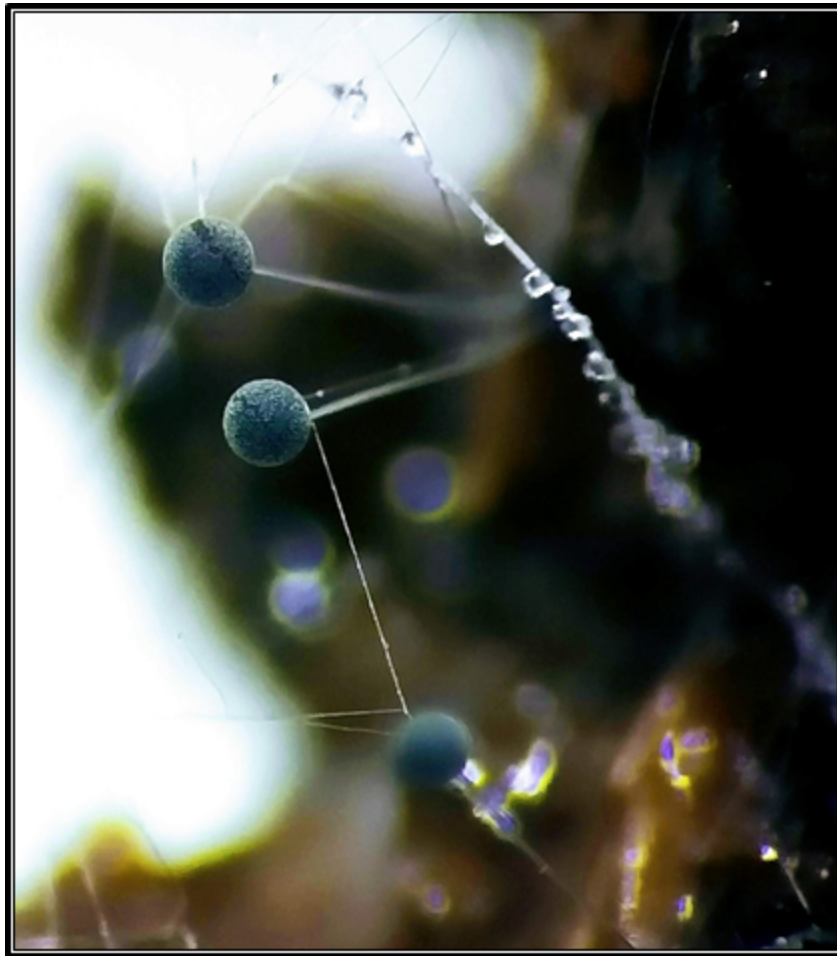
Fig 1. Sporangia [dark spheres] of unknown zygomycete -soil microfungi- 40x. The sporangia shown here are 0.07mm diameter on average. Photo by Jorge Montiel.

Names of importance in the history of microbial ecology
The history of microbiology can be viewed as occurring in phases. This history began with the microscope and Robert Hooke, who was a pioneer in the use of optical

instruments; he developed a primitive microscope to observe the unseen world in different sample types. With it, he contributed with the basic morphological descriptions for filamentous fungi (Karamanou, et al., 2010). Later, Antonie Van Leeuwenhoek (Delft, Netherlands 1632) improved the technology of the microscope by upgrading it into the compound microscope (or light microscope). He described the morphological traits of protozoa (paramecia and amoeba) and bacteria taxa, coining the term "animaculus" to refer to microorganisms (Karamanou, et al., 2010). Between the years 1800 and 1900, scientists such as Louis Pasteur began to study more deeply the biochemistry of bacteria using nutrient-rich substrates. Pasteur developed special laboratory equipment, and using an explicitly experimental approach, worked to understand the biochemical capabilities of microbes to develop concepts for medical immunology. Following Pasteur's work at the end of the 1800s, Robert Koch developed methods to study microorganisms as agents of disease, recognized today as the "Koch postulates". This methodology involved the inoculation of a healthy organism (host) with a given microorganism isolated from contaminated tissue and identifying the causal agent of a specific disease. We can consider this historical time period as the first phase of microbiology, where the main focus was on the biomedical aspects of microorganisms, their morphology, taxonomy and physiology.

Microbiology later focused on developing the field for industry. The Delft School of Microbiology, a prestigious research institute established in the hometown of Antone Van Leeuwenhoek, worked to improve core concepts in the study of modern microbiology, including themes such as microbial ecology, microbial community succession, microbial diversity, and microbial biogeochemistry. Mateus Beijerinck was a pioneer based at Delft. He studied free living microorganisms inhabiting lakes to understand their diversity and distribution in the landscape, while perfecting culturing methodologies for micro algae, fungi, protozoa, methanogenic bacteria and other specialist [extremophiles] microorganisms. In particular, his work attempted to determine the importance of specific environmental conditions on microorganismal diversity. His ideas were inherited by later generations of scientists (Ragon et al., 2012). Lourens Gerhard Marinus Baas-Becking is a central character in the history of microbial ecology. He was part of the The Delft School of Microbiology, but also had a background in botany. He explored interspecific relationships between microorganisms and larger organisms. He also compared the broad-scale biogeographic patterns of microorganisms and larger organisms, such as plants. Baas-Becking visited California, as part of his work on extreme environments and microbial community assembly (Ragon et al., 2012). His findings lead him to conclude that microorganisms are widely distributed across landscapes and 'selected out by ecology', a theory that has been rephrased in english as: 'everything is everywhere, and the environment selects' (Baas-Becking, 1932).

Nowadays, with the advancement of molecular tools (e.g. DNA sequencing), current research questions about microbial distributions can take innovative new approaches to understand distribution patterns (e.g., isolation by distance, biogeography), and the drivers of such distributions. It is now possible to examine microbial community assembly drawing on concepts developed for larger organisms (Fuhrman et al., 2008; Sonthiphand et al., 2014; Oono et al., 2017). As recent research has progressed, an emerging point of view is that both contemporary environmental factors and historical events likely contribute to current microbial diversity and its distributions (Fierer & Jackson, 2006; Zhou & Ning, 2017). However, there is still uncertainty and a need to create a theoretical framework for microorganisms, in order to explain both existing diversity and how it may change (Finlay, 2002; Finlay & Fenchel, 2004, Martiny et al., 2006; Zhou & Ning, 2017).

Microbial diversity
The microbial diversity living in the soil, water column, and symbiotically in larger organisms of vernal pool ecosystems is poorly described. However, vernal pools could be excellent systems for studying the drivers of microbial diversity and community composition. Vernal pools encompass soil/sediment, water, and macrobiota (e.g. plants), in a continuum where microorganisms could travel and disperse, or the contrary, these three compartments could have specific community composition regardless of their proximity and complementarity.

In freshwater ecosystems, microbial communities are likely to be dominated by similar microbial taxa. For example, in lakes from the Great Masurian Lake System in Poland, representatives of the phylum Actinobacteria, Proteobacteria, Cyanobacteria, Planctomycetes, Verrucomicrobia and Bacteroides dominate; Actinobacteria were the most abundant, with 20% abundance in highly eutrophicated to more than 40% in less eutrophicated lakes (Kiersztyn et al., 2019). High-altitude lakes in Yosemite, California are also dominated by the same six groups and Archaeas are found as part of this freshwater community; Actinobacteria have the highest abundances in most of these lakes, and at class level Betaproteobacteria present high abundances. Here, the altitude is the main driver for community composition among lakes (Hayden & Beman, 2016). Alkali pools in Hungary are dominated by the phyla Deinococcus-Thermus, Cyanobacteria, Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes, Fibrobacteres, Bacteroidetes, Gemmatimonadetes and Lentisphaerae (Jones et al., 1998). In ephemeral cave pools (karst pools) the highest abundances belong to Actinobacteria and Betaproteobacteria (Shabarova,et al., 2014). Carrino-Kyker et al. (2008) characterized the eukaryotic and bacterial microbiota from Ohio snowmelt "vernal pools", finding that these pools where rich in Alphaproteobacteria and Betaproteobacteria, along with lower levels of Actinobacteria, Acidobacteria, Flavobacteria and Gammaproteobacteria. The microbial eukaryotes identified in these pools belonged to

the fungal groups Ascomycota, Basidiomycota, and Chytridiomycota. They revealed that microbial community richness differs among substrates (soil, detritus, and water), and although they observed clear differences, the authors do not specify what taxa belonged to each substrate.

The degree to which microbes are shared among vernal pools compartments remains to be determined. The microbial community in vernal pools might have an overlap between water, soil, and the microbiome living in symbiosis with larger organisms. Vernal pools may support rare endemic taxa adapted to the unique conditions in vernal pools, which may be complemented by microbial taxa observed in other aquatic and terrestrial ecosystems. Generally, soil microbiomes are very variable across samples, dependent on the region, soil origin and contemporary environmental conditions. In soil, pH is known to be a very important driver for microbial diversity and community composition across landscapes (Tedersoo et al., 2014; Delgado-Baquerizo et al., 2018). Vernal pools transition from a stage of water saturation to extreme desiccation (aquatic to terrestrial), which might lead to shifts in microbial communities across seasons. On the other hand, such transition between seasons might produce taxa adapted to this environmental change, with the ability to evolve in terms of ecological niche (Sexton et al., 2017). As in larger organisms inhabiting vernal pools, some microbes may have specific adaptations, for example, desiccation-adapted algae persist dormant on the dry stream-beds until flow resumes (Sabater et al., 2017). Microbes in vernal pool water must either disperse into pools from surrounding perennial aquatic environments or have a dormant stage in soil/sediment as a strategy to colonize vernal pools. In this vein, microbes are either adapted to the vernal pool environmental conditions, or selected by the environment (Baas-Becking, 1932).

The identification of microbial taxa in nature is challenging, however a number of studies have performed biodiversity analyses in ephemeral aquatic ecosystems (Table 1). Colburn (2004) summarized the microbiome (eukaryotes and prokaryotes) inhabiting soil and water in the glaciated vernal pool ecosystems of eastern North America. In Ohio, Carrino-Kicker et el. (2008) assessed the diversity of microbial eukaryotes and prokaryotes, using denaturing gradient gel electrophoresis (DGGE). In Portugal, de Carvalho et al. (2014) studied the microbial diversity in a group of mediterranean "temporary ponds", using culturing techniques for samples collected in water and sediments. Such observations made by DGGE and culturing methods might not accurately reflect the total diversity of bacterial and fungal communities. For California, Carper (2013) determined some bacterial groups inhabiting water in a small group of vernal pools in Sacramento valley using the terminal restriction fragment length polymorphism technique [t-FRLP] and next generation sequencing. These studies show that some bacteria taxa from California seem to be shared with eastern North America,

yet nothing is known in regards to free living microbial eukaryotes or microbial communities living in symbiotic association with plants or other larger organisms.

Table 1. Summary of microbial taxa found in vernal pools and similar temporary wetlands, from Carrino-Kicker et al. (2008), Carper (2013), de Carvalho et al. (2014).

Taxa	Author	ID Method	Location	Habitat	Taxonomic group (phylum)
Rhizophydium elyensis	Sparrow 1957	DGGE	Ohio	Not determined	Fungi/Chytridiomycota
Physoderma dulichi	Johns 1957	DGGE	Ohio	Not determined	Fungi/Chytridiomycota
Leptodontidium orchidicola	Sigler & Currah 1987	DGGE	Ohio	Not determined	Fungi/ascomycota
Chalara cylindrospermum	Hughes 1958	DGGE	Ohio	Not determined	Fungi/ascomycota
Troposporella fumosa	Karst. 1892	DGGE	Ohio	Not determined	Fungi/ascomycota
Dothidea sambuci	(Pers.) Fr. 1823	DGGE	Ohio	Not determined	Fungi/ascomycota
Xylaria sp.	Hill 1789	DGGE	Ohio	Not determined	Fungi/ascomycota
Acidomyces richmondensis	Baker, Lutz, Dawson, Bond & Banfield 2004	DGGE	Ohio	Not determined	Fungi/ascomycota
Leotia lubrica	(Scop.) Pers. 1797	DGGE	Ohio	Not determined	Fungi/ascomycota
Hyphodiscus hymeniophilus	(P. Karst.) Baral 1993	DGGE	Ohio	Not determined	Fungi/ascomycota
Sporobolomyces	Kluyver & Niel 1924	DGGE	Ohio	Not determined	Fungi/basidiomycota
Achlya bisexualis	Coker & Couch 1927	DGGE	Ohio	Not determined	Chromista/Oomycota
Chrysocapsa paludosa	(West & West) Pascher 1913	DGGE	Ohio	Not determined	Algae/chrysophyceae
Uroglena sp.	Ehrenberg 1834	DGGE	Ohio	Not determined	Algae/Synurophyceae
Acidobacterium capsulatum	Craig 2009	DGGE	Ohio	Not determined	Acidobacterium
Terriglobus roseus	de Novoa & Williams 2004	DGGE	Ohio	Not determined	Acidobacterium
Erythrobacter sp. 1	Shiba and Simidu 1982	DGGE	Ohio	Not determined	Alphaproteobacteria
Erythrobacter sp. 2	Shiba and Simidu 1982	DGGE	Ohio	Not determined	Alphaproteobacteria
Methylobacterium extorquens	Vuilleumier 2009	DGGE	Ohio	Not determined	Alphaproteobacteria
Asticcacaulis sp.	Poindexter 1964	DGGE	Ohio	Not determined	Alphaproteobacteria
Sphingomonas sp.	Yabuuchi et al. 1990	DGGE	Ohio	Not determined	Alphaproteobacteria
Rhodobacter sp.	Imhoff et al. 1984	t-RFLP and next generation sequencing	CA Sacramento	water	Alphaproteobacteria
Rhizobiales spp.	Kuykendall 2006	t-RFLP and next generation sequencing	CA Sacramento	water	Alphaproteobacteria

Rickettsiales spp.	Gieszczykiewicz 1939	t-RFLP and next generation sequencing	CA Sacramento	water	Alphaproteobacteria
Variovorax paradoxus	Davis 1969	DGGE	Ohio	Not determined	Betaproteobacteria
Variovorax dokdonensis	Yoon et al. 2006	DGGE	Ohio	Not determined	Betaproteobacteria
Rhodoferax sp.	Hiraishi et al. 1992	DGGE	Ohio	Not determined	Betaproteobacteria
Rhodoferax sp.	Hiraishi et al. 1992	t-RFLP and next generation sequencing	CA Sacramento	water	Betaproteobacteria
Janthinobacterium sp.	De Ley et al. 1978	t-RFLP and next generation sequencing	CA Sacramento	water	Betaproteobacteria
Polynucleobacter sp.	Heckmann and Schmidt 1987	t-RFLP and next generation sequencing	CA Sacramento	water	Betaproteobacteria
Amphora delicatissima	Ehrenberg, 1844	DGGE	Ohio	Not determined	Diatom chloroplast
Flavobacterium bacterium	(Bergey et al. 1923) Kuo et al. 2013	DGGE	Ohio	Not determined	Flavobacteria
Flavobacterium columnare	(Bernardet & Grimont 1989) Bernardet et al. 1996	DGGE	Ohio	Not determined	Flavobacteria
Pseudomona spp.	Migula 1894	DGGE	Ohio	Not determined	Gammaproteobacteria
Pseudomona spp.	Migula 1894	t-RFLP and next generation sequencing	CA Sacramento	water	Gammaproteobacteria
Emticicia sp.	Saha and Chakrabarti 2006	t-RFLP and next generation sequencing	CA Sacramento	water	Sphingobacteria
Family-Chthoniobacteraceae	Sangwan et al. 2004	t-RFLP and next generation sequencing	CA Sacramento	water	Spartobacteria
Prosthecobacter sp.	(Staley et al. 1976) Staley et al. 1980	t-RFLP and next generation sequencing	CA Sacramento	water	Verrucomicrobia
Rhodococcus hoagii (R.equi)	(Morse 1912) Kampfer et al. 2014	Culturing	Portugal	Not determined	Actinobacteria
Micrococcus luteus	(Schroeter 1872) Wieser et al. 2002	Culturing	Portugal	Not determined	Actinobacteria
Kocuria rhizophila	Kovacs et al. 1999	Culturing	Portugal	Not determined	Actinobacteria
Sphingobacterium spiritivorum	(Holmes et al. 1982) Yabuuchi et al. 1983	Culturing	Portugal	Not determined	Bacteroidetes
Bacillus cereus	Frankland & Frankland 1887	Culturing	Portugal	Not determined	Firmicutes
Bacillus marisflavi	Yoon et al. 2003	Culturing	Portugal	Not determined	Firmicutes
Bacillus megaterium	de Bary 1884	Culturing	Portugal	Not determined	Firmicutes
Bacillus pumilus	Meyer and Gottheil 1901	Culturing	Portugal	Not determined	Firmicutes

Bacillus thuringiensis	Berliner 1915	Culturing	Portugal	Not determined	Firmicutes
Brevibacillus laterosporus	(Laubach 1916) Shida et al. 1996	Culturing	Portugal	Not determined	Firmicutes
Staphylococcus xylosus	Schleifer and Kloos 1975	Culturing	Portugal	Not determined	Firmicutes
Achromobacter xylosoxidans	(Yabuuchi and Ohyama 1971) Yabuuchi and Yano 1981	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Aeromonas caviae	(Eddy 1962) Popoff 1984	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Aeromonas hydrophila	(Chester 1901) Stanier 1943	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Alcaligenes faecalis	Castellani & Chalmers 1919	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Comamonas terrigena	Hugh 1962	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Edwardsiella tarda	Ewing and McWhorter 1965	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Klebsiella aerogenes (Enterobacter aerogenes)	(Hormaeche and Edwards 1960) Tindall et al. 2017	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Escherichia coli	(Migula 1895) Castellani & Chalmers 1919	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Ewingella americana	Grimont et al. 1984	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Kluyvera intermedia	(Izard et al. 1980) Pavan et al. 2005	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Neisseria sicca	(von Lingelsheim 1908) Bergey et al. 1923	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Pantoea agglomerans	(Beijerinck 1888) Gavini et al. 1989	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Pseudomona fluorescens	Migula 1895	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Pseudomona putida	(Trevisan 1889) Migula 1895	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Pseudomona syringae	van Hall 1902	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Serratia liquefaciens,	(Grimes and Hennerty 1931) Bascomb et al. 1971	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Serratia odorifera,	Grimont et al. 1978	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Stenotrophomonas maltophilia,	(Hugh 1981) Palleroni & Bradbury 1993	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Yersinia intermedia,	Brenner et al. 1981	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Yersinia kristensenii	Bercovier et al. 1981	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae
Yokenella regensburgei	Kosako et al. 1985	Culturing	Portugal	Water and soil	Proteobacteria/entero bacteriaceae

All organisms have the potential to create symbiotic associations, but microbial symbioses may confer special advantages to the host and result in significant

repercussions to ecosystems overall. For example, some microbial endophytes live inside plant tissues without causing any disease to the host plant, while enhancing the capacity of plants to survive stressful environmental conditions (Rodriguez et al., 2008). It has also been shown that endophytes may deter grazing (Azevedo et al., 2000). Little is known about the temporal and spatial distribution patterns of endophytes. In contrast to free living microorganisms (those that live in the water and soil), endophytes are less explored by ecologists because they live inside the plant and are difficult to access. Recent research has shown that host specificity is an important driver for the distribution of endophytes (Fonceca-Garcia et al., 2016). On the other hand, it seems that endophytes also display biogeographic patterns that are common in larger organisms. For example, endophytes have been shown to have distance-decay patterns, meaning that the difference of taxa in two communities may be best explained as consequence of the spatial distance between sample sites (Vaz et al., 2014; Oono et al., 2017). The distribution of endophytes has been addressed in different habitat types, such as conifer forest and grasslands (Loro et al., 2012; Brathen et al., 2015, Carrell & Frank, 2015; Carper et al., 2018). Ecological studies of microbial endophytes have focused mainly on trees and grasses, wherein altitude has been shown to be an important driver for endophytic microfungal diversity. Shrubs and aquatic plants are less studied (Kivlin et al., 2017), with the vernal pool flora highly understudied in terms of these microbial symbionts. Microbial endophytes can be tested under the same frameworks as in free living microorganisms in vernal pools, with emphasis in spatial, altitudinal, latitudinal, or anthropogenic gradients.

Existing information on symbiotic microorganisms in vernal pools is limited, with just a few observations made on samples of grasses from the genera *Orcuttia* and *Tuctoria* (Keeley, 1988). This study detected that roots of these grasses were colonized by microbial-fungal organisms under stressful conditions. The observations made by Keeley (1988) did not test if such symbioses have novel effects on these plants, for example alleviating stress in vernal pools. When considering vegetation as a complementary compartment where microbes can live in the vernal pool ecosystems, theories related to the microbial niche expansion and the resilience of microbial communities in the ecosystem can be extensively applied. Such an approach would be especially relevant in light of global climate change.

Vernal pools as good system to test theories of microbial biogeography
Understanding organismal biogeography has been a longstanding goal in ecological sciences, for example, Alfred Russel Wallace's seminal work about communities from the Malayan Archipelago (Goldhor, 1964; Gallardo, 2013). Because of their small size however, much less is known about microbial diversity and distribution. Additionally, the drivers that shape microbial communities are poorly understood. Microbial ecologists have begun to study microbial distribution patterns in some microbial groups. For

example, Bryant et al. (2008) have documented the effect of elevation on microbial diversity, with observed effects profoundly different between prokaryotic to eukaryotic microorganisms. This suggested that these groups of microorganisms are not responding the same way to the environment. Carrino-Kyker et al. (2011) examined the biodiversity of (non-Mediterranean) vernal pools across a gradient of anthropogenic disturbance, and demonstrated an increased diversity of fungi in urban areas in comparison with more rural areas.

Classical biogeographical distribution patterns, such as latitudinal distributions or distance-decay and temporal dynamics, have not been thoroughly studied in microorganisms. Vernal pools are ideal environmental systems to test the applicability of these theories – developed through study of larger organisms – to the microbial world. Due to the geographical orientation of the Mediterranean-type climate region along the Pacific Coast of western North America where vernal pools occur, it is possible to establish a latitudinal transect of study sites. Along this latitudinal gradient, vernal pool complexes are patchily distributed on the landscape, yielding "archipelago-like" potential study sites with different proximities at diverse spatial scales, locally and regionally. Finally the extreme seasonal changes within vernal pools over the course of a year, with pronounced wet periods and dry periods, produce "temporal islands" during which suitable habitat may be available (or not) for microbes. Finally, the physical morphology of vernal pools play a role in this temporal isolation, as deeper pools are more likely to have more frequent and longer hydroperiods compared to shallow pools.

Distance decay
An important property of vernal pools is that they are isolated habitats, "water islands" that occur in complexes surrounded by completely terrestrial ecosystems. Such isolation allows the study of the effects of how the distance between pools influences the microbial species assemblage. In larger organisms, similarity of species between ecological communities typically decreases with increasing distance (Fig 2); this phenomenon is known as distance decay (Nekola & White, 1999). In ecology, distance decay can be measured using metrics of similarity between natural communities situated in two or more sites; this approach provides a basic descriptor of how biological diversity is distributed in the landscape. Hyden & Beman (2015) evaluated similarity between microbial communities as a function of distance in Yosemite high-altitude lakes; they reported a strong distance-decay pattern. Beisner et al. (2006) used data from eighteen lakes, showing that the variability in community structure of less easily dispersed species (zooplankton and fish) are better predicted than bacteria and phytoplankton by the spatial distribution of lakes and their connections on the landscape. Zinger et al. (2014) focused on distance decay of bacterial communities in marine ecosystems, by comparing the open ocean habitat and coastal mangrove habitats. Mangrove communities occur at different distances from each other, while the ocean pelagic zone

is continuous. They concluded that more heterogeneous environments with spatially isolated habitats (e.g. mangrove communities) are more likely to show a distance decay pattern between habitat patches. For vernal pools at Sacramento valley, sites that were close to each other, showed similar microbial community composition (Carper, 2013). Niche theory predicts that community similarity decreases as a result of species differences and environmental filtering, irrespective of geographic proximity (Tilman, 2004). On the other hand, the neutral theory predicts that the decay of community similarity is caused by spatially limited dispersal, independent of environmental differences between sites (Hubbell, 2001). As mentioned above, vernal pools are "island-like" at both local and regional scales, and as such provide a good system to examine the distance-decay patterns in microbial communities.
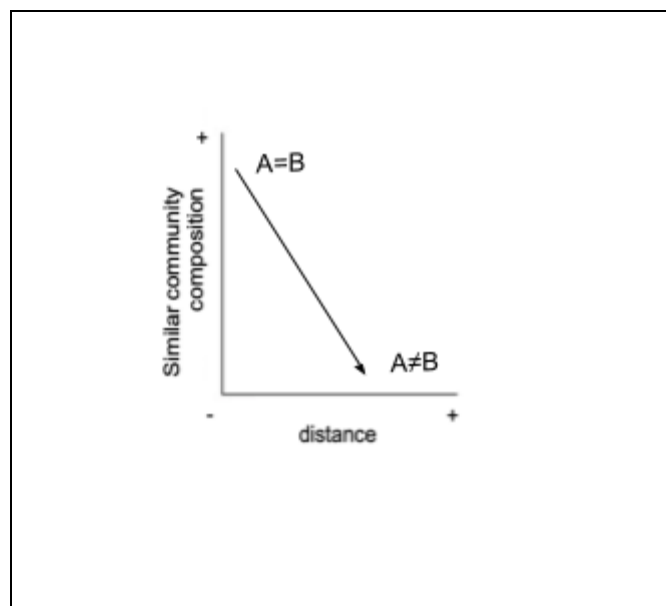
Fig 2. Distance decay. Natural communities are similar as a function of distance between them. As distances increase between A and B [sites], the natural communities are expected to become more dissimilar.

Latitudinal gradient
The latitudinal diversity gradient is a biogeographical pattern that has been studied for more than 200 years in natural communities (Fuhrman et al., 2008). The existing paradigm states that diversity increases from the poles to the equator, and communities are expected to be different among sites along this gradient. It is not clear whether this pattern results from a longer, more stable period of diversification in the tropics, higher speciation rates in the tropics, or lower extinction rates in comparison with temperate regions (Mittelbach, 2007). Regardless, studies have described similar latitudinal diversity patterns among many different groups of organisms (Schiaffino et al., 2016; Hyde et al., 2016; Caldwell, 2017).

A classic example of the latitudinal diversity gradient can be found in planktonic marine bacteria, which increase in diversity from the poles toward the equator (Fuhrman et al., 2008). Newsham et al. (2016) also documented a temporal-spatial gradient using a latitudinal transect. The authors showed taxonomic diversity of soil fungi increases towards the equator, a roughly 20-27% increase in taxon richness for every 2.5° C increase in temperature. Western North America vernal pools are common in California's Central Valley, but the ecosystem type extends latitudinally along the west coast on mesas and plains from southern Oregon to northwestern Baja California, following the extent of the California Floristic Province. Therefore, vernal pools follow a natural precipitation and temperature gradient across this region from north to south, a distribution pattern suitable to test questions related to climate change, and others applicable to microbial communities (Fig 3).
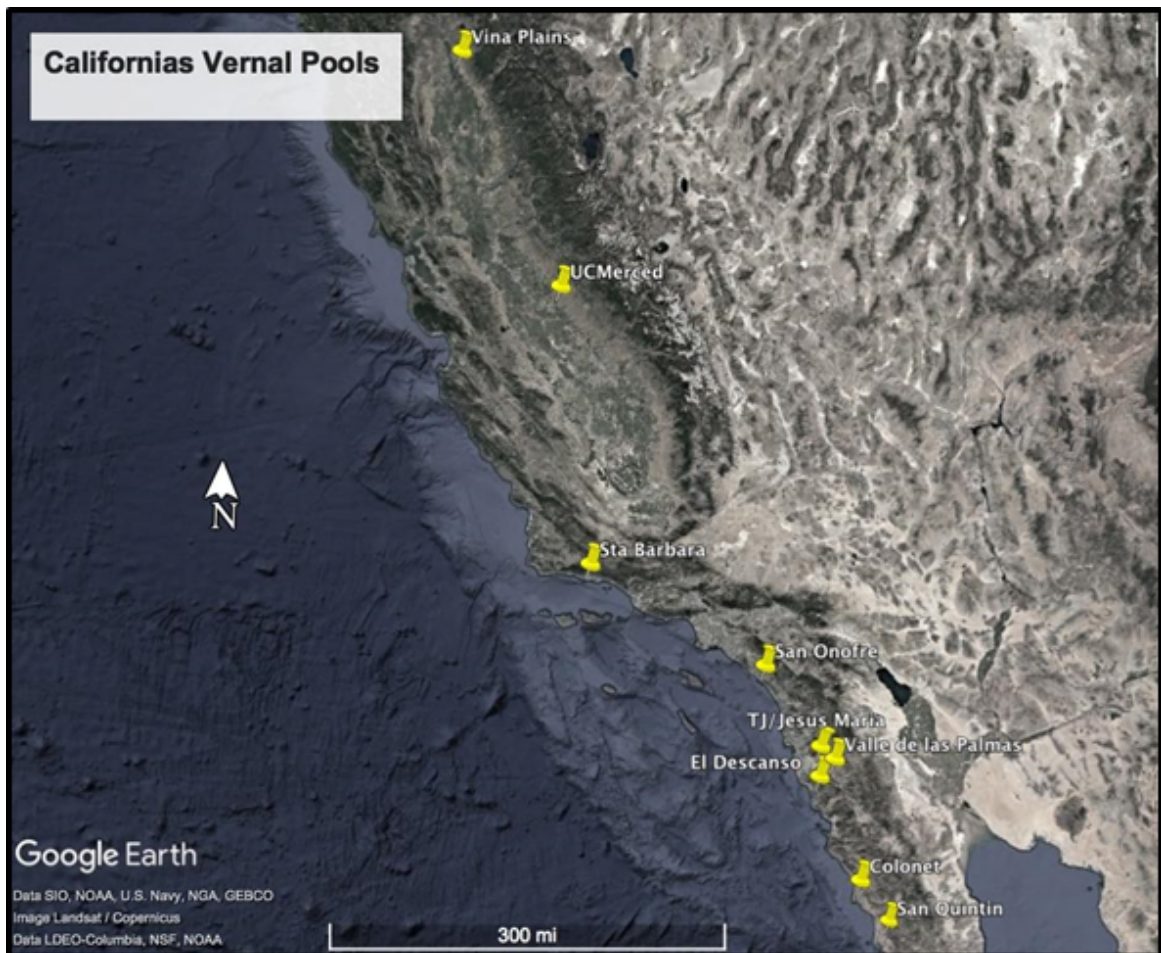
Fig 3. Some vernal pool complexes explored in 2016 (in yellow) following a latitudinal transect along the California Floristic Province, from Baja California to "Alta California".

Ecological succession

Seasonal events are considered significant drivers for microbial diversity in terrestrial (e.g., forest, Shigyo et al. 2019) and aquatic ecosystems (e.g., ocean, Giovanonni & Vergin, 2012). Ecological succession can be considered as the cyclical temporal pattern regarding species turnover across time (Clements, 1920 reviewed in Troll, 2003). Pastemak et al. (2013), stated that in soils of arid, semiarid, and Mediterranean ecosystems, some microbial orders such as actinobacteria differed between summer and winter seasons. Moisture, temperature, inundation, and other cyclical environmental factors are likely to explain the observed patterns of microbial community assembly. Shabarova et at. (2010) observed variation in the abundances of microbial groups across time in cave pools (karst pools). They argue that the variation in microbial community composition they observed was a consequence of inundation period differences and changes in water chemistry. Microbial communities from the same season (e.g., winters in different years), harbor large proportions of recurrent populations (Shabarova et at. 2013). When Li et at. (2015) studied the temporal distribution of plankton in a lake system, they found a seasonal successional pattern where winter months correlate, separate from summer months. Similar results were revealed in Ohio snow melt vernal pools, where summer time shifted to a different microbial community composition, in comparison to winter time (Carrino-Kycker, 2008).

Over the course of a year, as vernal pools experience successive periods of inundation due to different rain events, rainwater inputs into flooded vernal pools might produce shifts in microbial communities. In freshwater lakes, seasonal inputs of water produce a dramatic shift in bacterial communities, which has been attributed to resulting physical and chemical changes (Crump et al., 2016). Inundation events are known to have a direct influence on plant growth, therefore plants also might contribute to seasonality in microbial communities, with potential effects both above- and below-ground (Williams et al., 2013). Additionally, seasonal disturbances such as animal grazing and anthropogenic activities might influence microbial communities as well (Pasternak et al., 2013; Carrino-Kicker et al., 2011; 2013).

A very intriguing concept, contrary to succession is, the "climax" in natural communities, a concept stated by the plant ecologist Frederic Edward Clements in 1920, meaning a stationary or final assembly of species in a given habitat (Troll, 2003), the absence or very minimum successional patterns. Groups of microorganisms have been detected to remain as a "core" group that occupy a given habitat regardless of temporal variation (Shabarova et at., 2013). While this concept of "climax community" is highly debatable because of the implicit heterogeneity of the environment at different scales across the landscape, it is likely that the vernal pool microbial community includes a "core" set of taxa regardless of the substantial environmental shifts. Community composition studies across time are not very well explored because of the difficulty of maintaining a sampling

effort over the long term. Therefore, many studies do not pursue ecological questions over extended periods of time.

Conclusion

Microbiology is a vast field in the natural sciences, with many aspects yet to be explored. Throughout history, technological advances have formed successive bridges toward understanding microbiology in an ecological context. Despite the neglect of microorganisms in vernal pools studies to date, current technological advances such as high throughput DNA sequencing represent an opportunity to deeply explore the biodiversity of microbial prokaryotes and eukaryotes inhabiting such a unique ecosystem.

The drivers of microbial diversity and distributions remain poorly elucidated, and ecosystems such as vernal pools could play an important role in expanding our understanding of the processes that govern the microbial world. Vernal pools could be seen as a miniature Amazon rainforest, where both continuous and discontinuous patterns occur due to natural environmental changes on the landscape. Since the environmental variation in vernal pools occurs at a small scale relative to other ecosystems, vernal pools are vastly easier to sample and comprehend. Findings of studies in vernal pool ecosystems can be extrapolated to larger ecosystems; in this case results of studies focusing on microbial diversity could be applied to understand the ecology of similar freshwater ecosystems.

Ongoing and future research on vernal pool microbial diversity should concentrate on simple but foundational ecological questions that would permit the integration of microorganisms into the broader context of global organismal biodiversity. For example: What microbial taxa live in vernal pools? Are microbial communities shared among the soil, water column, and plant compartments of vernal pools? How do microbial communities vary across spatial distances? Do microbial communities vary in regards to environmental gradients? Do microbial communities have more variation temporally or spatially? What is the role of microbes when living in symbiosis with vernal pool plants? Are any microbial taxa shared among all plant species in a pool or pool complex? Do symbiotic microbes conform to classical biogeographical patterns? What are the drivers for microbes living in symbiosis with vernal pool plants?. And to visualize the microbial community across temporal scales, especially in regards to global change scenarios: Do microbial communities in vernal pools have successional patterns? How do microbial communities in vernal pools transition from an aquatic environment during the wet phase to a non-aquatic environment during the dry phase? Is a core community prevalent within different vernal pool complexes?

Today, research is focusing on the microbiome more than ever, highlighting the importance of microbes and the symbioses they form. As mentioned earlier, microorganismal diversity has been neglected in studies of vernal pool ecosystems for a variety of reasons. The historical challenges to study this unseen world represented a significant barrier in microbial ecology. Nevertheless, vernal pools are an intriguing, extreme environment, encompassing interesting cases of diversity, endemicity, and adaptations of the residents and their symbiotic partners. Microorganisms are important for ecosystem processes, the study of microbial diversity, before global change alters its current state is a matter to be prioritized. Finally, by understanding the links between macroorganisms, microorganisms and the environment, we will be closer to understanding the drivers that originate and maintain the diversity of living organisms.

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Chapter 2. Archaeal and bacterial diversity and distribution patterns in Mediterranean vernal pools of Mexico and the western USA

Abstract

Biogeographic patterns in microorganisms are poorly understood, despite the importance of microbial communities for a range of ecosystem processes. Our knowledge of microbial ecology and biogeography is particularly deficient in rare and threatened ecosystems. We tested for three ecological patterns in microbial community

composition within ephemeral wetlands—vernal pools—located across Baja California (Mexico) and California (USA): 1) habitat filtering; 2) a latitudinal diversity gradient; and 3) distance-decay in community composition. Paired water and soil samples were collected along a latitudinal transect of vernal pools, and bacterial and archaeal communities were characterized using 16S rDNA sequencing. We identified two distinct microbial communities, with one community present in the soil matrix that included archaeal and bacterial soil taxa, and another community present in the overlying water that was dominated by common freshwater bacterial taxa. Aquatic microbial communities were more diverse in the north, and displayed a significant but inverted latitudinal diversity pattern. Aquatic communities also exhibited a significant distance-decay pattern, with geographic proximity explaining 9%, and precipitation explaining 16%, of community variation. Collectively these results indicate greater sensitivity to spatial and environmental variation in vernal pool aquatic microbial communities than in soil microbial communities. We conclude that vernal pool aquatic microbial communities can display distribution patterns similar to those exhibited by larger organisms, but differ in some key aspects, such as the latitudinal gradient in diversity.

Introduction

Microorganisms play multiple ecological roles in terrestrial and aquatic ecosystems, from acting as symbionts and parasites to regulating biogeochemical cycles. Quantifying how microbial communities assemble and how microbial taxa are distributed across ecosystems is therefore important to our understanding of ecological equilibrium. As such, a central question in microbial ecology is the degree to which microbes follow ecological patterns displayed by 'macroorganisms' (Martiny et al, 2006, Hanson, 2012; Zhou & Ning, 2017, Gilbert et al, 2018; Dickey et al, 2021). Traditionally, microorganisms were thought to be widely distributed in all ecosystems due to their small size and high dispersal rates, with the environment responsible for "selecting" microorganisms according to their metabolic attributes (Vinogradskij, 1889; Becking, 1934). Niche theory predicts community composition as a result of environmental filtering, and irrespective of geographic proximity (Tilman, 2004)—for example, salinity, temperature variation, oxygen limitation, and other particular conditions can strongly influence microbial community assembly in natural habitats (Zhang et al, 2019). However, it is equally clear that a combination of historical events and contemporary factors are important in determining diversity and community composition, and that microbial communities can display broad biogeographic patterns (Fierer & Jackson, 2006; Martiny et al, 2006; Zhou & Ning, 2017). Ultimately, microbial communities distributed within and across different ecosystems may be shaped by different assembly processes to differing degrees, providing insight into microbial community assembly in comparison to larger organisms.

Vernal pools are ideal systems in which to examine microbial community assembly given the inherent attributes of these ecosystems. Reflecting the climatic extremes typical of "Mediterranean" ecosystems (moist and cold winters followed by hot and dry summers), vernal pools can form where water collects in shallow depressions with a high clay content and a deeper cemented layer—which prevents water percolation to the subsoil. Defined as temporary wetlands, and characterized by consecutive aquatic and terrestrial phases, vernal pools display seasonal transitions from completely flooded to totally desiccated soils (Zedler, 1987; Fig 4). During winters with sufficient precipitation, vernal pools fill with rainwater, creating water 'islands' embedded in a surrounding terrestrial ecosystem. Vernal pools then progressively retract and eventually dry out in warmer months as evapotranspiration exceeds precipitation. Vernal pools therefore encompass both aquatic as well as soil habitats—which both vary strongly over the year, and may interact in various ways (e.g., via species that transition between the different environments, or via fluxes of carbon and nutrients). How microorganisms colonize these ecosystems, and tolerate the variations within them, directly addresses the roles of geography versus environment in affecting community assembly.
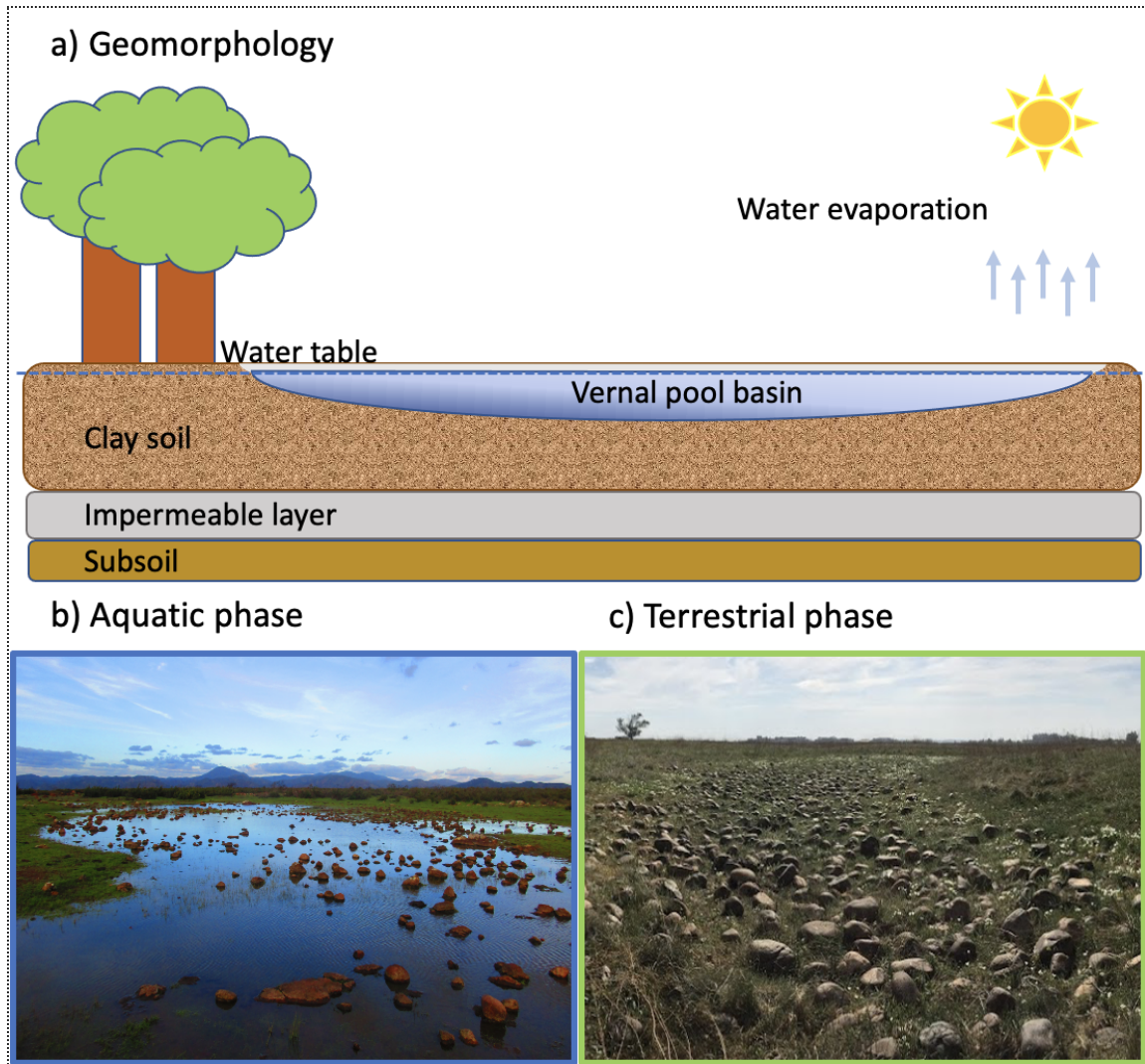
Fig 4. The physical attributes and climatic conditions that create Vernal pools - temporary wetlands with contrasting phases. a) Vernal pool general geomorphology, b) aquatic phase, characterized by flooding and soil saturation and, c) terrestrial phase, characterized by water evaporation and subsequent desiccation

In soil, seasonal fluctuations from inundation to aridity likely create strong environmental variations. Under one extreme, saturated soils often reach anoxic conditions that may affect the distribution of microorganisms (Comeau, et at., 2012). On the other extreme, dry and hot conditions during Mediterranean summers may require adaptations—for example, desiccation-adapted microalgae may persist dormant on dry stream beds until water flow resumes (Timoner et al., 2014). During the rainy season, inundated vernal pools resemble permanent aquatic ecosystems; however, the strong seasonality of Mediterranean vernal pools shares attributes with other ephemeral aquatic ecosystems like seasonally flooded deltas, wetlands (pitlands), or floodplains (Mccarthy et al., 2003;

Parolin, 2004). Freshwater ecosystems are typically inhabited by similar microbial groups (i.e., 'typical freshwater bacteria'; Zwart et al. 2002) regardless of geographic location and characteristics, but with significant spatial and temporal variation within these groups (Newton et al., 2011). Whether similar freshwater bacterial groups colonize filled vernal pools—or whether they are inhabited by more specialized groups—is unknown. In either case, these communities may be used to examine biogeographic patterns in microorganisms.

In classical ecology, the latitudinal paradigm states that diversity gradually increases from the poles to the equator following a latitudinal gradient (Keinetel, 2015; Montiel et al., 2019), and microbial ecologists have begun to study this pattern of biodiversity. The distribution of vernal pools across the California province represents a latitudinal transect of ~2400 kilometers, providing an opportunity to examine spatial patterns in diversity and community composition. In particular, vernal pools can be considered habitable islands for microbes, and microbes may show geographical patterns in diversity and community composition (Beisner et al., 2006; Finkel et al., 2012; Hayden & Beman, 2016; Newsham et al.,2016; Schiaffino et al., 2016; Montiel et al., 2019). Unresolved for the latitudinal gradient hypothesis, is whether the gradient results from a longer, more stable period of diversification in the tropics, higher speciation rates in the tropics, or lower extinction rates in comparison with temperate regions, based on the ecology of larger organisms (Mittelbach, 2007). Microbial ecology research is still extensively needed to determine the presence and causes behind latitudinal patterns. For example, Fuhrman et al. (2008) detected a significant increase in planktonic marine bacterial richness from the equator to the poles in the open ocean, but with notable variability in diversity at lower latitudes. Following a transect from the tropics to the polar circles within lakes of South America and Antarctica, Schiaffino et al. (2016) detected an increase in biodiversity of microbial eukaryotes with decreasing latitude. On land, Newsham et al. (2015) also detected an increase in biodiversity of soil microfungi from colder to warmer regions along a transect from the south polar circle to the tropics. These studies indicate that both aquatic and soil microbial communities may follow latitude-diversity gradients.

Ecological studies have also observed a 'distance-decay' pattern in many natural communities, in which community similarity decreases with increasing distance between locations (Finkel et at., 2012). Distance-decay patterns can be explained by the neutral theory that predicts community composition as a result of geographic proximity, where community similarity is driven by spatially limited dispersal, independent from environmental differences between sites (Nekola and White, 1999, Hubbell, 2001); on the other hand , environmental conditions play a key role by "filtering" some taxa, and microbial communities that are closer together in space may experience similar environments. The degree to which distance-decay relationships are shaped by dispersal capabilities (spatial distance), environmental differences, or a combination of

both, may differ among ecosystems. For example, community dissimilarity among microbial communities in alpine lakes can be partly explained by the distance between sites (Hayden & Beman, 2016), whereas temporary cave-pool microbial communities are explained by the sunlight exposure and environmental properties of the water (Shabarova & Pernthaler, 2010). For microbial communities in vernal pools of the North American continent, there is a lack of studies focusing on these ecological features.

Under the premise that microorganisms can show distribution patterns similar to those exhibited by larger organisms, we tested multiple hypotheses corresponding to specific paradigms established in classical and contemporary microbial ecology: (i) First, given the amphibious nature of other organisms inhabiting vernal pools—which have aquatic and terrestrial life cycles, and transit from soil to the water column (Zedler, 2003)—we hypothesized that microbial taxa in vernal pools may also transition between the soil matrix and water column. If this is true, we will observe measurable overlap in microbial composition between the soil matrix and the overlying water column. Alternatively, the soil matrix and water column may represent two adjacent but distinct habitat types, with distinct environmental properties that select for specific communities occupying two different niches. (ii) Considering that vernal pools are subject to strong environmental selection, we hypothesized that water saturation and desiccation events will drive shifts in community composition from flooded pools to dry pools. Niche theory (Tilman, 2004) states that the environment is the strongest driver for species to persist or recruit; in this context, moist and drought conditions may play a role in structuring microbial communities. If this is true, we expect to observe two different microbial communities in saturated versus unsaturated soils, according to the seasonal-environmental variation in vernal pools. (iii) Considering that vernal pools are distributed latitudinally, we hypothesized the existence of a gradual increase in diversity of microorganisms along a transect from southern sites in Mexico to northern sites in the USA. (iv) In parallel, considering that vernal pools act as scattered or clustered habitat "islands" (Zedler, 2003), we hypothesized that microbial communities will present spatial distribution patterns reflected in the community composition from each vernal pool. Thus, a "distance-decay" pattern at local and regional scales is expected, where isolated vernal pools will have less similar microbial communities as a function of the space between pools. (v) Finally, local environmental selection is likely to drive community composition as a result of the specific environmental properties across localities. We hypothesize that environmental variation in temperature and precipitation may be significant drivers of community similarity, explaining diversity between pools, sites, and regions, regardless of spatial proximity. Alternatively, dispersal capacity of microorganisms may be important in community assembly. If this is true, we expect to observe a relationship based mostly on spatial distances.

Methods

Study area and sampling design

As a Mediterranean climate region, the study area is characterized by winter precipitation events and dry and hot summers. We considered a latitudinal transect of ~1300km, covering part of the geographical extension of the North American Mediterranean climate regime and the distribution of its vernal pools. The sampling occurred in regions from Baja California, Mexico and California, USA (Figure 5a), and each location varied in temperature and precipitation; temperature differences within the region range between 16º-18**º** Celsius, and precipitation varies depending on subregions, but overall precipitation increases with latitude (Table 2). The vernal pools studied here belong to an "archipelago complex" (i.e., clusters of pools) or solitary vernal pools, often located at flat ground on top of coastal mesas or valleys. Due to federal regulations, vernal pool soils were not possible to access in the USA. From north to south, locations were annotated alongside with their climate and geographical position: In California, USA, 1) Vina Plains, 2) Merced, 3) Santa Barbara; in Baja California, Mexico, 4) Mesa de Jesus Maria, Tijuana 5) El descanso, 6) Valle de las palmas, 7) San Antonio del Mar, 8) Medina (Colonet mesa) and 9) San Quintin.

To address our research interest regarding environmental selection and distribution patterns, we considered the first 15 centimeters from the soil surface to have a good representation of the soil microbiome (Fierer, 2003). We collected one sample of 0.2 grams of dry soil per pool in summer August 2016, representing an individual pool for a total of 11 different pools; as a counterpart, we collected 0.2 grams of "wet-soil" from the same pools, but saturated with rainfall during the winter 2017, for a total of 11 pools. Unfortunately, some vernal pools were lost due to urbanization during our sampling period between summer and winter. Both sample types were stored in 2 ml centrifuge tubes with dry ice before processing in the laboratory. In parallel, we also collected 100 milliliters of water per vernal pool during the winters of 2016 and 2017, for a total of 27 water samples analyzed in this study; some pools in Valle de las Palmas and San Antonio del Mar regions were sampled twice during the year 2017 corresponding to different rain events. We filtered the microorganisms from the water samples using Waltham brand® filters of 0.23 ɳm pore size. After filtration the filters were preserved in lysing tubes with a sucrose EDTA lysis solution and transported to the lab for DNA extractions (Hayden and Beman, 2016). Equipment was sterilized in between samples with 5% bleach, 100% ethanol and rinsed with MilliQ water five times. Soil and filters were processed for DNA extractions with QIAGEN DNeasy® extraction kits. Final DNA aliquots with 50 microliters per vernal pool were diluted to a final concentration of 20 nanograms per microliter for further amplification and sequencing.

Table 2. 30 year annual average temperature and precipitation of vernal pool regions and ID of the vernal pools sampled. Temperature (temp) is in Celsius and precipitation (precip) is in millimeters. Data was obtained from BIOCLIM.

Vernal pool sites	Sample type collected	Temp	Precip	Longitude	Latitude
Vina Plains (i)	Water	16.2	719	-121.982942	39.901494
Vina Plains (ii)	Water	16.2	719	-121.983788	39.899747
Merced i	Water	16.3	370	-120.417208	37.376927
Merced ii	Water	16.3	370	-120.417668	37.377302
Merced iii	Water	16.3	370	-120.415969	37.375948
Santa Barbara i	Water	15.2	468	-119.86534	34.415124
Santa Barbara ii	Water	15.2	468	-119.866172	34.415841
Santa Barbara iii	Water	15.2	468	-119.867498	34.415982
Santa Barbara iv	Water	15.2	468	-119.868427	34.414142
Tijuana 'Jesus Maria Mesa'	Water, wet soil, dry soil	16.9	277	-116.833829	32.50431
El Descanso 'Mesa'	Water, wet soil	16.2	269	-116.871812	32.177932
(†)Valle De Las Palmas i	Dry soil	16.6	282	-116.73994	32.39779
(†)Valle De Las Palmas ii	Dry soil	16.6	282	-116.74147	32.39682
Valle De Las Palmas (iii)	Water, wet soil	16.3	340	-116.64541	32.36915
Valle De Las Palmas (iv)	Water, wet soil, dry soil	16.3	340	-116.64582	32.36913
Valle De Las Palmas (v)	Water, wet soil, dry soil	16.3	340	-116.64631	32.36937
San Antonio del Mar i	Water, wet soil, dry soil	16.6	242	-116.28739	31.09416
San Antonio del Mar ii	Water, wet soil, dry soil	16.6	242	-116.28722	31.09355
San Antonio del Mar iii	Water, wet soil, dry soil	16.6	242	-116.28787	31.09249
San Antonio del Mar iv	Dry soil	16.6	242	-116.29275	31.05852
(†)San Antonio del Mar v	Dry soil	16.6	242	-116.293954	31.027036
(†)San Antonio del Mar vi	Dry soil	16.6	242	-116.293954	31.01739
Medina i (Colonet mesa)	Water	16.6	242	-116.28147	31.030867
Medina ii (Colonet mesa)	Water, wet soil	16.6	242	-116.282222	31.030835
Medina iii (Colonet mesa)	Water, wet soil	16.6	242	-116.283296	31.030569

San Quintin 'Cerro las Torres'   Water, wet soil   18.9   179   -115.914153   30.584009

† *vernal pools destroyed due to urbanization*

Microbial Community Analysis
We examined dry soil, wet soil and water samples via 16S rDNA amplicon sequencing targeting the regions V4 - V5 (115F[Parada]: GTG**Y**CAGCMGCCGCGGTAA; 926R[Quince]: GGACTAC**N**VGGGTWTCTAAT), to capture bacterial and archaeal diversity (Beman et al, 2020). Analysis of the amplicon sequence variants (ASVs) was performed using the Qiime2 platform. Demultiplexing and pairing sequences indicated that the lowest and the highest ASV representations per sample were 714 and 66432, respectively.

Qiime2, PASTv4.0 and R studio packages were used for statistical analyses and graphics (Bolyen et al, 2019; Hammer, 2001). Alpha diversity metrics included Richness(observed taxa), Shannon (richness/evenness), Simpson (richness/abundances), Pielou(evenness) and ACE(richness and sampling coverage). The Kruskall-Wallis test was used to analyse differences in alpha diversity metrics between dry soil, wet soils and water samples. Beta diversity was analyzed using Bray-Curtis and unweighted Unifrac metrics of community dissimilarity; environmental information was included in community analyses using PCA and NMDS ordination methods. PERMANOVA and ANOSIM (999 permutations), were used to correlate dissimilarity matrices for grouping significance. Beta diversity values were correlated (r) with latitude and with spatial distances between sites. Mantel tests (rho) were performed to correlate taxa abundance similarity matrix values with temperature, precipitation and geographical distances matrices.

Results
Microbial community patterns across vernal pools
Analysis of microbial communities within vernal pools spread throughout California, USA and northern Baja California, Mexico revealed differences between soil samples and water samples (Fig. 5). PERMANOVA results between soil and water both showed significant differences in microbial communities (Table 3).

Table 3. Group significance by Permutation analysis of variance (PERMANOVA) and Analysis of similitud (ANOSIM).

PERMANOVA (bray-curtis)	2 groups determined	R = 0.227	P = 0.001
PERMANOVA (Unweighted-Unifrac)	2 groups determined	R = 0.158747	P = 0.001
ANOSIM	Soil - water	Wet soil - soil	Water - wet soil

Bray-curtis	R=0.98, p=0.001	R=0.21, p=0.002	R=0.97, p=0.001
Unweighted-Unifrac	R=0.89, p=0.001	R=0.28, p=0.001	R=0.90, p=0.001

Alpha diversity analyses of 22 soil samples, including dry soils and wet soils, was similar for all metrics of richness and abundance—suggesting that dry and wet soils host similarly diverse microbial communities despite geographic differences across sites (Fig.5b). Beta diversity analysis showed less variability in soil microbial communities in comparison with aquatic microbial communities (Fig. 5a). Soils seem to be one single habitat type defined by our taxa composition, however significant differences between dry and wet soils communities were detected with ANOSIM (Table 3). These findings suggest that conditions in vernal pools select for fairly consistent microbial communities in soils. Microbial adaptations to survive long periods of desiccation and the transition from flooding to desiccation could result in low variation from one phase to another (Timoner et al, 2004).
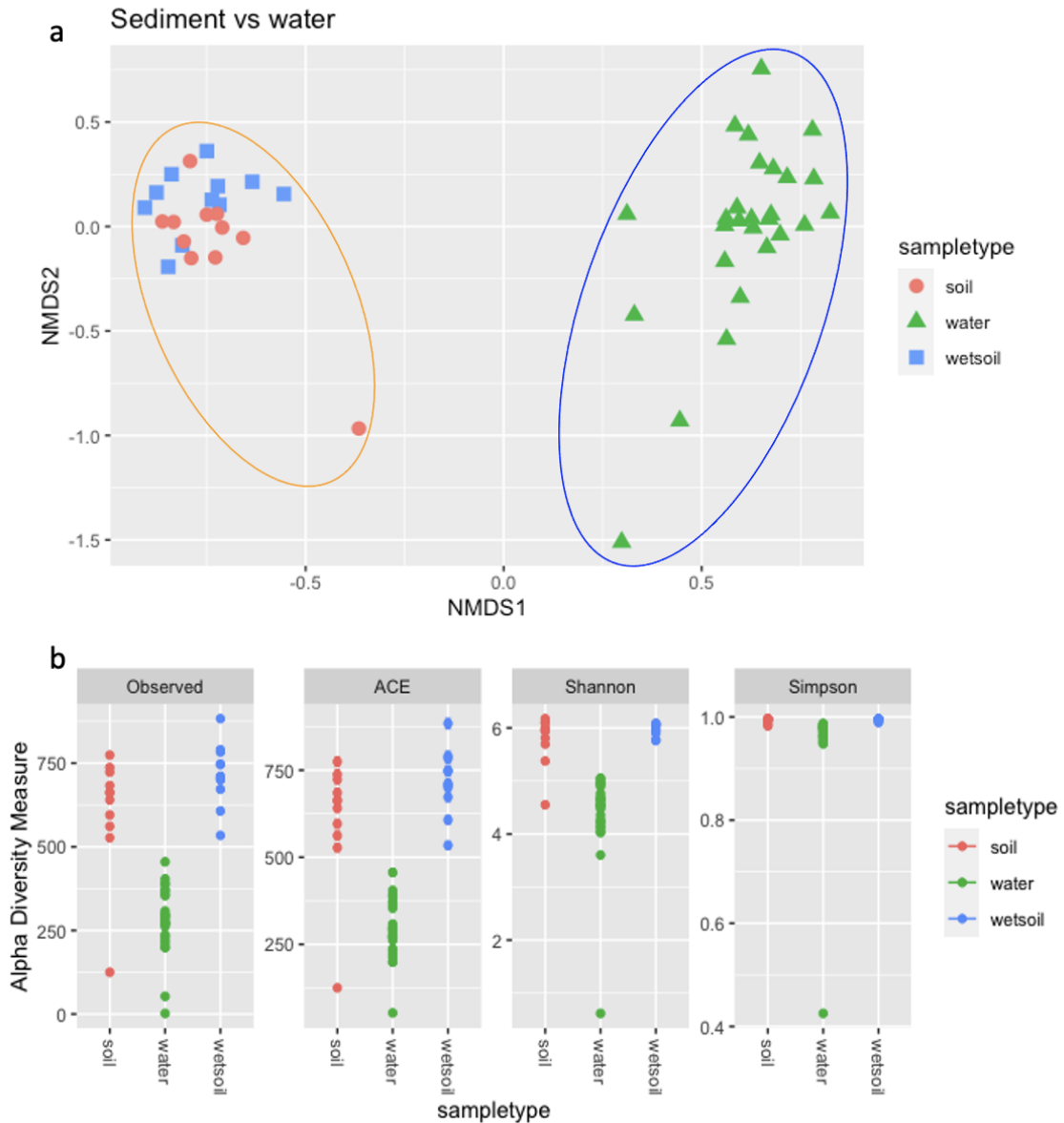
Fig 5. Microbial community analysis. a) Beta Diversity: *NMMS* ordination analysis based on Bray-Curtis similarity coefficients, soils and water determine microbial communities by environmental filtering; soil samples appear more homogeneous that water samples. b) Alpha diversity by sample type. Soils (dry and wet) shown higher diversity values in comparison with the water column: soil-water H=18.9,P = 0.000013; wet soil-water H=22.5, P=0.000002; soil-wet soil H=0.13, P=0.71

Microbial taxa in soil and water
Bacteria were present in both soils and water, but with marked differences between sample types (Fig. 6). We found 42 phyla in total, and the most abundant were Proteobacteria and Bacteroidetes. Water was dominated by a few phyla, primarily

Bacteroidetes and Proteobacteria, followed by Verrucomicrobia and Actinobacteria in lower proportions. Soil was dominated by Proteobacteria, followed by Actinobacteria, Acidobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Verrucomicrobia, Planctomycetes and Gemmatimonadetes. Our analysis revealed Fusobacteria as a rare phylum associated only with water samples; rare phyla associated with soil samples were AD3, BHI80139, GOUTA4, Kazan 3B28, MVP21, OP3WS2, WS3,WS4, WS6, WWE1 (Fig. 6a).

Archaea were detected almost exclusively in soils, comprising the phylum Crenarchaeota, Euryarchaeota and Pavarcheota. Archaea being mostly limited to soils suggests that aquatic archaea are less likely to inhabit vernal pool waters. At the class level, Archaeal diversity was dominated by Thaumarchaeota followed by Methanomicrobia, Thermoplasmata, MCG, and Parvarchaea (Fig. 6b). Methanobacteria and Parvarchaea were found specifically in two of the most northern sites of the study, Merced and Vina Plains, suggesting geographic community variation (which we specifically evaluated below). Overall, alpha diversity metrics indicated that soils have higher diversity than water.
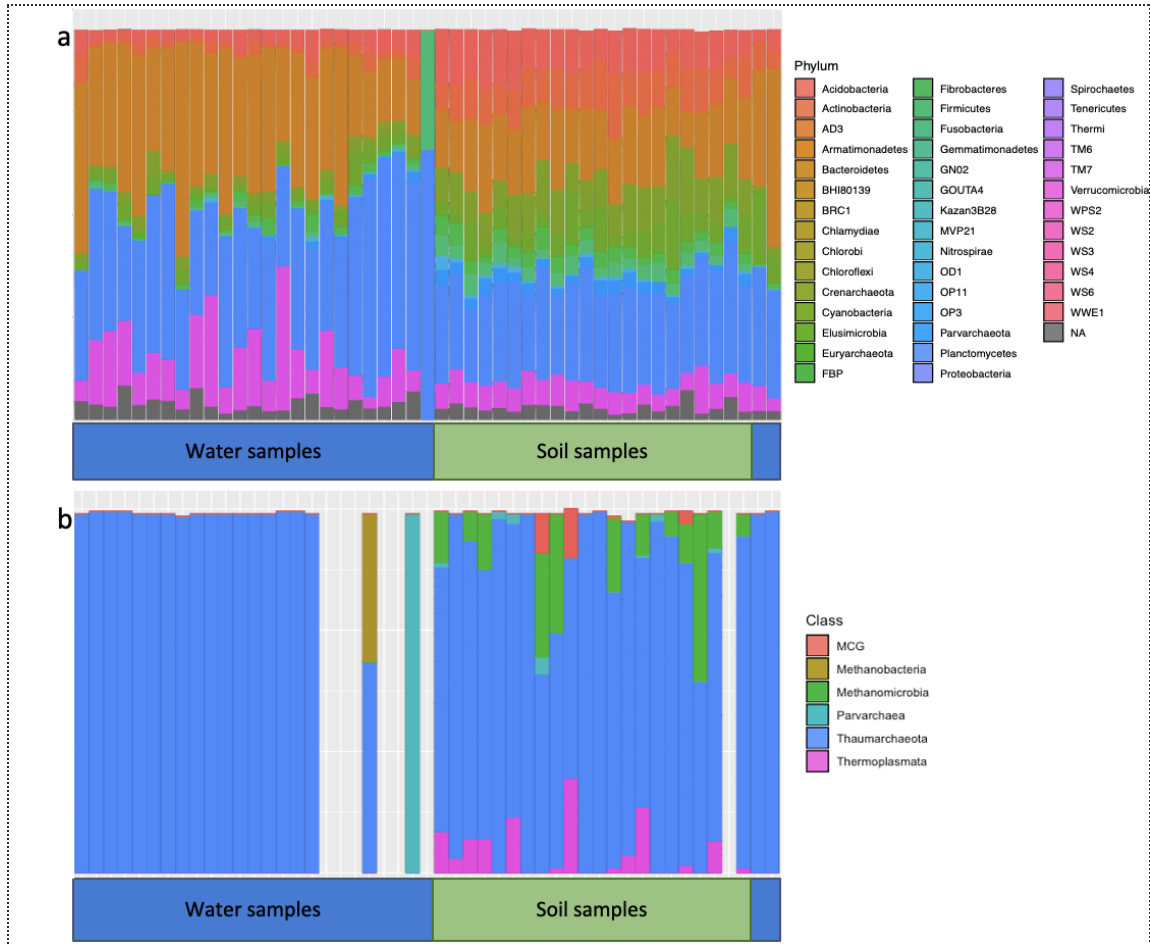
Fig 6. Bacterial and Archaeal diversity profile for water and soil samples; taxa abundances more evenly distributed in soil samples in comparison with water samples. a) Bacteria taxa at phylum level, b) Archaea taxa at class level.

The latitudinal diversity gradient in vernal pool microbial communities
The general increase in biodiversity from the poles to the equator is what defines the concept of the latitudinal diversity gradient, and community composition is expected to transition gradually in sites along this gradient. We found a significant latitudinal diversity gradient in vernal pools microbes; however, this was inverted and only applied to aquatic communities (Fig. 7). In addition, only diversity metrics based on evenness (Pielou and Shannon) displayed this significant pattern.

a) Vernal pool region and latitudinal extension
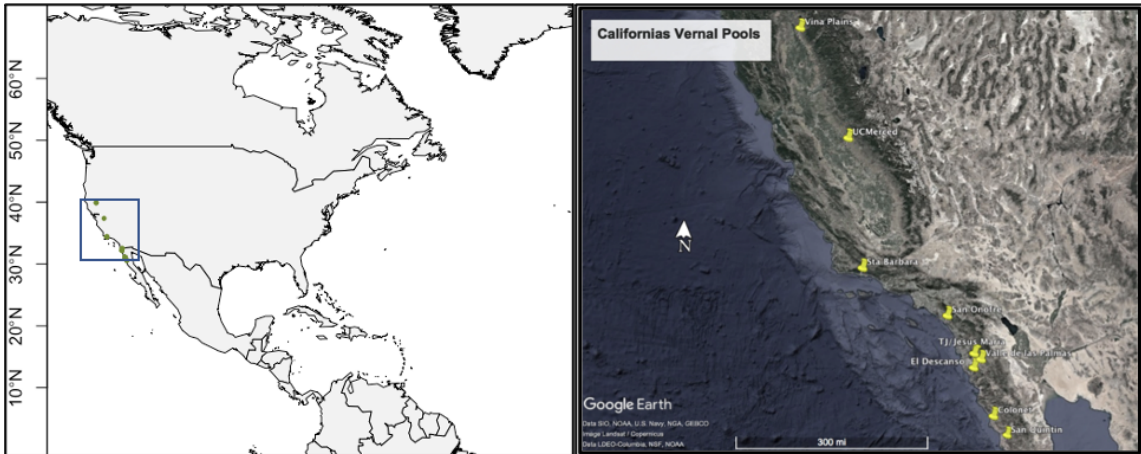
b) Correlation model (Pearson r) for diversity evenness and latitude
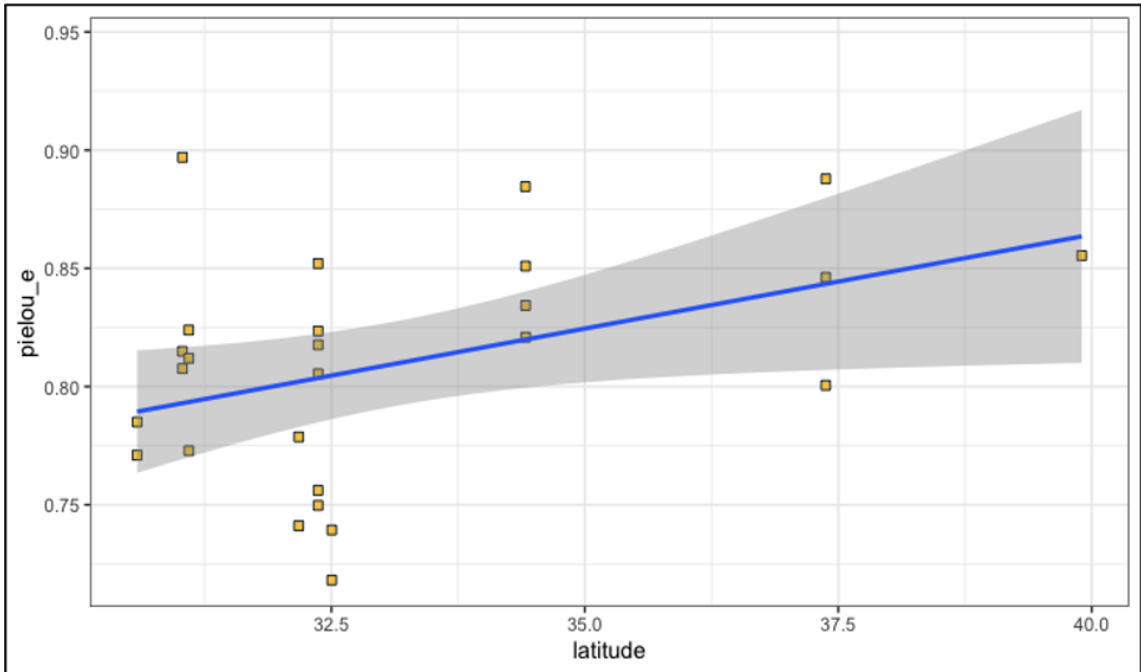
c) Synthesis of correlation coefficients between diversity metrics and latitude

	OTU's richness	Shannon	Pielou	Ace
Significance p	0.95	0.055	0.036	0.785
Distribution t	-0.054941	2.0131	2.2114	-0.27562
Pearson's coef	-0.01121413	0.38	0.411	-0.05617155

Fig 7. Diversity along a latitudinal gradient is inverted for aquatic prokaryotes in vernal pools. a) sampling sites across mediterranean climate from the parallel 30ºN to 40ºN; b) Scatter plot indicating higher diversity evenness at vernal pools in higher latitudes; c) Correlation coefficients for the diversity-latitude relationship

Distance decay: dispersal capabilities vs environmental selection
Our beta diversity analysis demonstrated that aquatic communities varied among sites (R^2=0.5625; P<0.01 Fig. 8), suggesting that community isolation is driven by dispersal limitation or environmental differences between sites. To test whether dispersal limitation (geographical distance) or environmental filtering were drivers of community composition, we analysed the correlation (rho) between climatic parameters, spatial distances, and community dissimilarity indices.

Community variation was tested for correlations with mean annual temperature and precipitation. The Mantel test based on Spearman's rank correlation (permutations = 9999) between mean precipitation and taxa distribution (Bray Curtis dissimilarity) showed significant correlation across all samples (r: 0.1269, P= 0.0249). Our results showed stronger correlation with water samples only (r: 0.4324, P= 8e-04), meaning that about 16% of the variance in water microbial communities is explained by precipitation. On the other hand, geographical distance (r: 0.32, P=0.02)—which addresses the influence of dispersal limitation—also significantly explained the assembly of communities (Table 4). A combination of the amount of local precipitation and geographical distance between sites was therefore significantly related to community composition. We acknowledge longer precipitation events lead to longer hydroperiods in the vernal pools, allowing aquatic microbial taxa more opportunity to disperse to some degree. Our results demonstrate that a combination of environment and spatial distances shape vernal pool microbial communities. This pattern has been found in freshwater bodies, where the existing pattern of dissimilarity (community isolation) is a product of both environmental differences and the spatial distance between sites (Shabarova & Pernthaler, 2010; Hayden & Beman, 2016).
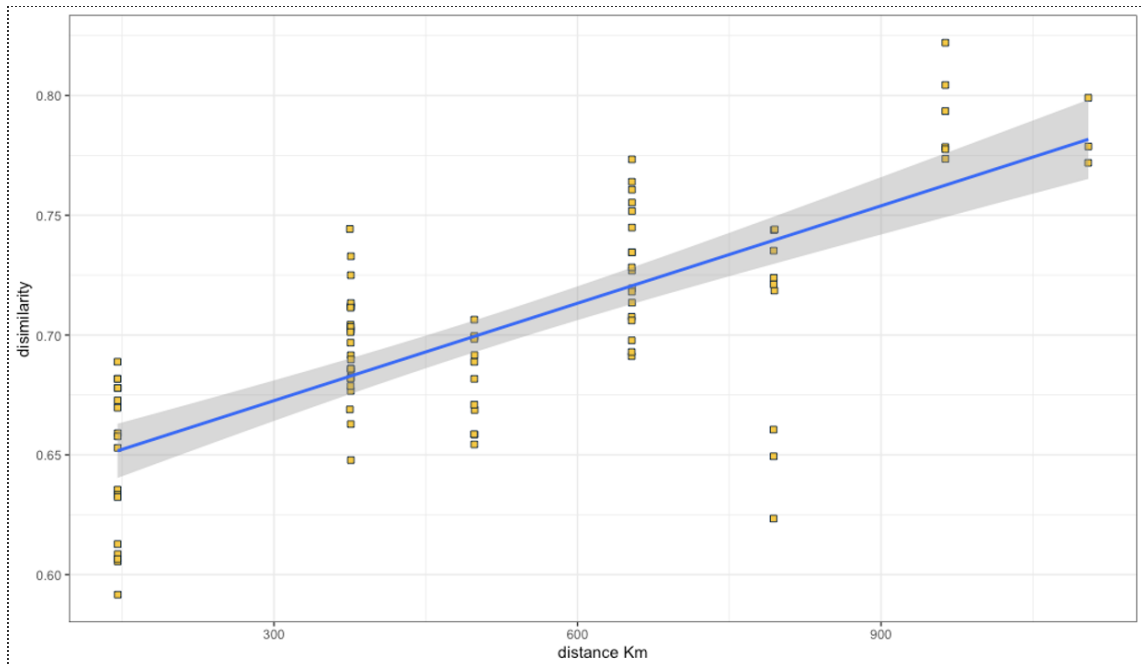
Fig 8. Dissimilarity in aquatic microbial communities in vernal pools increases with distance between sites; each point represents a specific value given by the Bray-Curtis dissimilarity index between two vernal pool microbial communities at varied distances

Table 4. Mantel test summary: Correlation between similarity matrix based on total abundances of microbial taxa, and precipitation or geographical distances (spatial distances).

Matrix analysed	(rho)	Significance P	Permu
precipitation/total.abund	r: 0.1269	P= 0.0249	9999
precipitation/water.abund	r: 0.4324	P= 8e-04	9999
geographical.distance/water.abund	r: 0.3203	P= 0.0203	9999

Discussion
Taxa identities in water
We found that vernal pool microbial communities in water were dominated by 'typical' freshwater bacterial taxa (Zwart et al. 2002, Newton et al. 2011). These groups are commonly found across a wide range of freshwater ecosystems, and include various groups, especially within the Bacteroidetes and beta-proteobacteria—which were dominant overall in water samples (Fig. 2). Vernal pool waters included, for example, multiple groups of Comamonadaceae within the betaproteobacteria, which are important constituents of many freshwater ecosystems (Kasalický, 2013; Nuy et al, 2020). Actinobacteria are also common in freshwater (Newton et al. 2011), but were less abundant overall in vernal pool water. These findings suggest that common freshwater microbes are able to colonize vernal pools each year in spite of the ephemeral nature of the pools. We evaluated whether this results in significant biogeographical patterns, particularly in vernal pool water.

Lack of significant biogeographic patterns in vernal pool soil communities
A main goal of this research was to determine if microbial communities in vernal pools show distribution patterns previously recorded in other microbial systems and for larger organisms. We detected several patterns in microbial communities (e.g., a significant latitude-diversity gradient and distance-decay), but these patterns were only significant for aquatic communities. In the case of soil, our results indicate that conditions in vernal pool soils select for consistent microbial communities across distant locations. However, several factors might have also obscured our ability to detect significant patterns in soil, if present. One issue is the presence of relic DNA coming from extracellular or non-intact cells, which can account for 40% to 80% of prokaryotic DNA in soils, and can persist in soil for weeks to years (Lennon et al, 2017). Another possibility is that

precipitation events have an impact on the microbial community by "resetting" a well established microbial community. In combination with physicochemical properties (i.e. cation exchange capacity and pH), this could have a direct effect on the recovery of eDNA (Carini et al., 2016, Lennon et at., 2017). Increased sampling in future studies, and including other molecular techniques such as metatranscriptomic sequencing, may help reveal additional patterns, especially with soil microbial communities.

Implications of vernal pool microbial communities resembling other freshwater ecosystems
We expected that vernal pool variability (desiccation to inundation) could potentially select for unique taxa, but we found that mostly typical freshwater bacteria inhabited vernal pool water. How exactly vernal pools are populated by common freshwater taxa is an interesting question worthy of additional study and experimentation. Freshwater bacteria are notably widespread (Zwart et al, 2002), and the attributes of vernal pools may help provide insight into their broad geographic distribution and the connectivity between freshwater ecosystems via animals or air. For example, possible transport pathways could involve larger organisms that use the vernal pools as a place of rest and breeding, such as migratory birds. Additionally, rain might play an important role in transporting microorganisms (Evans et al, 2006). On the other hand, vernal pools have a cemented layer preventing interchangeable flows from subsoil to surface, which reduces the possibility of groundwater connectivity. Considering the impact of anthropogenic activities over the landscape—where some vernal pools have been transformed into stock ponds, and other human-made water reservoirs are located nearby—anthropogenic activities may also be relevant in the context of microbial distributions and its alterations. Finally, some taxa may also disperse widely by wind, and environmental constraints between habitats might shape microbial communities (Dueker et al, 2018).

Inverted Latitudinal Gradient
Some ecological explanations for such inverted gradients rely on specific interactions between taxa—for example, predation, and theories of energy around environmental systems (Morales-Castilla & Garcia-Valdez, 2014). We suggest that the most possible explanation for this relationship is that vernal pools in southern latitudes have shorter periods of inundation given the lower precipitation rates compared with more northern sites. As a result, dispersal may be diminished at the southern boundary due to the absence of water bodies. Further studies should address if surrounding water bodies or precipitation provide vernal pools with freshwater taxa. Based on the limited energy hypothesis behind latitudinal diversity—which states that sunlight is directly correlated with plant productivity and species biodiversity (Morales-Castilla & Garcia-Valdez, 2014)—another possible explanation is that higher primary production in Northern California is a reflection of longer growth periods, with inundated vernal pools lasting longer and favoring higher diversity. Moreover, the inverse prokaryotic diversity gradient observed among these study sites may be associated with eukaryotic diversity patterns,

which we did not evaluate. Interestingly, at the regional scale (California vs Baja California), some bacterial phyla were unique to vernal pools from northern latitudes

Conclusions

This research is the first formal attempt to characterize and quantify microbial communities in Mediterranean-climate vernal pools in North America, providing initial insight into the microbial ecology of these endangered ecosystems. Overall, our study indicates that environmental selection plays an important role in defining distinct vernal pool microbial communities. Aquatic communities in vernal pools exhibit a non-traditional latitudinal diversity pattern, which may be partially explained by precipitation patterns. Dispersal limitation is important as well, which means that a combination of spatial and environmental variation affect the assembly of vernal pool microbial communities. Whether such patterns are consistent over longer time periods, and what mechanisms are involved to assemble community differences observed here, are important future research avenues. Vernal pools are well-known for being inhabited by organisms adapted to both aquatic and terrestrial conditions (e.g., plants), with life cycles modified in order to survive shifts of inundation and total dedication in a short period of time. Occasionally this transition occurs quickly, with intermittent precipitation and evapotranspiration happening rapidly within days. Exploring these transitions at different temporal scales may provide insights about an "amphibious behavior" in microorganisms inhabiting these ecosystems—i.e., the ability to survive in both soil and water. In this case, this "taxa mixing" phenomenon is worth further study. Finally, additional spatial, temporal, and biological (e.g., plant and animal hosts and vectors) sampling may reveal new biological discoveries in these endangered vernal pool ecosystems.

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Chapter 3. Unraveling the bacterial communities associated with *Eryngium castrense* Jeps. (apiaceae) – an amphibious plant from vernal pool ecosystems

Abstract

Amphibious plants are defined by their ability to tolerate a wide range of hydrologic conditions, from completely saturated soils to progressively drier conditions. In the natural environment these specially adapted plants may serve as hosts for microbial communities that in turn influence the host, but these relationships remain mostly unknown. Microorganisms living in association with a host form part of a "holobiont," and the mechanisms influencing holobiont diversity and community composition need additional study in order to better understand organismal functions. We investigated diversity and community composition of the bacterial endophytes associated with the California native plant *Eryngium castrense* in its natural habitat. Root and shoot samples were taken during aquatic and terrestrial vegetative stages of the host plant. We collected specimens across a transect of five kilometers at the University of California Vernal Pools and Grassland Reserve, Merced, for the high-throughput sequencing analysis of the 16S rRNA region. Plant host compartments and the external environment influenced microbial communities living in association with *E. castrense*. Roots and shoots harbor distinct microbial communities, and significant diversity differences between the aquatic and terrestrial morphological stages of the plant host were also detectable, suggesting different potential sources of microbes: water and soil as a microbial source for root and air as a microbial source for shoot. Community similarity evaluated with beta diversity metrics suggested three main communities given by the morphology of the plant host and the plant compartments: aquatic roots, terrestrial roots and shoots. On the other hand alpha diversity metrics considering plant specimens (roots and shoots together) at different sites indicated no significant differences, with Proteobacteria and Bacteroidetes dominating the bacterial microbiome of the plant *E. castrense*. In addition, our results also suggest gradual increment in the abundance of some bacterial taxa from the moderate anthropogenic influence area in direction to the urban area.

Introduction

Plants that are uniquely adapted to the vernal pool environment are referred to as 'amphibious,' able to grow in both terrestrial and submersed aquatic conditions. Vernal pools—ephemeral bodies of water that provide habitat for specialized plants and animals—represent an extreme version of the climatic variation characteristic of many other ecosystems (Zedler, 2003). With seasonal saturation during winter and gradual desiccation conditions the rest of the year, the vernal pools (en español: charcas vernales) trigger evolutionary processes of dormancy and amphibious life cycles. In vernal pools, aquatic plants [e.g *Isoetes howellii]* became adapted to desiccation conditions and plants that originated in terrestrial taxa became aquatic [e.g., *Eryngium*

castrense]. Typically the *isoetoide* morphology (i.e grass-like tufts) of aquatic plants mirrors the aquatic period of the pools, typically during winter and early spring. Later in the season, after water evaporation and total desiccation of pools, plants acquire a terrestrial *weedy* morphology. Not only does the morphology of these plants transform, some species are capable of switching from CAM photosynthesis during aquatic phases, to C4 photosynthesis during the dry phases (Keeley, 1999).

Symbiotic microorganisms can radically change the capacities of the host, creating symbiotic microbial associations that are fundamental for plant health (Morelli, et al 2020). Microbial inhabitants of the rhizosphere and phyllosphere (those near or on plant tissue) are considered epiphytes, whereas microbes residing within plant tissues (the endosphere), whether in leaves, roots or stems, are considered endophytes. Microbes occupying these niches can establish beneficial associations of varying specificity with their host plants (Carrell & Frank, 2014; Turner et al, 2013; Fan, et al 2020; Wiewióra & Zurek, 2021). Beyond general systemic plant health, the microbial endophytic symbionts can induce niche breadth evolution via relief from environmental stressors (Rebman, et al 2009). The microbial symbionts living within the host tissues and the host are considered to be a single entity – a 'Holobiont.' Where several plant traits are co-regulated by the associated microbiome and the plant's genome, a paradigm is emerging in which interactions between plants and their associated microbiome are a means to generate new phenotypes with increased fitness under distinct environmental conditions (Baedke, et al 2020; Trivedi et al, 2020). In this context, researching the assembly of amphibious plant microbial communities living in vernal pools can enhance ecological theory and may even lead to the development of new biotechnology. To some degree, the plant microbiome holds interesting parallels to other host microbiomes (i.e the gut microbiome) with conceptually similar questions (Muller et al, 2016).

Selection imposed by plant habitats strongly shapes the diversity and composition of the microbiota, with different plant tissues such as leaves, roots, or flowers typically harboring unique taxa (Fitzpatrick, et al 2020). The Proteobacteria usually dominate samples, particularly those of the α and β classes. Other major groups include Actinobacteria, Firmicutes, Bacteroidetes, Planctomycetes, Verrucomicrobia and Acidobacteria (Turner et al, 2013). In addition to plant habitats, environmental factors can shape microbial communities such as soil versus air environments. For example, roots and leaves impose different selection on microbiota due to both their structural differences and exposure to soil versus air, respectively (Fitzpatrick et al, 2020). Also, variation across plant host genotypes or phenotypes seems to shape microbiota and to vary in different tissue habitats within plants and among environments, modulating microbe–microbe interactions and changing host fitness. (Fitzpatrick et al, 2020). Nevertheless, sometimes bacterial communities on leaves and roots can be surprisingly similar (Bai et al, 2015; Van der Heijden et al, 2016). After plant habitat, variation in the

abiotic and biotic environment can exert large direct and indirect effects on plant associated microbiota (Fitzpatrick, et al 2020).

Climate-driven geographical variation can also drive the composition of a microbiome (Oono, et al 2017; Harrison & Griffin, 2019), and as global change advances environmental variation, both abiotic and biotic, this variation can indirectly shape plant microbiota through plant responses (Suryanarayanan & Shaanker, 2021; Ware, et al 2021). North America has undergone a large-scale non-native plant invasion over the past 150 years. Most of the grasses that now cover the lands are not native. For example, since the 1800s, the grasslands in the San Joaquin Valley, California have been used for cattle or sheep grazing, followed by agricultural activity and urbanization in recent years. These changes are accompanied by human made surface waters convey, store, and redistribute hydrologic flows between vernal pools into ephemeral drainages that ultimately flow out of the region (Vollmar, 2002). With the natural landscape changed and vernal pools eliminated by agricultural and urban development, the influence of anthropogenic activities over the plant microbial associated communities remain unaddressed.
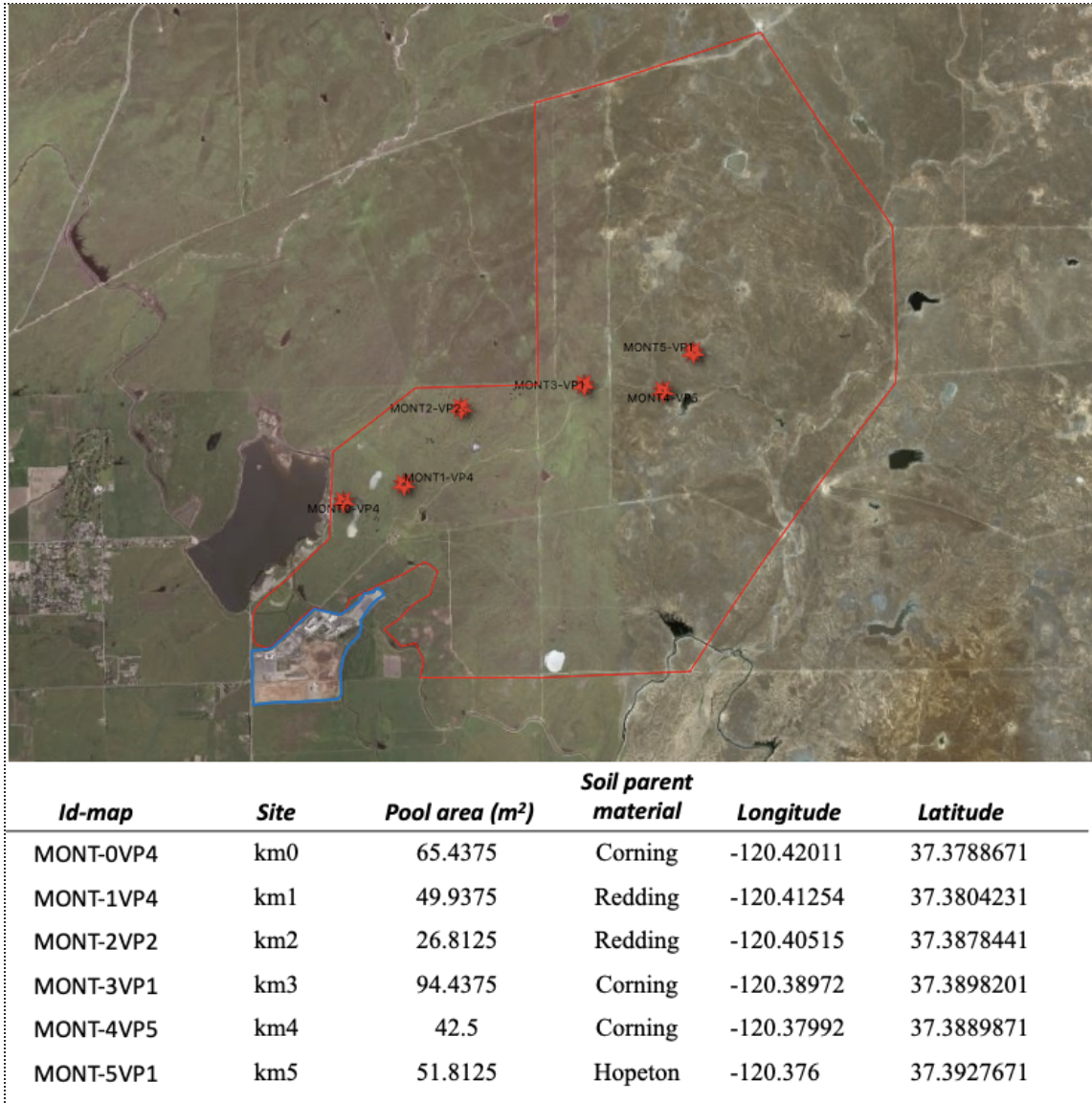
In this study we focused on the plant species Eryngium castrense, which is a vernal pool specialist, presenting an amphibious behaviour. This plant species is endemic of the vernal pools in California Central Valley, with several members of this genus in other regions of California USA and Baja California in Mexico. We are aiming to analyse the endophytic bacterial diversity and community composition of diffrent specimens and to explore the anthropogenic influence across a transect covering areas with major to minor anthropogenic influence. We hypothesize that the plant-associated microbiome is shaped by interactions among the plant host and with the surrounding environment. We ask specific questions based on our hypothesis. Given the structural differences of the plant we ask if the habitat within the host matter for microbial community assembly; How does the microbiome from roots and shoots compare? Is there any significant difference? Considering the amphibious behavior and the importance of the exposure to sun or air/water or other features of the environment we ask, How does the morphology of the amphibious host plant play a role in species assembly? Are the endophytic taxa defined by the aquatic or terrestrial stages of the plant host? In parallel we explore the importance of the heterogeneity of the landscape. Are there differences in diversity and composition of the endophytic microbial taxa associated with different vernal pool plants in the same area? Is the core community across plant specimens detectable? Finally, given the Anthropogenic influence Are there any signs of variation from vernal pool plants closer to the urban area in comparison with those inhabiting vernal pools with moderate human influence? How does endophytic microbial diversity correlate across an urban-rural gradient?

Methods

Study area

The study area is located in the heart of the San Joaquin Valley, a flat open area comprising 250 miles long and 50 miles wide, flanked on the east by the high Sierra Nevada and on the west by the Coast Range mountains. The climate of the region is Mediterranean characterized by cool, wet winters and hot, dry summers; rainfall annual averages range from 230-380 mm with 90% of the precipitation occurring from November to April. The western and eastern boundaries of the region delimit a distinct topographic and biogeographic unit of undulating terrain topography from above the historic San Joaquin River floodplain to the base of the Sierra Nevada foothills. This area supports the largest block of unfragmented vernal pool habitat remaining in California, characterized by low slope basins with undulating *mima mound* topography that typically support a high density of vernal pools.

The UC Merced Vernal Pools and Grassland Reserve is the 39th reserve in the UC Natural Reserve system. This portion of moderate anthropogenic impact encompasses 6,500 acres and is home to several of native plant species adapted to the climatic extreme conditions (Fig 9). An estimate of 6,202 vernal pools is contained, with 32 large vernal pools, 7 playa pools, 84 swale wetlands, and mima mound topography. The Reserve was historically and is still currently used for livestock grazing and contains a number of artificial stock ponds that capture and retain water for longer periods than the vernal pool complexes dispersed throughout the site. Other landscape alterations including fences, dirt roads, berms, and other water source developments that have occurred over the years are part of the ranching operations. Initial road and stock pond development likely occurred between 1918-1948 (University of California, 2018); today, the area is part of a fast growing urban settlement and the main campus of the University of California, Merced is adjacent to the buffer zone of the reserve, where some restored vernal pools complexes are present.

Id-map	Site	Pool area (m²)	Soil parent material	Longitude	Latitude
MONT-0VP4	km0	65.4375	Corning	-120.42011	37.3788671
MONT-1VP4	km1	49.9375	Redding	-120.41254	37.3804231
MONT-2VP2	km2	26.8125	Redding	-120.40515	37.3878441
MONT-3VP1	km3	94.4375	Corning	-120.38972	37.3898201
MONT-4VP5	km4	42.5	Corning	-120.37992	37.3889871
MONT-5VP1	km5	51.8125	Hopeton	-120.376	37.3927671

Fig 9. Map of the UC-Merced Vernal pools and Grassland Reserve (red polygon) adjacent to UC Merced campus (blue polygon), and landscape properties of the sampling sites.

E. castrense is a plant species characterized by its amphibious life cycle, during the first months of development the plant resembles aquatic grasses (isoetoide morphology Fig 10a), erected over the vernal pool water surface, and in March-April becomes a terrestrial spiny-weedy plant (Fig 10b), flowering throughout the hot summer. This plant species has well differentiated tissues –roots and shoots, and differentiated phenotype as a result of the metamorphosis from aquatic to a terrestrial, according to the flooding and desiccation conditions. Colloquially known as coyote thistle, *E. castrense* is a member of the carrot family 'Apiaceae'. They are glabrous ascending herbaceous plants,

multi-branched and spiny, with lanceolate leaves, spiky on the edges. The inflorescence size ranges between 8 to 15 mm and the sepals are 3mm lanceolated with sharp spines, the flowers have small oblanceolate petals, light blue to light purple colored (Baldwin & Goldman, 2012). This plant species is abundant inside the limits of UC Merced's Vernal pools and grassland reserve.
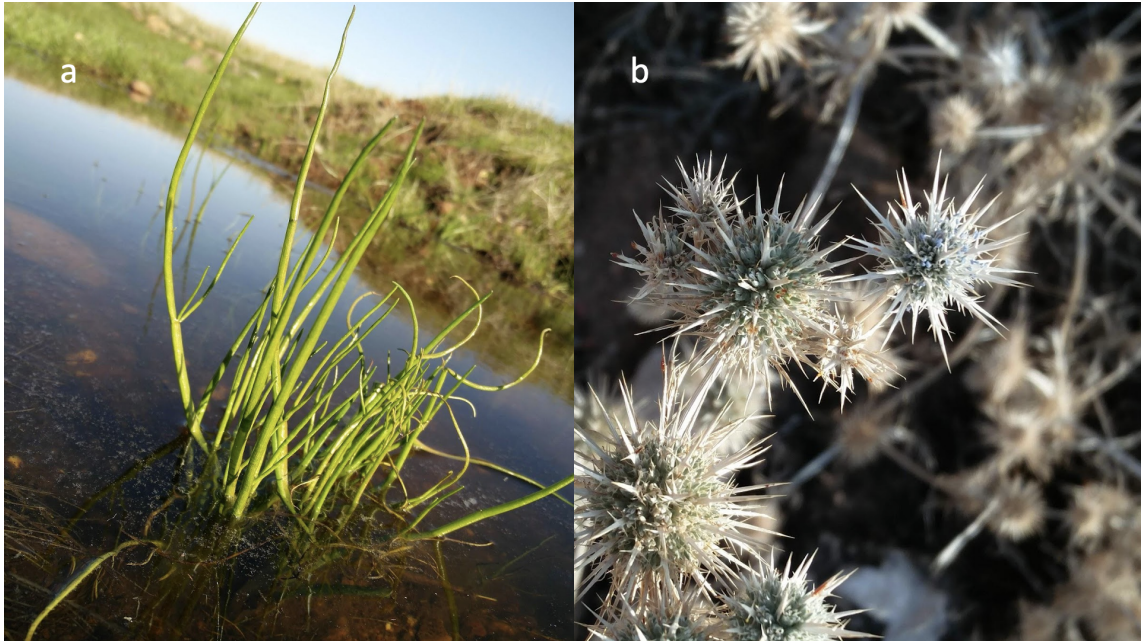
Fig 10. *Eryngium castrense* Jeps. is a California native plant, specialized to live under aquatic and desiccated stressed environments, it grows in Mediterranean temporary wetlands –vernal pools: a) aquatic morphology - Isoetoide; b) terrestrial morphology - Weedy

Sampling
Sample collections of specimens of the plant *Eryngium castrense* were at five vernal pools corresponding with specific sites. Sites were selected across a transect of 5km starting from UC Merced campus boundary (zero Kilometers= KM0), to five kilometers in direction to east, each site determined by a kilometer (KM1, KM2, KM3, KM4, KM5). Collections were done in two seasons, during the aqua-period occurred in winter 2018, comprising 20 specimens (four individuals per pool); followed by a collection of 25 specimens (five individuals per pool) in late spring 2018, for a total of 45 specimens; this sampling comprised the root and the shoot for separate and the aquatic to terrestrial morphology of the plant speciements.

Sterilization
Sterilization was performed at the symbiosis laboratory following previous methodologies (see Guzman, et al 2020), following a mechanical procedure: two rinses with distilled water for two minutes each, to separate from the biological sample the soil aggregates.

Ethanol 75% one rinse 1 minute ; Milli Q water one rinse 2 minutes, Bleach 5% 1 minute, and Milli Q water one rinse 5 minutes. After the sterilization, roots and shoots were separated with an autoclaved blade, and pulverized using liquid nitrogen and a mortar. From the pulverized plant tissue, 0.2g were measured and processed with Qigen PowerSoil extraction kit, a total of 95 samples were prepared for sequencing.

Library preparation

Previous to PCR amplification and library preparation, extracted DNA aliquots were analysed for concentration and purity in NanoDrop. PCR mix was prepared for 50ul per sample, as follow: water 31.8 ul; Buffer1x 31.8ul, dNTP(0.2uM) 1ul, 926R_Adapter (0.4uM) 2ul, PNA chloroplast (1.2uM) 0.6ul, PNA mitochondria (1.2uM) 0.6ul, BSA (0.2 mg/ml) 1ul, Taq(0.1 U/ul) 1ul, 515F_Barcode primers (10uM) 2ul, DNA template 5ul. Barcoded primers targeting the 16s rRNA V4-V6 coding regions were used for the analysis of the bacterial endophytes (Quince, et al 2011; Apprill, et al 2015; Parada, et al 2016).Termocycler was programed as follows: 94°C for 3 min; 35 cycles at 94°C for 45 sec, 78°C for 10 sec, 50°C for 30 sec, 72°C for 1 min, 72°C for 10 min and 4°C. Blockers for mitochondria and chloroplast (Lundberg et al, 2013) were added to each PCR master mix. Final amplification products were analyzed for quality using QuBit. Sequencing was achieved on the illumina miseq platform at UC Davis Genome Center.

Analysis

Data analysis started after receiving the sequences demultiplexed in *.fastq* files from UC Davis Genome Center. The platform Qiime2 (v.2021.4) was used for the analysis, and R studio (v.1.3.1093) packages Qiime2R-v0.99.1, Phyloseq-v1.34.00, ggplot-v3.3.3 and vegan 2.5-7 for additional analysis and figures. After demultiplexing, two samples with zero sequence counts were dropped from the analysis, DADA2 was used as quality control to filter chimeric reads from the analysis (Callahan et al, 2016), samples varied greatly in amplicon sequence variants abundance, with the majority of samples reaching the asymptote at ~400 reads (Fig 11). Samples were rarefied to 500 sequence reads; low sequence reads were found in four samples, corresponding to terrestrial samples (weedy shoots) from the sites KM4 and KM5, these samples were dropped from the main results. Chloroplast and mitochondria sequences were filtered for the diversity analysis. Richness and abundances were normalized for relative abundances at phylum level and for the alpha diversity analysis.
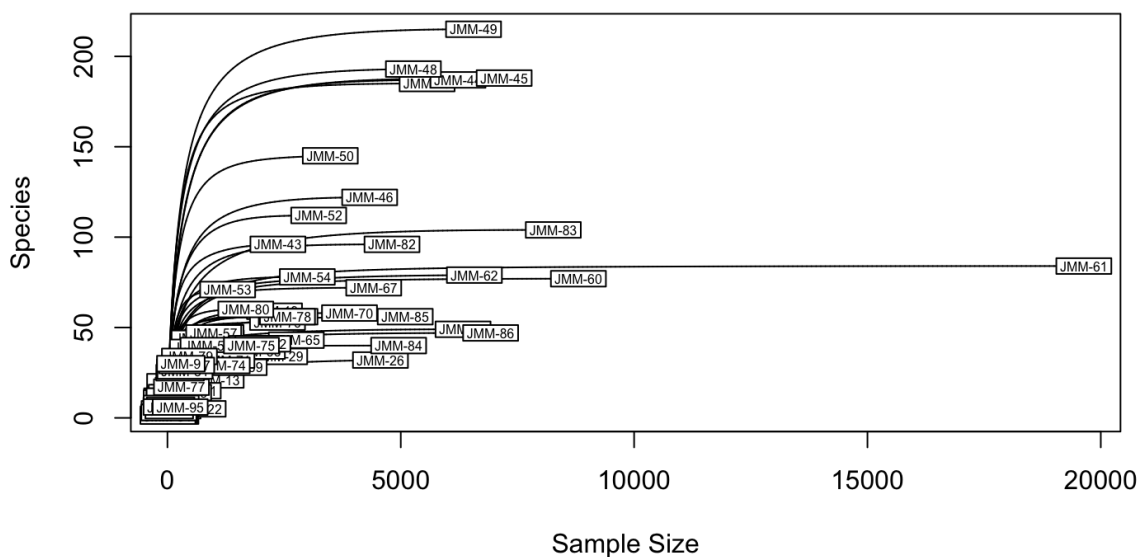
Fig 11. Amplicon sequence variances (species) accumulation curve.
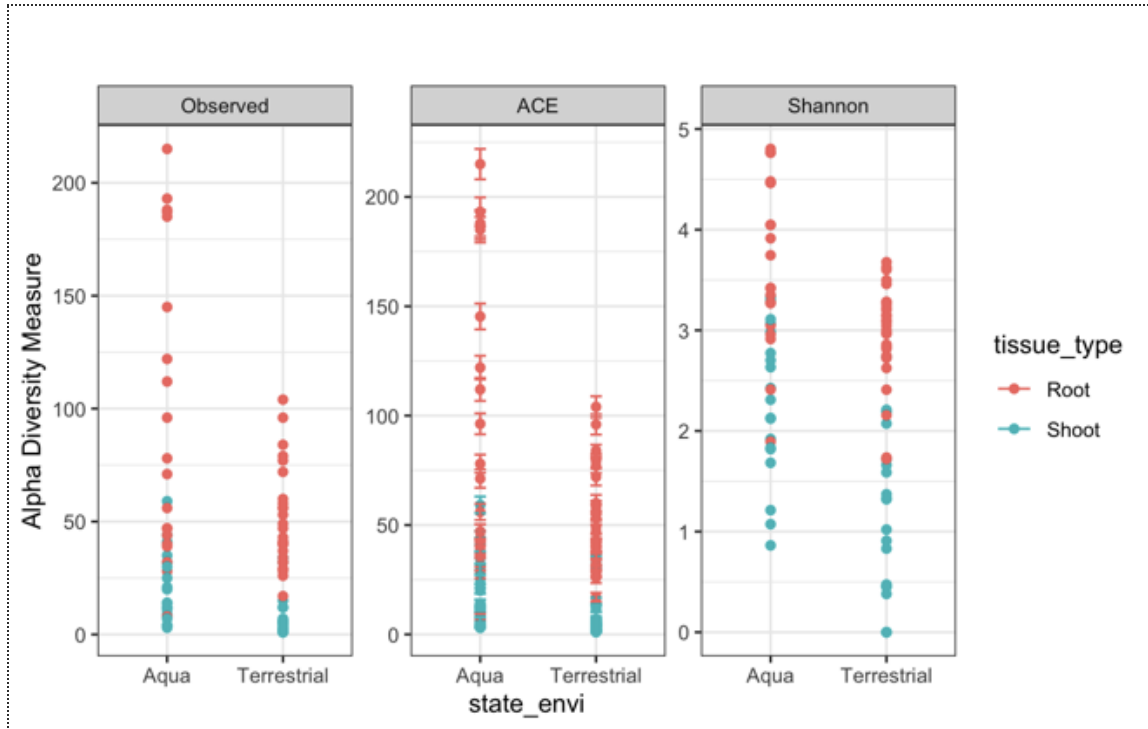
Diversity measures

Richness, abundances and evenness were analysed by tissue type (roots and shoots) and the plant morphology, aquatic and terrestrial. For the analyses a *sampling depth* of 500 (rarefied) was chosen in order to preserve as many samples as possible, specifically samples from the shoots of the weedy morphology (terrestrial form) with low sequence reads. In parallel we also analyzed some diversity under a sampling depth diffrent than 10 and 4000 to contrast. We focused on diversity at ecological niches: a) habitat/tissue type: roots and shoots and, b) plant host morphology: isoetoide or weedy plant. Additionally, diversity by site was analyzed to understand the anthropogenic influence. Beta diversity and community composition was analyzed using unweighted-unifrac diversity, comprising abundances and phylogenetic distances as a metric of disimilarity between samples.

Results

Microbial composition and diversity across plant tissues, and aquatic vs. terrestrial morphologies

Roots from the aquatic and terrestrial morphology allocated higher relative taxa abundances when contrasted with shoots, and diversity differences were supported by alpha diversity examinations between roots and shoots showing significant differences (Kruskal-Wallis Observed-Richness H=11.34,*P*=0.0007;Shannon H=6.88,*P*=0.008; ACE H=48.3472,*P*=3.5705e-12). Overall the aquatic morphology of the plant (isoetoide morphology) presented higher abundances, with Shannon diversity scores –which combines evenness and richness, revealed higher diversity in roots from the aquatic

morphology of *E. castrense* (Fig 12).

Fig 12. Alpha diversity measurements for bacterial endophytes associated with *Eryngium castrense*. Diversity scores are on relative abundances by sample.

Differences in abundances were clearly detectable at phylum level across morphologies, and taxa members from Bacteroidetes and Proteobacteria dominated in the study. Comparisons between plant habitats isoetoide-shoots, isoetoide-roots, weedy-shoots and weedy-roots showed differences in phyla abundances, Acidobacteria were more abundant in both shoots and roots from isoetoide morphology, Firmicutes and Actinobacteria were mostly present in weedy-roots. Important presence of Cyanobacteria in shoots might be an artifact given by the presence of chloroplasts in the shoots, as well as higher proportions of unknown taxa; other phylums such as Terenicutes and Verrucomicrobia were present in all sample habitats (Fig 13). At higher taxa resolution, dominant abundances belong to the class Alphaproteobacteria, Betaproteobacteria, Flavobacteriia, Gammaproteobacteria, Saprospirae [subPhylum],
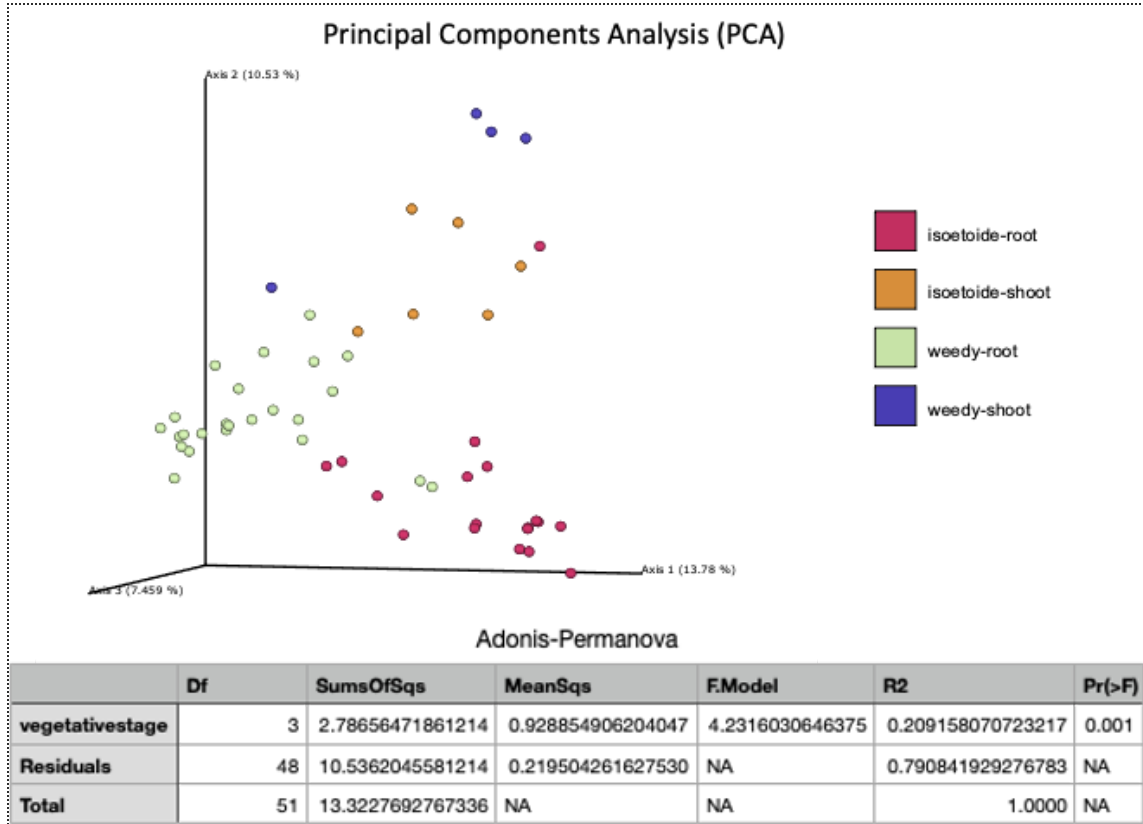
Sphingobacteria.
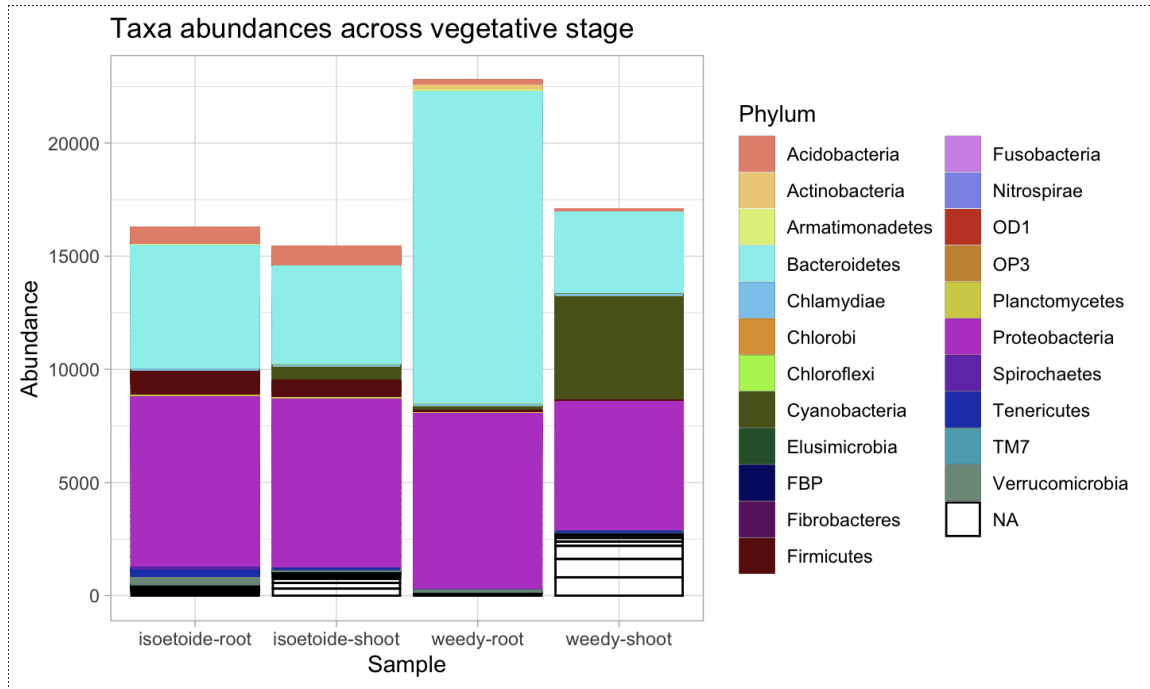
Fig 13. Microbial taxa comparison between isoetoide morphology and weedy morphology, abundances at phylum level.

Variations in community composition
Microbial community composition was examined in order to determine the potential endophytic communities within host habitat and the morphological stage of the host, aquatic or terrestrial. Disimilarity by sample analyzed with beta-diversity metrics unweighted-unifrac, and ANOSIM analysis demonstrated differences between root and shoots, and grouping was defined also by aquatic or terrestrial conditions. As reflected in the ordination analysis samples projected one community defined by roots of the terrestrial morphology (weedy community), roots of the aquatic morphology (isoetoide community), plus shoots combining the terrestrial morphology (the aerial part of the plant) and the aquatic morphology (tissues in contact with the water column), three communities defined by the plant compartments and the external environment (Fig 14).

Principal Components Analysis (PCA)

Adonis-Permanova

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
vegetativestage	3	2.78656471861214	0.928854906204047	4.2316030646375	0.209158070723217	0.001
Residuals	48	10.5362045581214	0.219504261627530	NA	0.790841929276783	NA
Total	51	13.3227692767336	NA	NA	1.0000	NA

Fig 14. Principal Components Analysis (PCA) and Adonis-Permanova significance, showing sample groups (communities) of bacterial endophytes in *E. castrense*.
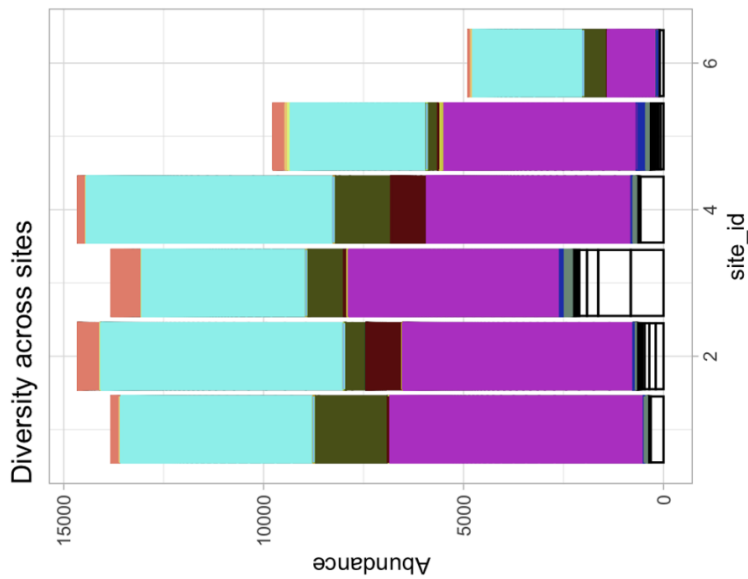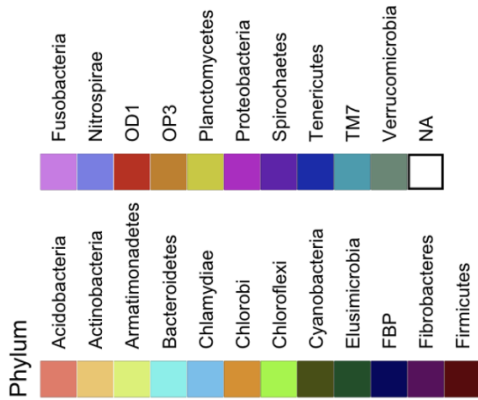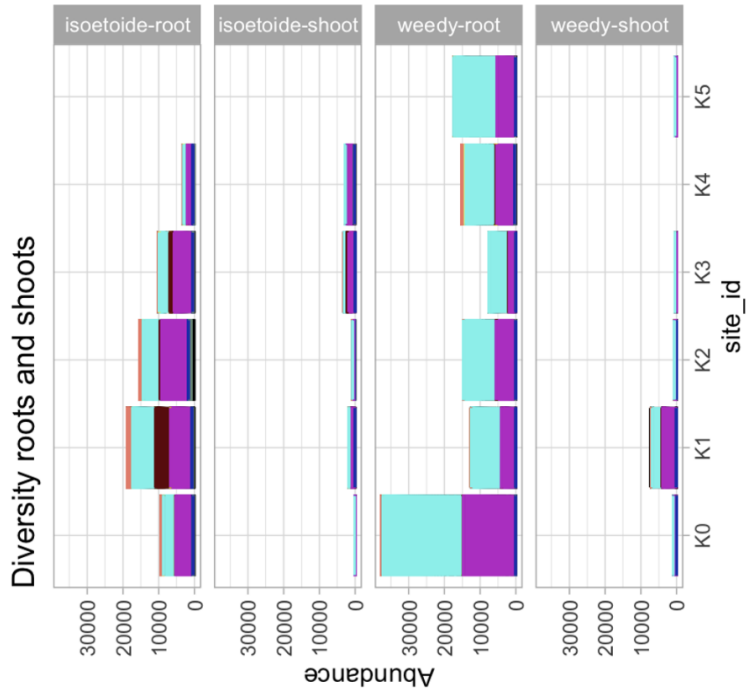
Microbial composition and diversity across sites and the Anthropogenic influence transect

At the phylum level, Bacteroidetes, Protobacteria, Acidobacteria, Cyanobacteria (and in lower proportions, Verrucomicrobia and Tenericutes) characterized the microbiome of *E. castranse* across sites. In the case of the sites K1 and K3, the phylum Firmicutes was abundant. K2 presented several unknown taxa. The only significant differences in alpha diversity across sites were for the K5 site when compared with K2, K3 and K4 (Table 5). Further analysis of each habitat- plant compartment showed in isoetoide roots a gradual decrease in abundances at phylum level across sites. With isoetoide roots more diverse in comparison with weedy roots, on the other hand, this habitat was dominated by just a few phyla. Isoetoide morphology roots projected an abundance decrease from site K1 to site K5 (Fig 15).

Table 5. Kruskal-Wallis significance metrics for Shannon Alpha Diversity

kruskal-wallis-pairwise-site_id

Group 1	Group 2	H	p-value	q-value
K0 (n=9)	K1 (n=12)	0.3232323232323180	0.5696715815879690	0.9308894967057020
K0 (n=9)	K2 (n=9)	0.09551656920077530	0.7572777496236270	0.9308894967057020
K0 (n=9)	K3 (n=11)	0.03607503607503300	0.8493606187801670	0.9308894967057020
K0 (n=9)	K4 (n=8)	0.0	1.0	1.0
K0 (n=9)	K5 (n=3)	3.0854700854700900	0.07899443583372190	0.29622913437645 70
K1 (n=12)	K2 (n=9)	0.3232323232323180	0.5696715815879690	0.9308894967057020
K1 (n=12)	K3 (n=11)	0.03409090909090650	0.8535135775742220	0.9308894967057020
K1 (n=12)	K4 (n=8)	0.5952380952380880	0.4404006981390060	0.9308894967057020
K1 (n=12)	K5 (n=3)	2.083333333333340	0.14891467317876100	0.446744019 53628200
K2 (n=9)	K3 (n=11)	0.03607503607503300	0.8493606187801670	0.9308894967057020
K2 (n=9)	K4 (n=8)	0.14814814814815200	0.7003113729368860	0.9308894967057020
K2 (n=9)	K5 (n=3)	3.7692307692307700	0.05220363534131460	0.261018176706573
K3 (n=11)	K4 (n=8)	0.027272727272723800	0.868830196925322	0.9308894967057020
K3 (n=11)	K5 (n=3)	4.4181818181818200	0.03555790569992090	0.261018176706573
K4 (n=8)	K5 (n=3)	4.166666666666660	0.04122683333716380	0.261018176706573

Diversity roots and shoots

Diversity across sites

Phylum

Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chlamydiae
Chlorobi
Chloroflexi
Cyanobacteria
Elusimicrobia
FBP
Fibrobacteres
Firmicutes
Fusobacteria
Nitrospirae
OD1
OP3
Planctomycetes
Proteobacteria
Spirochaetes
Tenericutes
TM7
Verrucomicrobia
NA

Fig 15. Phylum level diversity and abundance of bacterial endophytes associated with E. castrense across sites.

At higher resolution 27 taxa represented the 90 percent of sequences recorded in the study. Overall, site K0 showed high abundances of these 27 taxa, and the most abundant sequence variants belonged to the families Oxalobacteraceae, Weeksellaceae and Chitinophagaceae (Fig 16). Across the transect of five kilometers, these taxa abundances seem to vary accordly with the distance from the urbanization influence zone (K0 = UC Merced campus boundaries).
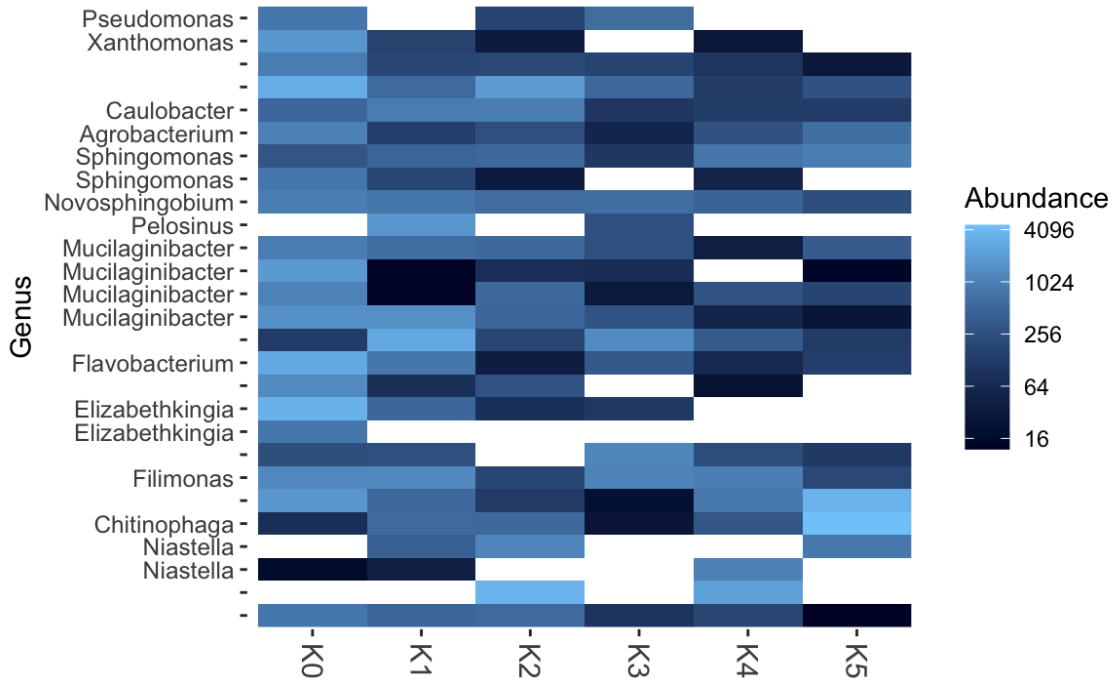
Fig 16. Most abundant bacterial endophytes taxa (genus level) for *E. castrense.* Sites (x axis) followed a transect of five kilometers from high to low anthropogenic influence.

Discussion

The microbiome from roots and shoots

The results of our analysis were consistent with other studies regarding microbiomes in other plant species. Our results suggest that roots and shoots differed consistently as a result of tissue properties, chemical processes, or the exposure to sun and air. In the case of *E. castrense*, roots communities suggest that the exposure to water saturation and soil low oxygen concentrations influenced the microbial species to assemble, despite living inside the plant tissues. Most of the abundant taxa found in the study live in environments that intersect aerobic and anaerobic habitats, usually soils or aquatic sediments, although some may be parasites or commensals. On the other hand, considering the amphibious behavior and the morphology of the host plant shoots, is likely that the exposition to the water column versus the air in a particular scenario with

contrasting conditions for the microbial composition, nevertheless our results in beta diversity ordination were not totally significant given the low quality of samples.

Rafare and sampling depth affected microbial community analysis
In this study, we highlight that roots and shoots define different endophytic bacterial communities, and in addition, the morphology isoetode and weedy differentiated root endophytes accordly to the aquatic and terrestrial environment. In the case of shoots it was complicated to address whether morphological changes given by the amphibious behaviour determine two separate communities –isoetoide shoots and weedy shoots. We attribute these deficiencies in addressing the shoots communities to PCR amplification contamination, such as the amount of sequences from chloroplast in our samples, obscuring the real diversity and abundances of the microorganisms in shoots. Samples dropped from the analysis after quality control represented a majority of the plant shoots. Such circumstances were likely to create an artifact regarding beta diversity and the ordination analysis. In addition, during the rarefare proces, low reads represented a potential bottleneck given the sampling depth selected for the analysis and the number of samples dropped for the analysis. With a sampling depth of 10 our beta diversity analysis showed four well delimited group based on sample dissimilarity with the principal component analysis, alternatively sampling depth of 4000 served to corroborate that, isoetoide roos, isoetoide shoots and, weedy roots and shoots belonged to differentiated groups (Attachments Fig 1).

Core community across sites and anthropogenic influence
The microbiome composition across sites suggests that taxa are heritable or present some degree of endemicity within the plant species E. castrense, however this statement is applicable to plants within the reserve boundaries. Similar diversity across individual plants (both roots and shoots together) could also suggest that microorganisms have the same source of transmission. Comparisons between water column and soils versus plant tissues are required in future studies to understand the transmission sources for microbial endophytes. On the other hand, in our study a discrete pattern of gradual decrease in abundances regarding those 27 most abundant taxa (Fig 8) and the decreasing microbial abundances of isoetoide roots (Fig 7) suggest the existence of an anthropogenic gradient. We correlated the total abundances against longitud (geoposition) with no significant correlation and performed a Mantel test between spatial distances and community similarity matrices explaining only the 4% based on Pearson correlation. Perhaps a large-scale study including different organisms can reveal more clearly the influence of urbanization and anthropogenic activities over the microbiome, in the case of the plant microbiome. Other plant species should be considered alongside with the amphibious plant *Eryngium castrense* to have a larger picture about the microbial endophytes within vernal pool plants.

Conclusion

We conclude roots and shoots from aquatic and terrestrial morphology in Eryngium castrense harbor specific microbial communities, with different taxa composition and diversity. The host and the external environment determine the assembly of microbial species, similar to other other microbiomes in other ecosystems. These communities are typical microbial endophytes from plants, presumably sensitive to anthropogenic activities derived from urbanization, our results also suggest gradual influence in the abundance of some bacterial taxa, given by the distance from the urban-anthropogenic influence. The interactions and mechanics underlying this microbial-plant association remains very complex and the need for future studies is still recommended, especially in such an interesting ecosystem as vernal pools and its amphibious plants. This study is the fist of its kind, in exploring the microbial symbionts of an endemic amphibious plant for the 'carrot' family.

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Attachments
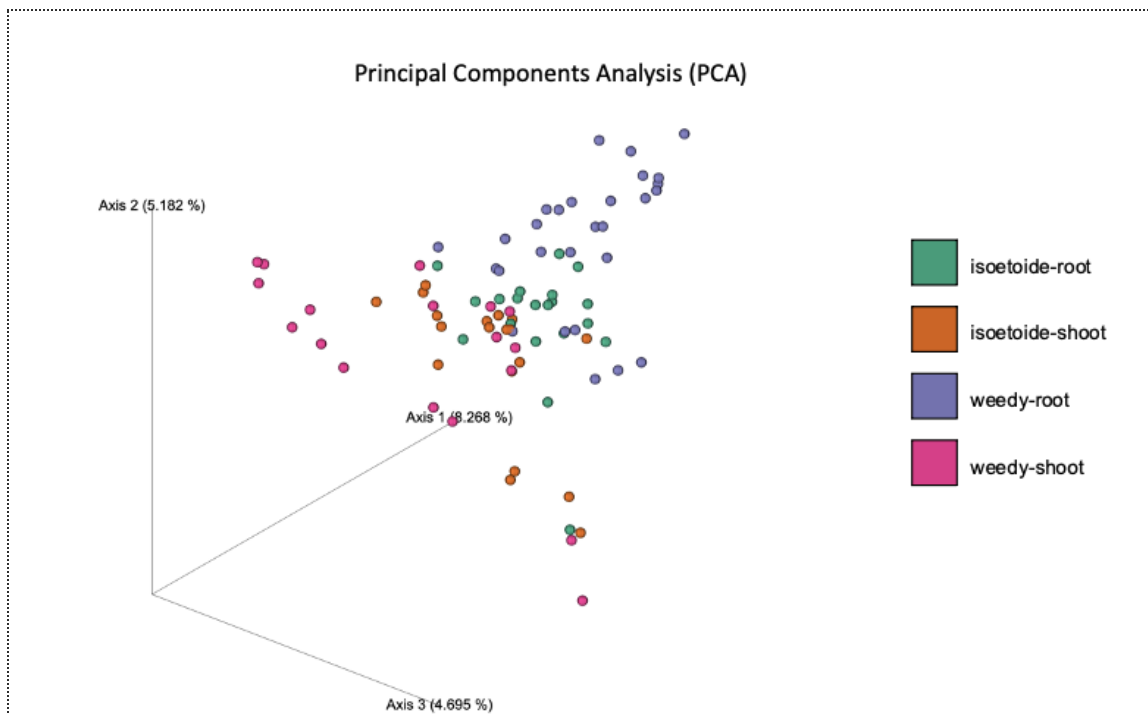
Fig 17. Ordination analysis (PCA) showing four main groups at sample depth (rarefaction) 10 sequences per sample. Given the low reads in samples from weedy-shoots at higher rarefaction differences are not detectable.