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Spatiotemporal Changes of Microbial Community Assemblages and Functions in the Subsurface

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by

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DEDICATION

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ABSTRACT

The subsurface hosts diverse microbial community assemblages and functions. These communities play an important role in biogeochemical cycling and groundwater purification. Many physicochemical factors affect microbial communities and can cause short-term or long-term perturbations. Subsurface microbes are susceptible to anthropogenic changes in the environment, which can be caused by nutrient inputs or municipal groundwater extraction. Despite the importance of the subsurface microbiome, these microbial communities are poorly characterized. This dissertation describes the characterization of spatiotemporal drivers of subsurface microbial communities through a variety of techniques that include eDNA analyses, bioinformatics, hydrochemical analyses, stable isotope geochemistry, and multivariate statistics.

Three coastal sinkholes and three coastal wells were used to characterize the spatiotemporal variation in microbial communities and hydrochemistry in the subsurface. Hydrochemistry appears to play an important role in determining the types of microbes that inhabit the different groundwater zones of a stratified sinkhole called Hospital Hole. Different groundwater zones in Hospital Hole hosted unique microbial community assemblages and estimated metabolic functions, but taxa implicated in sulfur and nitrogen cycling were identified in all zones. Seasonal patterns of microbial community assemblages and potential metabolic functions were not identified. A single physicochemical parameter was not implicated in microbial community change for the different zones. Hurricane Irma did not appear cause a large perturbation in the microbial communities in Hospital Hole, suggesting that local hydrogeology is important to subsurface microbial community changes. Similarities between the hydrochemistry and microbial communities of three coastal sinkholes and three coastal wells suggest that microbial communities from sinkholes could be used as a model for aquifer

microbial community interactions. These findings also suggest that wells may become highly sulfidic like the sinkholes if sulfate and organic material are available. Differences in the microbial communities between and within sediment, biofilm, and water column samples in three different sinkholes demonstrated subsurface heterogeneity. These communities varied in community composition and the abundance of their estimated metabolic functions.

Surface runoff into the Monte Conca cave system in Sicily, Italy caused large perturbations in the microbial communities in a sulfidic spring pool. During the dry season, the microbial community in this sulfidic spring pool was comprised primarily of sulfur oxidizing bacteria. These sulfur oxidizers could utilize sulfide present in the spring pool and the oxygenated air of the cave. Heavy rains in the wet season caused surface runoff into the cave. *Escherichia*, a fecal microbe, and *Lysinibacillus*, a soil-derived microbe, displaced the sulfur-oxidizing communities in the wet season. These taxa were likely derived when surface runoff carried these organisms from soils in the surrounding agricultural areas into the cave system. One sampling date appeared to show a transition between the wet and dry seasons when microbes from both seasons were present in the spring pool.

This dissertation identified several potential causes of spatiotemporal changes in subsurface microbial communities. These changes include the following: differences in groundwater hydrochemistry, differences in seasonal physicochemical parameters, hurricanes and tropical storms, and connectivity to the surface. Subsurface ecosystems appear to be vulnerable to anthropogenic inputs, which may contain *Escherichia* and other biological contaminants. This dissertation demonstrates the complexity of microbial communities within the subsurface and emphasizes the spatiotemporal causes of subsurface heterogeneity.

CHAPTER 1: INTRODUCTION TO SUBSURFACE MICROBIAL ECOLOGY

Microbes within the subsurface

The continental subsurface, which extends from 8 m to about 5,000 m in depth, is one of the five major habitats in which prokaryotic cells reside on Earth (Flemming and Wuertz 2019). It is estimated that anywhere from 20-80% of these cells occur in biofilms, though the microbial community within groundwater is primarily comprised of unattached cells. Biofilms are more abundant in fine-grained sediments (Griebler et al. 2002, Albrechtsen 1994) but can form in the pores and conduits of karst (e.g. Macalady et al. 2007, Pronk et al. 2009) and contain multiple species (Escudero et al. 2018). The presence and thickness of biofilms has been shown to influence porosity and permeability (Leon-Morales et al. 2004), and trap particulate matter (Coombs et al. 2010).

The microbial communities within the subsurface consist of diverse prokaryotic and eukaryotic assemblages (Magnabosco et al. 2018, Barton et al. 2007, Banks et al. 2010). Most of these microbes are chemosynthesizers and can metabolize reduced compounds (e.g. Sarbu et al. 1996, Pohlman 2011). Surface connectivity and nutrient inputs can influence whether these environments become eutrophic or oligotrophic (Pohlman 2011) and affects microbial assemblages and nutrient cycling. Surface microbes, including soil bacteria and fecal coliforms, may be brought into the subsurface by water flowing into caves, bat guano, or recharge areas (Lavoie et al. 2017, D'Angeli et al. 2017, Modra et al. 2017). Humans can impact subsurface microbial communities by throwing coins into pools of water (Mateo-Mederos et al. 2018), leaving food (Griffin et al. 2014), and installing light fixtures (St. Clair et al. 1981).

Microbes play an important role in subsurface biogeochemical cycles (Flemming and Wuertz 2019) and groundwater purification (e.g. Amano et al. 2017). Subsurface communities

can have diverse metabolic functions (Barton et al. 2007) including carbon, nitrogen, and methane cycling (Engel et al. 2003, Jones et al. 2008, Chen et al. 2009, Spilde et al. 2005). Iron and manganese deposits (Northup et al. 2007, Spilde et al. 2005), biomineralization (Busquets et al. 2014), biocorrosion (Vidal et al. 2013), speleogenesis (Engel et al. 2004, Macalady et al. 2006, Gray and Engel 2013), and the formation of some speleothems (e.g. cave pearls, helictites, pool fingers, and moon milk) within caves have been attributed to microbial processes (Hose et al. 2000, Engel et al. 2004, Northup et al. 2000, Tisato et al. 2015, Maciejewska et al. 2017, Melim et al. 2018, Macalady et al. 2007, Portillo and Gonzalez 2010, Guido et al. 2017).

There are many physicochemical factors that affect cave microbial communities. Large increases in rainfall, such as those by hurricanes and tropical storms, can alter aquifer discharge and can form a new microbial assemblage (Lakey and Krothe 1996, Pronk et al. 2006, Menning et al. 2018). Changes in physicochemistry, including those from tidal cycles, seasonal turnover events, and flow reversals, can induce changes within microbial communities (Menning et al. 2015, Garman et al. 2011, Rubelmann 2014) but only if the system is perturbed (León-Galván et al. 2009). Density gradients of physicochemistry can cause water column stratification and may create heterogeneity of microbial community abundance and structure within the different hydrochemical zones (Alcocer et al. 1999, Lin et al. 2012). Differences in physicochemistry (Gonzalez et al. 2011, Menning et al. 2014) and light penetration (Jones 1995) can create significant different in microbial communities.

Short-term (Landesman and Dighton 2011, Landesman et al. 2019, Gunnigle et al. 2017) and long-term (Lipson et al. 2004, Siboni et al. 2016, Ward et al. 2017) seasonal variation in subsurface microbial community assemblages may occur. Environmental parameters have a greater impact in inducing seasonal patterns than community interactions (Gilbert et al. 2012). Seasonal reoccurrence of physicochemical parameters can cause recurring microbial assemblages (Bosshard et al. 2000, Camacho et al. 2000, Dimitriu et al. 2008, Ward et al. 2017), but the physicochemistry can have different effects on the prokaryotic and eukaryotic

members of the community (Franklin and Mills 2003, Lehours et al. 2005, Oliverio, Aas et al. 2019).

Anthropogenic impacts

Anthropogenic processes can impact subsurface microbial assemblages and functions. Municipal groundwater extraction can accelerate subterranean seawater intrusion (Werner et al. 2013), and caves and fractures can be used as channels for intruding seawater (Calvache and Pulido-Bosch 1997, Beddows et al. 2007, Vera et al. 2012, Xu et al. 2016). Saltwater intrusion can alter the hydrochemistry of subsurface fractures and cave systems (Scharping et al. 2018) and may impact the microbial communities. Some management agencies have sought to remediate water depletion and coastal saltwater intrusion by injecting wastewater effluent to create a saltwater intrusion barrier between freshwater and saltwater aquifer regions (e.g. Fackrell et al. 2016).

Agriculture, deforestation, mining, urbanization, tourism, military activities, and wastewater injection can introduce contaminants into the subsurface (De Waele 2009, Hernández-Terrones et al. 2011, Arcega-Cabrera et al. 2014, Fackrell et al. 2016). Subsurface contaminants may include chemical contaminants, such as pesticides, pharmaceuticals, heavy metals, and fertilizers (Ekmekci 2005, Metcalfe et al. 2011), or biological contaminants, primarily fecal bacteria and enteric viruses (Paul et al. 1995, Lipp et al. 2001, Celico et al. 2004). Contaminants may be introduced via point and non-point sources. Point sources are from an identifiable source, such as sewage pipe leaks and waste storage in sinkholes (Ekmekci 2005, Katz 2012) whereas nonpoint sources are diffuse and include contaminated surface runoff and septic tanks (Alcocer et al. 1999, Ekmekci 2005). Exposed or unconfined bedrock may allow for point and non-point source contaminants to directly infiltrate into the groundwater (Lipp et al. 2001, Celico et al. 2004, Katz 2012). Fractures and tidal pumping may transport contaminants long distances (Paul et al. 1995 Katz 2012).

Government agencies have implemented a number of management practices to combat subsurface contamination. In Florida, government agencies have investigated the impaired first-magnitude springs and have implemented 13 restoration projects to limit the total daily load of nutrients to these springs (Protect and Restore Springs). The types of contaminants were examined to determine the percentages of nutrient sources to these springs. For example, the estimated percentage of nitrogen loading sources to the Weeki Wachee spring are broken down as follows: 30% from septic, 28% from turf fertilizer, 17% from farm fertilizer, 10% from livestock, 10% from atmospheric, and 5% from wastewater treatment facilities (Florida Department of Environmental Protection 2018). Concerns regarding the potential contamination of the subsurface by septic and wastewater treatment systems have caused Florida water management districts to allot money for mitigating these contaminants. This money can be used for upgrades to wastewater treatment facilities, the installation of sewage systems to homes that previously had septic systems, or by reimbursing homeowners for the installation of nitrogen removal technologies to their septic systems (Florida Department of Environmental Protection 2018, Protect and Restore Springs). Other government agencies around the world have implemented similar conversation techniques to protect the subsurface, such as the formation of the Monte Conca nature reserve (Ministero dell'ambiente e della tutela del territorio e del mare 2010).

Groundwater microbial communities are relatively stable (Farnleitner et al. 2005, Pronk et al. 2009), so these communities can be used to detect anthropogenic disturbances (Paerl et al. 2003). The types of nutrients inputted into an ecosystem can cause different species to dominate (Paerl et al. 2003). For example, excess of organic carbon can lead to the depletion dissolved oxygen, create anoxia, and can promote anaerobic microbes (Iliffe et al. 1984, Gonzalez et al. 2011) from the development of terminal electron accepting processes (see Schedel and Trüper 1980, Hoor 1981, Sublette and Silvester 1987, Lovley 1991, Lovley and Chapelle 1995). The residence times of some contaminants are of interest since they may alter

food webs and persist in the groundwater for some time (e.g. Ekmekci 2005, Cao et al.). Antibiotics have been detected in aquifers (Metcalfe et al. 2011), which may affect microbial community assemblages or cause antibiotic resistance to occur. Groundwater microbes have been implicated in bioremediation of groundwater, but the processes involved in aquifer storage and recovery programs in coastal, saline groundwater karst are not well known (see Meckenstock et al. 2015, Fackrell et al. 2016).

Rationale

Despite the importance of the subsurface microbiome, the community composition and function, causes of change, and potential for bioremediation in coastal karst regions is poorly characterized. In this study, prokaryotic communities from groundwater in a variety of coastal cave, sinkhole, and aquifer ecosystems were used to characterize the spatiotemporal drivers of community change in subsurface microbial communities. Since all study areas were near the coast, references to groundwater and aquifers throughout this dissertation are primarily referencing the saline or brackish groundwater present from coastal mixing processes. Karst caves and sinkholes were used as model systems to (1) determine whether distinct groundwater regions host similar microbial assemblages and ecological functions, (2) examine the heterogeneity of microbial communities in different subterranean locations, (3) identify the impacts of surface runoff on cave microbial communities, and (4) determine patterns of microbial assemblages and potential metabolic functions are similar in different groundwater zones. These aims help to understand the microbial diversity within the subsurface and how surface connectivity can determine microbial assemblages. This study seeks to address a general assumption from some management practices that the subsurface microbes are homogenous and to provide context to the complexity of subsurface microbial ecology.

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CHAPTER 2: MICROBIAL FUNCTION AND HYDROCHEMISTRY WITHIN A STRATIFIED ANCHIALINE SINKHOLE: A WINDOW INTO COASTAL AQUIFER INTERACTIONS

Note to reader: This chapter has been published (Davis, M.C.; Garey, J.R. Microbial Function and Hydrochemistry within a Stratified Anchialine Sinkhole: A Window into Coastal Aquifer Interactions. Water. 2018,10, 8, 972) and is used with permission from the publisher. All supplemental materials referenced herein are available online.

Abstract

Anchialine sinkholes provide insight into coastal aquifer systems and coastal mixing processes. Aquifer microbial community function is usually inferred from hydrochemical information, but there are few direct studies of microbial communities in the Floridan Aquifer. Hospital Hole is a 43 m-deep stratified sinkhole under the Weeki Wachee River, FL, with three distinct brackish layers: a hypoxic layer, a chemocline and a sulfidic anoxic layer. Illumina sequencing and bioinformatic tools were used to reconstruct metabolic functions and interactions of microbial communities in each layer. Each layer appears to originate from different parts of the coastal mixing zone and has a distinct microbial community with unique functions, which are influenced by the respective hydrochemistry. Sulfide oxidation and nitrate reduction are the most abundant functions. Syntrophy between methane oxidizers, methanogens and sulfate reducers is present. Similarities between the hydrochemistry and potential connectivity of Hospital Hole and the Floridan Aquifer coastal mixing zone suggest that microbial communities of Hospital Hole could be a surrogate for the coastal mixing zone of the aquifer in the absence of direct studies. Understanding how groundwater microbial communities

react to saltwater intrusion and nutrient flux will be useful in predicting how coastal aquifer regions might react to anthropogenic change.

Introduction

Karst landscapes make up 20 percent of the world's landmass. Human populations within Northwest Australia, the Caribbean, Eastern Europe and the Southeastern United States obtain municipal water from karst aquifers (Ford and Williams 2007). The Floridan Aquifer is one of the most productive aquifer systems in the world, with billions of liters of water withdrawn for human use daily. Studies of this and similar aquifers are important because anthropogenic activities cause a decline in overall water quality. In coastal karst systems, saltwater can infiltrate into the freshwater layer of the aquifer, and other contaminants such as nitrate from agricultural run-off or wastewater infiltration may be present (Sprinkle 1989).

Microbial processes affect hydrochemical cycling and contaminants within the groundwater. Microorganisms utilize several pathways within both the sulfur cycle and the nitrogen cycle, which are often coupled. Some sulfur-oxidizing Bacteria link sulfur oxidation with dissimilatory nitrate reduction to ammonia. Hydrogen sulfide may cause sulfur oxidation to couple with denitrification instead of dissimilatory nitrate reduction to ammonia (Burgin and Hamilton 2007). Anaerobic degradation of organic material is often limited to small organic substrates (Amon and Benner 1996). Nutrient availability regulates organic matter degradation in the aquifer, which is carried out by acetogenic bacteria, methanogens or sulfate reducers (Lovley and Chapelle 1995).

The Floridan Aquifer has two distinct layers, the Upper Floridan Aquifer and the Lower Floridan Aquifer, which are separated by at least one confining unit. The Upper Floridan Aquifer is characterized by freshwater, which can be discharged by springs or recovered from wells and utilized as a municipal water source (Sinclair 1978). Deeper regions of the Upper Floridan aquifer can contain saltwater, but in coastal regions, saltwater infiltrates the shallower and

normally freshwater regions of the Upper Floridan aquifer. Sulfate is also present within this system, either from saltwater intrusion, or from dissolution processes of gypsum within the aquifer. Phosphate and nitrate from fertilizers can infiltrate the Upper Floridan Aquifer in unconfined regions. The primary geological formations within the Upper Floridan Aquifer in the study region are the Ocala Limestone and the Avon Park Formation (Sprinkle 1989).

Most studies of the Upper Floridan Aquifer have inferred the presence of microbial communities from hydrochemical analyses. Only a few direct studies of microbial diversity within karst systems have been carried out. In the Edwards Aquifer in Texas, microbial community structure varied with hydrochemical stratification (Gray and Engel 2013), and in the Wakulla spring system in North Florida, a spatial-temporal distribution of the microbial communities was observed (Moss et al. 2011). The ability to study microbial processes within aquifers is limited by technical difficulties in sampling subsurface water. Sinkholes are prevalent within karst systems and common to West-Central Florida. These sinkholes allow access to Upper Floridan Aquifer water and may provide insight into the hydrochemical processes within the aquifer. Coastal saltwater wedge intrusion into the freshwater aquifer causes stratification that produces anchialine systems (Coke and Stoessell 2006), which can extend into coastal water-filled sinkholes. Analysis of water samples from these coastal sinkholes may provide insight into microbial processes within coastal aquifers.

This study focuses on Hospital Hole, a coastal stratified anchialine sinkhole located underneath the Weeki Wachee River in Florida. Although it may have similarities to the coastal aquifer mixing zone, it does not represent the entire Floridan Aquifer. Distinct layers of water appear to be derived from parts of the Upper Floridan Aquifer via active conduits (Sinclair 1978). Hospital Hole is easily accessible by SCUBA divers and thus offers an easily studied window into the coastal aquifer mixing zone. The proximity to the coast and the presence of saltwater suggests that this sinkhole is influenced by coastal mixing processes, as shown in Figure 2.1, which demonstrates the location of Hospital Hole in relation to the coastal mixing zone. Hospital

Hole appears similar to Jewfish Sink, an offshore sinkhole in West-Central Florida. The microbial community structure and function of this offshore sinkhole (Garman et al. 2011) appear connected to the coastal mixing zone of the Upper Floridan Aquifer, and thus, there could be similarities between the microbial community in Jewfish Sink and Hospital Hole. We hypothesize that each distinct layer of Hospital Hole hosts a distinct microbial community that fulfills an ecological function and service to the system. Studies of Hospital Hole and other coastal sinkholes can identify and characterize microbial processes that occur within the coastal mixing zone. The primary focus of this paper is to characterize the microbial communities and their function within this sinkhole to determine if these communities could be similar to those within the coastal mixing zone of the Upper Floridan Aquifer. Hydrochemical analyses were utilized in order to understand some of the nutrients utilized by the microbes within Hospital Hole.

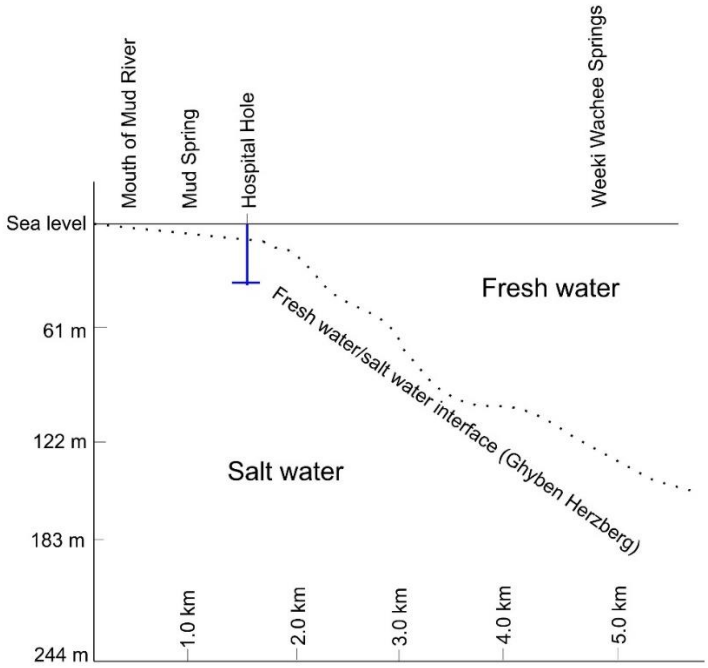

Figure 2.1. Diagram of the freshwater-saltwater interface near the study region. The blue line indicates the depth of Hospital Hole in relation to the freshwater/saltwater coastal mixing zone. Image recreated from Sinclair (1978).

Materials and Methods

Site Description

Hospital Hole is a submerged, anchialine sinkhole in the Weeki Wachee River, FL, as shown on the map in Supplemental Figure S2. It is located in West-Central Florida 1.4 km from the Gulf of Mexico. It is 44 m in maximal diameter and about 44 m in depth, depending on tide (Sinclair 1978). There are four layers within this system, as shown in Figure 2.2. The first is the surface river layer, which is no more than 3 m deep. The hypoxic layer is located between a 3-m and 21-m depth. At 21 m, there is a mixing zone between the hypoxic and anoxic layers, which contains particulate matter that looks cloudy and opaque and varies in thickness, from 6 m (Sinclair 1978) to as thin as 3 cm. This mixing zone is referred to as the chemocline throughout the paper because of the distinct hydrochemical differences (e.g., salinity, sulfide, nitrate, temperature and pH) in the hypoxic layer above to the anoxic layer below, which is outlined in the Results and Discussion sections. The bottom is the anoxic layer, which extends to the bottom of the sinkhole and is characterized by the absence of light. There is a horizontal vent at 21 m that can discharge water into Hospital Hole (Sinclair 1978).

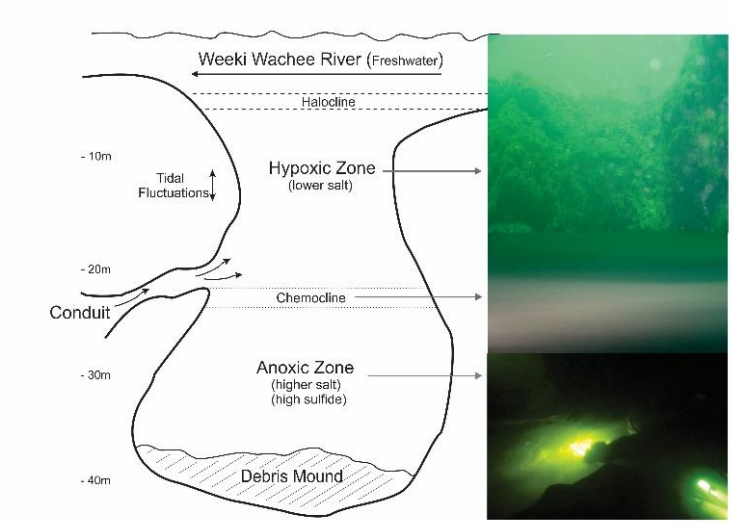

Figure 2.2. Profile view of the Hospital Hole site. Layers and boundaries within Hospital Hole are designated in this profile diagram. The direction of the river flow is southwest. Photographs taken by divers of the corresponding three layers within this study are shown.

Sampling Strategies

The overall strategy was to collect five replicate samples per layer for both hydrochemical and DNA sequencing analysis. Scientific divers collected water in five 500-mL bottles for DNA analysis and in five 125-mL glass bottles for chemical analysis (sulfide, sulfate, phosphorus, nitrite, nitrate, alkalinity and ammonia). Samples from the surface layer were collected at a depth of less than 1 m; from the hypoxic layer at 15 m; from the chemocline at roughly 21 m (depending on tide); and from the anoxic layer at 27 m. A Hydrolab DS5 accompanied divers on each dive in order to collect pH, salinity, temperature, depth and dissolved oxygen (OTT Hydromet, Loveland, CO, USA). All samples and data collections were made in March 2016, except for dissolved oxygen, which was from December 2015.

Hydrochemical Analysis

Depth, pH, temperature, conductivity, dissolved oxygen and salinity were collected at 30-second intervals using the Hydrolab DS5X (Hydromet). Water samples were collected by divers at each layer (four depths), transported on ice and analyzed in the lab. Nitrite (HACH, Loveland, CO, USA; Method 10207), nitrate (HACH; Method 8171), ammonia (HACH; Method 10205), total phosphorus (HACH; Method 8190), alkalinity (HACH; Method 10239), sulfide (HACH; Method 8131) and sulfate (HACH; Method 10248) were performed on our HACH DR 3900 spectrophotometer. We used the protocols from the manufacturer with some modifications; we used nanopure water for all necessary sample dilutions. We were unable to measure organic carbon with HACH total organic carbon kits because high chloride interfered with the spectrophotometric method available.

Biological Analysis

Water samples were collected and filtered (500 mL) through 0.22-µm filters. Illumina sequence analyses of three replicates from each layer (hypoxic, chemocline and anoxic) were carried out.

Environmental DNA was extracted from filters using a phenol/chloroform procedure and used for PCR amplification with pro341f and pro805r primers (Takahashi et al. 2014). PCR

products were purified using AmPure XP beads (Illumina, San Diego, California, USA). Illumina 300-bp paired-end sequencing was done commercially (Applied Biological Materials Inc., Richmond, BC, Canada) from three replicate DNA samples from each layer (hypoxic, chemocline, anoxic) of the sinkhole. Sequences were analyzed using Mothur software (Schloss et al. 2009) using their MiSeq standard operating procedures in which paired sequences were combined and ambiguous or chimeric sequences and those of unexpected length or poor quality removed. Sequences were compared to the Silva Version 128 database for initial identification. Bacterial and Archaeal sequences were clustered to operational taxonomic units (OTUs) of 97% identity. Bacterial and Archaeal sequences were clustered separately. For Archaea, the bootstrap cutoff for classification was 50; Bacteria followed the Mothur protocol using a cutoff of 80.

The top 100 Bacterial OTUs for each layer were identified, used as a GenBank query, and the nearest identified sequence match was used to provisionally identify each operational taxonomic unit (OTU). Due to the overlap of these OTUs in each layer, a total of 186 OTUs were investigated. Several sequences identified OTUs were given the same provisional identification. For example, OTU 1 and OTU 6 were both identified as *Sulfurimonas*, and references to *Sulfurimonas* included both OTUs. Archaea were processed similarly, but because there were far fewer Archaeal sequences, the top 25 OTUs for each layer were identified and a total of 54 OTUs investigated.

The potential metabolic function of each OTU was assigned by a review of the literature for each identified prokaryote. If a provisionally-identified OTU had more than one metabolic function (e.g., sulfur oxidizing and nitrogen reducing), it was scored in both categories. OTUs identified as fecal Bacteria, pathogens or previously found in wastewater treatment plants were categorized together. Facultative anaerobes and aerobes were categorized in the “facultative” category. Microbes listed as anaerobes were obligate. The aerobes category included obligate and microoxic microbes. Halotolerant and halophilic microbes were listed together. Sulfur

reducers included sulfur disproportionation and dissimilatory sulfate reduction functions. Sulfur oxidizers included microbes that oxidize any sulfur compounds. Nitrogen reducers included microbes that carry out denitrification, nitrogen fixation and dissimilatory nitrogen reduction. Nitrogen oxidizers included microbes that can utilize nitrification and anaerobic ammonia oxidation. Methanogens included microbes that were obligate methane producers.

The relative abundance of sequences with a particular metabolic function (sequence abundance) was calculated independently in each layer. For each layer, the number of sequences with a provisional function was divided by the total number of sequences in the layer and converted to a percent. The 186 most abundant OTUs that represented the 100 most abundant Bacterial sequences in each layer were analyzed for function and represented 75,415 sequence or 30% of the sequences in our dataset. Archaea and Bacterial sequence abundance were calculated separately. The 55 most abundant Archaeal OTUs that represented the 25 most abundant sequences in each layer were analyzed for function and represented 10,651 sequences or 16.7% of the sequences in our Archaeal dataset.

Statistical Analysis

Statistical analyses of the replicate microbial community and hydrochemical data were performed using Primer v7/Permanova + statistical software (Primer-E Ltd., Albany, New Zealand). Abiotic data were transformed (log X + 1), normalized and clustered using Euclidean distance (Menning et al. 2017). The OTU abundance of each replicate was used for the top 2000 OTUs for Bacteria and the top 200 OTUs for Archaea in our dataset for Primer-E biological analysis. Biological data were square-root transformed and clustered using Brays–Curtis similarity (Menning et al. 2007). Biological and abiotic replicate data were analyzed using principal coordinate analysis (PCoA). A Bio-Env (BEST) analysis was performed to determine relationships between biotic and abiotic datasets (Menning et al. 2017). Principal coordinate analyses were utilized in order to avoid multicollinearity, non-linearity and non-normality within

the biological and abiotic data. Rarefaction curves were produced using PAST3 software for each layer by summing across the replicates (Hammer et al. 2001).

Results

Hydrochemical analysis showed vertical stratification within Hospital Hole (Table 2.1). Alkalinity values were lowest in the surface layer and highest in the anoxic layer. The anoxic layer had higher ammonia and nitrate concentrations than the other layers. Phosphorus concentrations were highest in the surface layer. The chemocline layer had the highest concentration of nitrite. Sulfate was highest in the anoxic layer and was lower in the upper layers.

Table 2.1. Hydrochemistry of Hospital Hole.

	Alkalinity (mg/L)		Ammonia (mg/L)		Nitrate (mg/L)		Nitrite (mg/L)		Sulfate (mg/L)		Sulfide (µg/L)	
Surface	145	±30	0.601	±0.008	0.440	±0.11	0.005	±0.001	0	±0	7	±1
Hypoxic	192	±38	0.434	±0.079	0.640	±0.15	0.000	±0.001	800	±158	1	±1
Chemocline	153	±28	0.356	±0.038	0.800	±0	0.197	±0.048	1000	±58	200	±6
Anoxic	206	±23	0.659	±032	5.080	±0.15	0.000	±0.001	1000	±55	30,000	±7972

	pH		Salinity (ppt)		Temperature (°C)		Dissolved oxygen (mg/L)		Phosphorus (mg/L)	
Surface	7.91	±0.02	0.44	±0.05	23.93	±0.00	6.25	±0.02	0.50	±0.16
Hypoxic	7.47	±0.16	10.09	±3.89	24.15	±0.10	0.24	±0.53	0.16	±0.0
Chemocline	7.42	***	11.75	***	24.18	***	0.01	***	0.15	±0.20
Anoxic	7.27	±0.55	14.90	±0.7	20.5	±1.04	0	±0.00	0.28	±0.00

Asterisks (***) indicate that only one measurement was made in the chemocline layer during the 30-s sampling interval of Hydrolab, so standard deviations could not be calculated for pH, salinity, temperature, and dissolved oxygen.

Hydrolab profiles also showed vertical stratification (Table 2.1). Salinity values at the surface were <1 ppt, increased to 10.09 ppt in the hypoxic layer and 14.90 ppt in the anoxic layer. Dissolved oxygen values were the highest in the surface layer (6 mg/L), were detectable in the chemocline and hypoxic layer and decreased to 0 mg/L in the anoxic layer. The pH was highest at the surface at 7.91 and decreased to 7.47 in the hypoxic layer and was 7.27 in the anoxic layer. Water temperature was generally 24 °C but dropped to about 20 °C in the anoxic

layer. The hydrochemistry PCoA plot (Figure 2.3) shows three distinct layers: the surface layer, the chemocline/hypoxic layers and the anoxic layer.

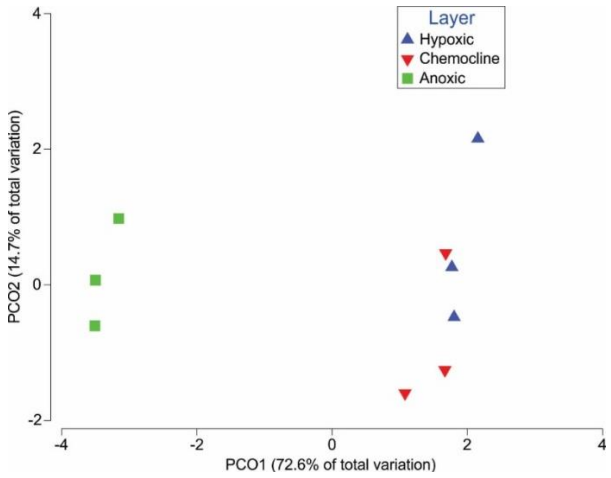

Figure 2.3. Principal coordinate analysis (PCoA) of the Hydrochemistry. This PCoA plot includes hydrochemistry data from Table 2.1. Three replicates from each layer are shown. The horizontal axis, principal coordinate 1 (PCO1), is related to pH, temperature, ammonia, salinity, nitrate and sulfide. The vertical axis, principal coordinate 2 (PCO2), is related to alkalinity, phosphorus and sulfate.

Mothur analysis of Illumina sequencing revealed 7432 distinct Bacterial OTUs representing 249,184 sequences and 2422 distinct Archaeal OTUs representing 10,651 sequences. Most of the sequences categorized by Mothur as “unknown” were found to be Archaea upon subsequent basic local alignment search tool (BLAST) analyses and so were included as Archaeal sequences in this study. Illumina sequences from the top 2000 Bacterial OTUs and the top 200 Archaeal OTUs were analyzed by principal coordinate analysis and coded by layer. The hydrochemical analysis is overlaid on the plot (Figure 2.4). Sequences from the anoxic layer clustered separately from the hypoxic layers. The chemocline separates as its own layer but appears to be similar to both the hypoxic and anoxic layers.

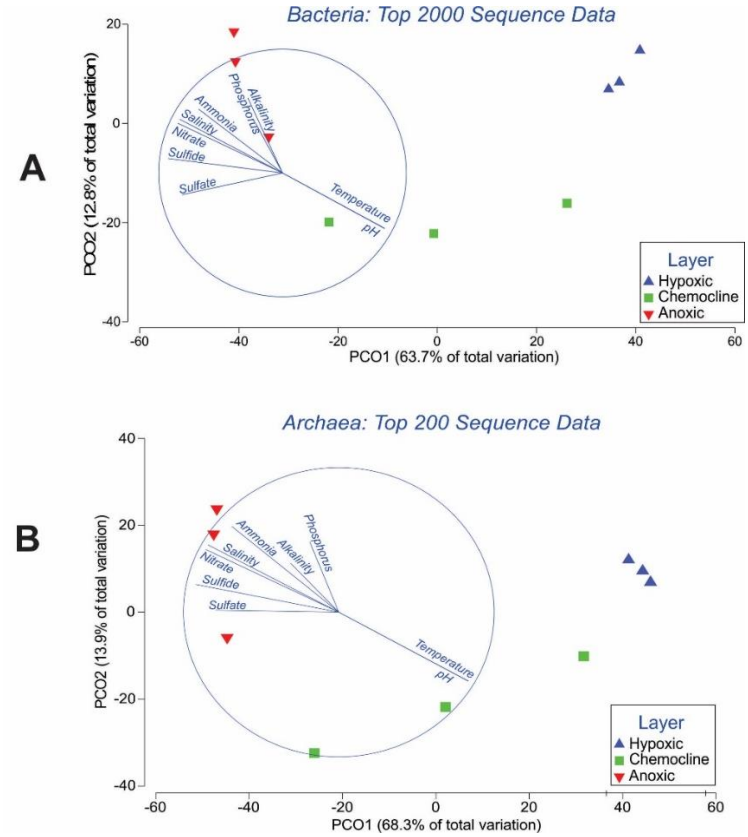

Figure 2.4. Principal coordinate analysis of the sequences. This PCoA shows the Illumina sequencing data with three replicates from each layer for (**A**) Bacteria and (**B**) Archaea. The hydrochemical data from Table 2.1 were used to produce the trend lines within the circles in the figure. These trends explain the biological variation as defined by PCO1 and PCO2.

Bioinformatic results are shown in the Supplemental Materials as a spreadsheet. These include the percent of match to closest identified genus and the known metabolic functions of the provisionally-identified genus.

The identity and function of the top 100 distinct OTUs in each layer were analyzed in detail and represent ~30% of the sequences. Archaeal sequences represented 4.1% of the total sequences identified. The 25 most abundant provisionally-identified genera in each layer are shown in Table 2.2.

Table 2.2. Abundant taxa present within Hospital Hole.

Bacteria					
Name	**Hypoxic**	**Name**	**Chemocline**	**Name**	**Anoxic**
Halioglobus	15,033	*Sulfurimonas*	10,604	*Sulfurimonas*	12,659
Escherichia	2994	*Halioglobus*	10,264	*Escherichia*	2433
Arcobacter	782	***Arcobacter***	5757	**Unknown**	1434
Sulfurimonas	703	***Alkalilimnicola***	1023	***Desulfocapsa***	1144
Prochlorococcus	182	***Emcibacter***	366	***Christensenella***	745
Oceanicola	148	Unknown	344	***Lentimicrobium***	624
Emcibacter	136	*Desulfocapsa*	275	*Arcobacter*	565
Alkalilimnicola	129	*Escherichia*	204	***Erysipelothrix***	469
Rhodobacter	109	*Lentimicrobium*	133	***Desulfosarcina***	354
Citreicella	87	*Sulfurovum*	129	***Mariniphaga***	304
Thalassobius	84	*Christensenella*	76	***Candidatus Aquiluna***	276
Unknown	56	*Desulfosarcina*	71	***Anammoximicrobium***	245
Clostridium	46	*Thalassobius*	65	***Ornatilinea***	168
Citreimonas	41	*Prochlorococcus*	51	***Claocimonetes***	158
Methylotenera	32	*Erysipelothrix*	48	***Sulfurovum***	156
Desulfocapsa	30	*Mariniphaga*	48	***Desulfotalea***	155
Hypnocyclicus	28	*Candidatus Aquiluna*	42	***Thermomarinilinea***	140
Pelobacter	26	*Dissulfuribacter*	40	***Sphingobacteriia***	139
Lentimicrobium	25	*Citreicella*	40	***Nibribacter***	129
Streptococcus	22	*Draconibacterium*	34	***Dissulfuribacter***	126
Dechloromonas	22	*Anammoximicrobium*	33	***Cryobacterium***	117
Nitrospina	18	*Cytophaga*	29	***Pelolinea***	112
Psychrobacter	17	***Lutibacter***	28	***Draconibacterium***	104
Thiobacillus	13	*Citreimonas*	28	***Cytophaga***	100
TOTAL	20,764	*TOTAL*	29,731	*TOTAL*	22,855
Archaea					
Name	**Hypoxic**	**Name**	**Chemocline**	**Name**	**Anoxic**
Halarchaeum	0	*Halarchaeum*	*9*	***Halarchaeum***	*54*
Haloparvum	2	*Haloparvum*	*0*	*Haloparvum*	*0*
Halorubrum	1	*Halorubrum*	*3*	*Halorubrum*	*16*
Methanobacterium	5	*Methanobacterium*	*5*	***Methanobacterium***	*66*
Methanobrevibacter	3	*Methanobrevibacter*	*4*	***Methanobrevibacter***	*69*
Methanocalculus	1	*Methanocalculus*	*12*	***Methanocalculus***	*115*
Methanocaldococcus	1	*Methanocaldococcus*	*5*	***Methanocaldococcus***	*55*
Methanococcus	6	*Methanococcus*	*72*	***Methanococcus***	*599*
Methanoculleus	0	*Methanoculleus*	*4*	***Methanoculleus***	*20*
Methanomicrobium	0	*Methanomicrobium*	*2*	***Methanomicrobium***	*31*
Methanoplanus	2	*Methanoplanus*	*0*	*Methanoplanus*	*0*
Methanosaeta	0	*Methanosaeta*	*2*	***Methanosaeta***	*36*
Methanosaeta	6	*Methanosaeta*	*3*	*Methanosaeta*	*1*
Methanosphaera	1	*Methanosphaera*	*1*	*Methanosphaera*	*1*
Methanospirillum	4	*Methanospirillum*	*2*	*Methanospirillum*	*0*
Methanothermococcus	3	*Methanothermococcus*	*5*	*Methanothermococcus*	*13*
Nitrosopulmilus	365	*Nitrosopulmilus*	*164*	*Nitrosopulmilus*	*6*

Roughly 20% of the Bacterial sequence abundance in each layer identified microbes that are known fecal Bacteria and/or known pathogens (Table 2.3). Facultative aerobes/anaerobes were present in all layers. Bacterial sequence abundance of obligate anaerobes was higher in the chemocline and anoxic layers than in the hypoxic layer. Sulfur oxidizers and nitrogen reducers were present in all layers, while photosynthetic Bacteria were only in the hypoxic layer (1.4%). Methane oxidizers were identified in the anoxic layer (4.7%). The Bacterial sequence abundance of sulfur reducers was highest in the anoxic layer. Microbes capable of iron

reduction and manganese oxidation were not identified in this system. Methanogens were present mainly in the anoxic layer (92.9%), and nitrifying Archaea were dominant in the hypoxic layer (90.8%).

Table 2.3. Sequence abundance of function by layer.

		Total per layer		Fecal/ WTP/ pathogen	Anaerobes	Aerobes	Facultative aerobe/ anaerobe	Halotolerant	Photosynthetic	Methane oxidizers
Bacteria	Hypoxic	20,947	Total	3956	931	651	19,037	16,715	299	39
			Percent	18.9%	4.4%	3.1%	90.9%	79.8%	1.4%	0.2%
	Chemocline	30,301	Total	6667	6576	725	22,396	23,111	71	277
			Percent	22.0%	21.7%	2.4%	73.9%	76.3%	0.2%	0.9%
	Anoxic	24,167	Total	5633	4937	666	16,363	15,033	6	1143
			Percent	23.3%	20.4%	2.8%	67.7%	62.2%	0.0%	4.7%
		Total per layer		**Sulfur reducers**	**Sulfur oxidizers**	**Nitrogen reducers**	**Nitrogen oxidizers**	**Iron reducers**	**Manganese oxidizers**	**Unknown organism**
	Hypoxic	20,947	Total	66	1847	17,104	7	8	0	59
			Percent	0.3%	8.8%	81.7%	0.0%	0.0%	0.0%	0.3%
	Chemocline	30,301	Total	502	17,616	28,137	35	42	0	407
			Percent	1.7%	58.1%	92.9%	0.1%	0.1%	0.0%	1.3%
	Anoxic	24,167	Total	2289	13,466	14,711	246	152	0	1882
			Percent	9.5%	55.7%	60.9%	1.0%	0.6%	0.0%	7.8%
		Total per layer		**Fecal/ WTP/ pathogen**	**Halotolerant**	**Aerobe**	**Anaerobe**	**Facultative**	**Methanogen**	**Sulfate reducers**
Archaea	Hypoxic	402	Total	19	373	368	32	0	32	0
			Percent	4.7%	92.8%	91.5%	8.0%	0.0%	8.0%	0.0%
	Chemocline	295	Total	20	193	166	119	9	121	0
			Percent	6.8%	65.4%	56.3%	40.3%	3.1%	41.0%	0.0%
	Anoxic	1083	Total	204	204	22	1006	54	1006	0
			Percent	18.8%	18.8%	2.0%	92.9%	5.0%	92.9%	0.0%
		Total per layer		**Nitrate reduction**	**Nitrification**	**Hydrogen sulfide formation**	**Nitrogen fixation**			
	Hypoxic	402	Total	3	365	4	10			
			Percent	0.7%	90.8%	1.0%	2.5%			
	Chemocline	295	Total	12	163	17	77			
			Percent	4.1%	55.3%	5.8%	26.1%			
	Anoxic	1083	Total	70	6	125	665			
			Percent	6.5%	0.6%	11.5%	61.4%			

Discussion

The Hypoxic Layer

The fresh river water flowing above the hypoxic layer is from Weeki Wachee Spring, several kilometers to the east. The hypoxic layer begins 2–3 m below the river surface where there is a halocline and salinity increases rapidly to 10 ppt (Table 2.1). Dissolved oxygen in the hypoxic layer varies from 0–1 mg/L and appears to be influenced by a vent that we observed

located at 22 m (Sinclair 1978) as indicated by a spike in oxygen around this depth (Table 2.1). Small contributions of dissolved oxygen from this and other vents appear to have selected for both aerobes and facultative aerobes/anaerobes (Table 2.3). The salinity and sulfate content suggests that water in the hypoxic layer is derived from the shallower part of the coastal mixing zone. Water with similar properties has been found in the Double Keyhole Spring system (Menning et al. 2017) and wells at least 160 m deep (Table 2.4), which penetrate the Avon Park Formation according to the well description by the South Florida Water Management District (SWFMD). Despite this comparison, we are currently unable to determine whether the sulfate is derived from gypsum dissolution from deeper within the aquifer or from saltwater from the Gulf of Mexico. The saline water in the hypoxic layer appears to select for halophilic microbes (Table 2.3).

Table 2.4. Comparative hydrochemistry.

Well identifier	Well depth (m)	Casing depth (m)	Distance to coast (km)	Collection Date	Sulfate (mg/L)	Alkalinity (mg/L)	Specific conductance (uS/cm)
20891	29	13	1.2	11 February 2016	6.7	211.3	1999
20890	49	41	1.2	11 February 2016	26.6	300.1	2100
20690	92	84	2.4	24 March 2016	193	146.9	4643
20124	138	135	2.8	3 February 2016	618	140.8	358
20893	159	147	4.0	11 February 2016	1100	122	3851
20939	160	154	0.8	24 March 2016	2750	113	50,246
20735	171	46	8.0	31 January 2001	2569	N/A	12,600
Hospital Hole hypoxic layer			1.4	28 March 2016	800	145	20,000
Hospital Hole anoxic layer			1.4	28 March 2016	1000	200	25,000

Although some light penetrates the hypoxic layer (Figure 2.2), photosynthetic Bacteria represented only 1.4% of the sequence abundance of the layer (Table 2.3), and eukaryotic macroalgae were not observed. This could be explained by the shady location of Hospital Hole (Supplemental Figure S2). The predominant photosynthetic Bacteria present include *Rhodobacter*, *Prochlorococcus* and *Roseobacter*. The fifth most abundant microbe within this layer is *Prochlorococcus*, which is commonly found in aerobic marine ecosystems (Table 2.2). *Rhodobacter* is an anaerobic phototroph (Supplemental Table S1), while *Roseobacter* is also commonly found in aerobic marine ecosystems. Freshwater photosynthetic Bacteria, especially

the kinds of Cyanobacteria commonly associated with Florida springs (Cowell and Botts 1994), have not yet been identified in the hypoxic layer.

The Anoxic Layer

The anoxic layer is below the particulate-containing chemocline layer and extends to the bottom of the sinkhole. Light does not penetrate into this region (Figure 2.2). The signature of this layer is increased hydrogen sulfide and salinity compared to the hypoxic layer. Because of its salinity and sulfate content, it is likely that this water originates deeper in the coastal mixing zone than the hypoxic layer. Water with similar properties has been observed in nearby coastal sinks such as Palm Sink (Garman 2010). The high concentrations of sulfide (~30 mg/L) and sulfate (~1000 mg/L) in Hospital Hole (Table 2.1) are comparable to some Bahamian blue holes (Bottrell et al. 1991). Despite comparisons of local wells to Hospital Hole (Table 2.4), we are unable to determine whether the source of the sulfate in the anoxic layer is primarily from dissolution of gypsum from deeper within the aquifer or derived from salt water from the Gulf of Mexico. Sulfur-reducing Bacteria were present in the anoxic layer (Table 2.3), and they are responsible for producing the hydrogen sulfide by utilizing carbon and sulfate (Gonzalez et al. 2011). The rarefaction analysis demonstrates that this community is more diverse than the communities within either the chemocline or hypoxic layers (Figure 2.5).

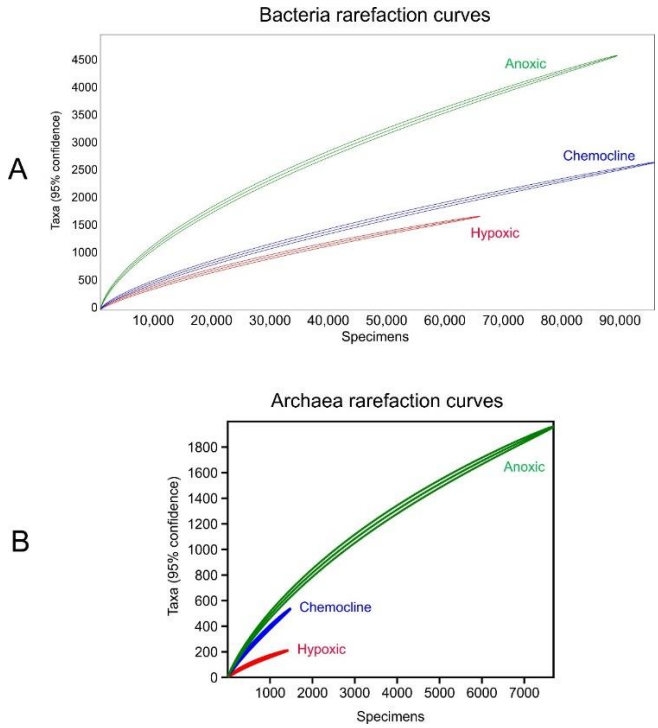

Figure 2.5. Rarefaction curves of the (A) Bacterial sequence abundance and (B) Archaeal sequence abundance for each layer are shown.

The Chemocline

The chemocline is defined as the boundary between the hypoxic and the anoxic layers. Particulate matter descends through the water column and forms a cloud-like zone at the bottom of the hypoxic layer (Figure 2.2) due to the higher density of the anoxic layer (Table 2.1). The thickness of the particulate layer likely depends on the degree of disturbance in this layer, such as from divers or from tidally-caused vertical mixing.

The hydrochemical and biological PCoA analyses each tell a unique story. Permutational multivariate analysis of variance (PERMANOVA) of the hydrochemical data from each layer shows that the chemocline is hydrochemically distinct from both the anoxic and hypoxic layers (p = 0.009). The hydrochemical PCoA suggests that the chemocline layer is most like the hypoxic layer because of its lower concentrations of alkalinity, phosphorus, ammonia and salinity (Figure 2.3). Lower concentrations of sulfide and sulfate in the chemocline further

distinguish it from the anoxic layer. PERMANOVA analyses of the microbial communities in the three layers also show that the chemocline is distinct from both the anoxic and hypoxic layers (p = 0.006). Examination of a PCoA analysis of microbial communities in each layer demonstrates that the chemocline is clearly separate from the hypoxic and anoxic layers (Figure 2.4) because it hosts a unique microbial community. The two most abundant chemocline microbes (*Sulfurimonas* and *Halioglobus*) in the chemocline were also present in the other layers, while microbes such as *Arcobacter* and *Alkalilimnicola* were found in the highest abundance within the chemocline (Table 2.2). Archaea within this layer were not unique and appeared similar to those in either the hypoxic or anoxic layers (Table 2.2). Sulfide concentrations measured in the chemocline layer (200 µg/L) were intermediate in value between the sulfide in the hypoxic layer and the anoxic layer (Table 2.1). The high variation of sulfide within the replicate samples in this layer is likely due to difficulty collecting samples from this relatively thin layer by the divers.

The chemocline in Hospital Hole demonstrates that ecological niches at interfaces host unique microbes and functions that would have been missed from investigating hydrochemistry alone.

Hospital Hole: The Ecosystem

Water bodies within the Upper Floridan Aquifer are often defined by their hydrochemistry, but their microbial communities are most often inferred from the hydrochemistry and not studied directly. We suggest that direct studies of the microbial communities in the Upper Floridan Aquifer may provide new and different views of aquifer ecosystem function. The water of Hospital Hole exists as three layers, each with its own unique hydrochemistry and biological function. Within this stratified system, there are interactions between the layers and interactions between the layers and the aquifer, although the connectivity of the system to the aquifer is not completely known. There are numerous vents that discharge water into Hospital Hole, especially in the hypoxic layer (Sinclair 1978). The largest vent brings in oxygenated water 18 m below the river surface within the hypoxic zone (Figures 1.2 and 1.3). The vents in the

hypoxic layer likely originate from the coastal mixing zone of the Upper Floridan Aquifer. Around these vents, divers have identified unique biofilms, which can be white, brown or red. Small vents at the bottom of the anoxic layer have been seen discharging 2–3 cm yellow globules, presumably containing sulfur and sulfur Bacteria. Some distinctive biofilms are not directly associated with vents. For example, the walls of the sinkhole in the deepest part of the anoxic layer hosts grey finger-like Bacterial mats similar to those observed in Jewfish Sink (Garman and Garey 2005). Other parts of the anoxic layer contain long thin filamentous mats with a white coating.

Nitrogen reduction is utilized by the majority of the analyzed microbes in this study (Table 2.3), but the specific pathways differ in each layer (Figure 2.6). Denitrification and nitrogen fixation were the most abundant types of nitrogen reduction, as suggested by the presence of ammonia in all layers (Table 2.1, Figure 2.6). Denitrification occurred in all three layers as evidenced by the abundance of *Sulfurimonas* and *Halioglobus* (Table 2.2, Figure 2.6). The high abundance of the Bacteria *Arcobacter* and the Archaea *Methanococcus* in the chemocline suggests that nitrogen fixation is a predominant function in that layer (Table 2.2, Figure 2.6). While *Arcobacter* is found in the hypoxic and anoxic layers, it is 10-fold less abundant than in the chemocline layer (Tables 1.1 and 1.2). Dissimilatory nitrate reduction to ammonia likely occurs mostly at the chemocline, due to the presence of *Alkalilimnicola* (Table 2.2, Figure 2.6). This microbe may explain why nitrite was nearly 20-times higher in the chemocline than other layers (Table 2.1). Both nitrogen fixation and dissimilatory nitrate reduction produce ammonia, but ammonia concentrations were lower in the chemocline than other layers (Table 2.1), suggesting a rapid cycling of ammonia. The OTUs representing Bacteria that can reduce nitrate to nitrite were identified in both the hypoxic and chemocline layers, but not the anoxic layer (Figure 2.6). *Prochlorococcus*, *Citreimonas* and *Alkalilimnicola* were responsible for this reduction and were within both layers (Table 2.2) suggests that this pathway is important for metabolism in the hypoxic and chemocline layers. Archaeal sequences

associated with nitrate reduction in the chemocline and anoxic layer are likely a minor component of the total Archaeal community (Table 2.3).

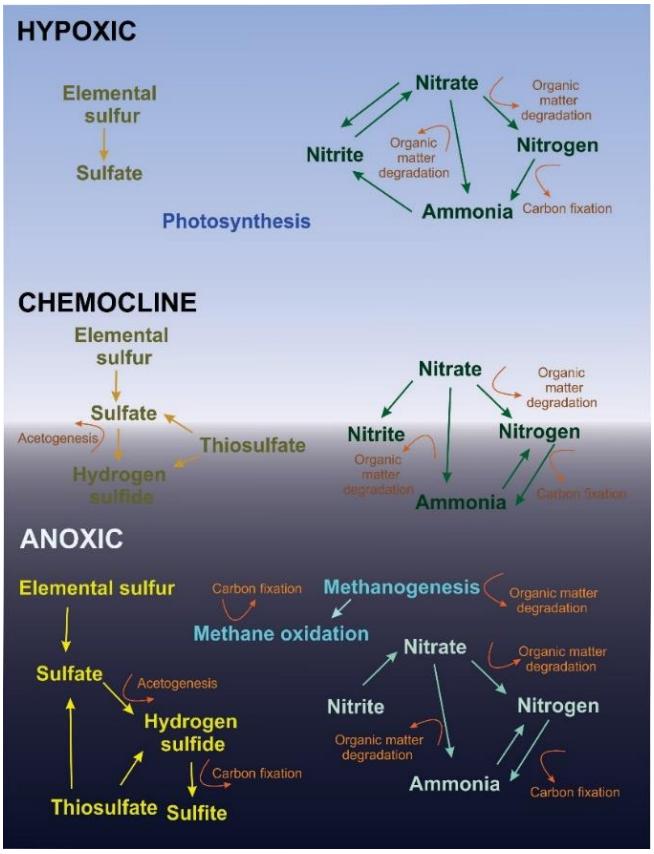

Figure 2.6. Microbial metabolism within layers of Hospital Hole. The metabolic pathways determined from the functional analysis are shown for each layer. Pathways associated with OTUs that were less than 1.0% of the sequence abundance were omitted.

The potential nitrogen oxidation pathways determined by sequence analyses differed between the hypoxic layer and the anoxic/chemocline layers. Within the hypoxic layer, nitrification was the only oxidation pathway identified, and 90.8% of the Archaeal sequence abundance was capable of nitrification, primarily by *Nitrosopumilus* (Table 2.3, Figure 2.6). The Bacteria *Nitrospira*, *Nitrospina* and *Acinetobacter* and the Archaea *Nitrosopumilus* appeared to be responsible for nitrification in the chemocline layer (Table 2.2). In combination with the nitrogen reducers, these Bacteria help complete the cycle of nitrogen metabolism within the hypoxic layer (Figure 2.6). The chemocline and anoxic layer did not have Bacteria that utilize

this type of oxidation (Figure 2.6). Instead, Bacteria in these layers utilized the anammox pathway, which is accomplished by *Anammoximicrobium* (Figure 2.6). The higher abundance of *Anammoximicrobium* compared to *Nitrospina* in this layer could be due to competition between these microbes for nitrite and ammonia (Kuenen 2007). It is likely that *Nitrospina* is dominant in the hypoxic layer due to the availability of (Kuenen 2007). Although Bacteria capable of nitrogen oxidation were identified within Hospital Hole, they were not very abundant (Table 2.3).

Nitrate values in Hospital Hole range from 0.44–5.1 mg/L while nitrate can range from 0–3.16 mg/L in the Upper Floridan Aquifer (Sprinkle 1989). We cannot completely explain why the anoxic layer has high levels of nitrate in the presence of sulfide (Table 2.1). We found evidence of nitrogen reducers in all layers, but they had a lower relative abundance within the anoxic layer, which could account for part of the higher nitrate. Other than hydrochemistry, the main difference between the anoxic layer and the hypoxic layer was that organic debris (e.g., tree branches, leaves) collected at the bottom of the sinkhole, suggesting that organic carbon is more readily available in the bottom anoxic layer. This is consistent with the extremely high levels of sulfide present in the anoxic layer. It is possible that the persistence of nitrate in the anoxic layer was related to the high sulfate concentration.

Nitrate reduction occurs throughout the Upper Floridan Aquifer, affecting both the carbonate chemistry of the groundwater and the carbon isotope composition (Sprinkle 1989) Dissimilative nitrate reduction and denitrification are important processes that aid in the removal of nitrate in the Upper Floridan Aquifer (Sprinkle 1989). Nitrogen cycling within Hospital Hole may be similar to that of the coastal mixing zone of the Upper Floridan Aquifer and provide insight into subsurface bioremediation of nonpoint nitrate pollution in coastal aquifer regions.

Sulfur oxidation and nitrogen reduction are often coupled (Burgin and Hamilton 2007), as evidenced by our sequencing analysis (Supplemental Table S1). Bacterial sulfur oxidizers represent 8.8% of the sequence abundance in the hypoxic layer and roughly 50% sequence abundance in both the chemocline and the anoxic layer (Table 2.3). This abrupt difference is

likely due to the presence of hydrogen sulfide (Burgin and Hamilton 2007), which links sulfur oxidation to dissimilatory nitrate reduction to ammonia. It has been suggested that sulfidic environments with high carbon allow for sulfur oxidizers to dominate (Burgin and Hamilton 2007). These sulfur oxidizers likely reduce nitrate to ammonia or carry out denitrification (Burgin and Hamilton 2007). The presence of hydrogen sulfide inhibits denitrification and promotes the reduction of nitrate to ammonia (Burgin and Hamilton 2007). Despite the high concentrations of hydrogen sulfide in the anoxic layer, denitrification may be present (Figure 2.6) as *Sulfurimonas* is capable of denitrification and was the most abundant microbe in the anoxic layer (Table 2.2).

Bacterial sulfur reduction is an important process within the anoxic layer (Figure 2.6). Sulfur disproportionation and sulfate reduction are associated with several Bacteria identified in the anoxic layer. For example, *Desulfocapsa* can carry out sulfate disproportionation, and other Bacteria found in the anoxic layer such as *Desulfosarcina* and *Desulfotalea* (Table 2.2) can reduce sulfur. Despite the low abundance of Bacterial sulfur reducers (Table 2.2), sulfide concentration was high within the anoxic layer (Table 2.1). According to the BEST analysis, sulfide accounts for the majority of the variance in all layers (r = 0.787). The presence of sulfur reducers likely accounts for the 30 mg/L of sulfide detected within this layer (Table 2.1). It has been reported in a similar system that Archaea contribute more to sulfur reduction than Bacteria (Garman et al. 2011), but this is not corroborated by the Archaeal functional analysis (Table 2.3), suggesting that a significant population of Archaeal sulfur reducers was present, but not detected by our sequence analysis.

One of the main processes within the aquifer is sulfur reduction (Sprinkle 1989), very much like in the Hospital Hole hypoxic layer. The low sulfide concentrations within the hypoxic layer (Table 2.1) were likely due to the relatively low abundance of Bacterial sulfur reducers (Table 2.3) and the occasional presence of dissolved oxygen. Typical sulfide levels within the Upper Floridan Aquifer are similar to those of the hypoxic layer of Hospital Hole, indicating that this layer is a component of the Upper Floridan Aquifer (Figure 2.6). While sulfide

concentrations were higher in the anoxic layer than normally found within the Upper Floridan Aquifer, the lack of available organic matter for sulfate reduction in the Upper Floridan Aquifer limits sulfate reduction (Sprinkle 1989). Thus, the anoxic layer of sinkholes like Hospital Hole could serve as a model for the response of the aquifer to eutrophication.

The methanogens (Table 2.3) appear to have syntrophic relationships with the methane oxidizers and the sulfate-reducing Bacteria (Oremland and Polcin 1982) in the anoxic layer. Sulfate-reducing Bacteria and methanogens can occur within similar anaerobic environments (Fry et al. 1997) and can develop syntrophic relationships (Coke and Stoessell 2006, Boetius et al. 2000, Thamdrup et al. 2002). Methanogens are known to be present in the Upper Floridan Aquifer (Sprinkle 1989), so the inferred interactions between methanogens, methanotrophs and sulfate-reducing Bacteria in Hospital Hole are similar to microbial interactions that occur within the Upper Floridan Aquifer (Thamdrup et al. 2002).

Despite the potential of organic matter degradation as seen in Figure 2.6, bulky organic matter (branches, leaves and twigs) was present at the bottom of the anoxic layer. We suspect that bulky organic materials are present in Hospital Hole because the microbial community cannot degrade these materials faster than their input into the system. Most anaerobes are limited to the utilization of small organic substrates (Amon and Benner 1996). Dissimilatory nitrate reduction allows Bacteria to degrade complex organic matter to carbon dioxide (Kellerman et al. 2012), but not necessarily plant material. Carbon fixation from methane oxidation (Figure 2.6) could allow these microorganisms to grow independently of the organic carbon input to the system (Leschine 1995). Another possibility is that the microbes that are able to degrade cellulose and other organic matter (Harrington et al. 2010) are not abundant within this system.

The microbial metabolism in Hospital Hole may elucidate mechanisms for remediating nitrate eutrophication within the coastal mixing zone of the Upper Floridan Aquifer. Nitrate concentrations in all layers were higher than the Florida Department of Environmental

Protection (FDEP) recommended concentrations (Boyer et al. 1999). Dissimilatory reductions of nitrate, denitrification and anammox reactions are microbially-mediated processes that transform nitrate within the groundwater systems (Burgin and Hamilton 2007). These processes have been identified in Hospital Hole and could be used to demonstrate how subsurface ecosystems respond to elevated nitrate caused by agricultural and wastewater infiltration into the aquifer.

Microbes provisionally identified as fecal/pathogenic Bacteria accounted for ~20% of the sequence abundance in all three layers of Hospital Hole (Table 2.3). *Escherichia* includes human and cattle fecal Bacteria, which can be pathogenic in humans (Supplemental Table S1). *Escherichia* could occur naturally or could originate from nearby septic tanks or faulty sewer systems and enter Hospital Hole through the subsurface. Other potentially pathogenic Bacteria, such as *Arcobacter, Clostridium* and *Vibrio*, are opportunistic pathogens that are found within aquatic systems naturally (Supplemental Table S1). *Arcobacter* is suspected to have natural reservoirs within similar aquatic systems (Supplemental Table S1). Potential animal pathogens such as *Erysipelothrix* (Supplemental Table S1) identified in Hospital Hole could originate from nearby farmlands. Studying the microbial communities in Hospital Hole or other sinkholes may aid in the identification of microbial contaminants (Blaschke et al. 2016, Farnleitner et al. 2005) that might be present in the coastal mixing zone of the Upper Floridan Aquifer.

The microbial communities within Hospital Hole are similar to other karst systems. Two genera, *Vulcanibacillus* and *Thiobacillus*, within Hospital Hole (Supplemental Table S1) were found in the Edwards Aquifer (Gray and Engel 2013). Sulfur reducers in the anoxic layer of Hospital Hole (Table 2.2), in Jewfish Sink (Garman et al. 2011) and the saltwater portion of Edwards Aquifer (Gray and Engel 2013) were all within the order Desulfobacterales. Jewfish Sink and Hospital Hole had similar microbes. *Arcobacter*, *Sulfurimonas*, *Desulfosarcina*, *Desulfofaba*, *Clostridium*, *Spirochaeata*, *Dehalococcoides*, *Cytophaga*, *Pelobacter* and *Desulfobacterium* were present within both Hospital Hole (Table 2.2) and Jewfish Sink (Garman

et al. 2011), which are 14 km apart. *Sphingomonas* was present in the Wakulla spring system in North Florida (Moss et al. 2011) and in Hospital Hole (Supplemental Table S1). Freshwater spring systems such as Wakulla springs likely do not share the same microbial communities with the coastal mixing zone, which may account for the lack of similarities between Hospital Hole. *Nitrospira* found in Hospital Hole is present in other karst systems including springs in Switzerland and Austria and the Frasassi cave system in Italy (Farnleitner et al. 2005, Macalady et al. 2006, Pronk et al. 2009). The microbial communities within Hospital Hole appear most similar to those of Jewfish Sink, suggesting a subsurface connectivity via the coastal mixing zone.

The chemocline thickness is an indicator of the amount of vertical mixing that occurs within Hospital Hole. This layer can be several centimeters to several meters thick as reported in the literature (Sinclair 1978) and observed by our divers. While tidal mixing may influence the chemocline, especially its depth and thickness, anthropogenic activity (it is a popular SCUBA diving site) is the likely the main culprit of mixing within Hospital Hole. The chemocline is likely the main interface between the hypoxic and anoxic layers, and its disruption could interfere with key microbial functions that are unique to this layer (Figure 2.6).

The bio-hydrochemistry of Hospital Hole reflects those of the coastal mixing zone of the Upper Floridan Aquifer. Aerobic respiration, nitrate reduction, sulfate reduction and methane production are typical processes found within deep aquifers, including the Upper Floridan Aquifer (Sprinkle 1989, Burgin and Hamilton 2007). The saltwater present is likely from the coastal mixing zone, and salinity increased with depth (Sacks and Tihansky 1996). Sulfate present in Hospital Hole likely originates from freshwater from the Upper Floridan Aquifer that has dissolved gypsum (Sacks and Tihansky 1996) or from seawater. Sulfide measurements in all layers (Table 2.2) were similar to other sinkholes and to the Upper Floridan Aquifer (Amon and Benner 1996, Garman 2010, Gonzalez et al. 2011). Using sinkholes as a surrogate of the coastal aquifer mixing zone does not specifically address microbe-rock matrix interactions, but it

is still able to elucidate how salinization of coastal aquifers affects some of the indigenous microbial communities.

Conclusions

The coastal karst system represented by Hospital Hole provides a unique window into the hydrochemical and biological processes that occur in the coastal regions of karst aquifers, including the Upper Floridan Aquifer. Each layer of Hospital Hole has unique hydrochemistry and microbial communities. The hydrochemistry of each layer affects the metabolic pathways that the microbial communities utilize. Sulfur oxidation and nitrogen reduction are the predominant metabolic functions and are often coupled. The hydrogen sulfide in the anoxic layer of Hospital Hole appears to be from both sulfur-reducing Bacteria and Archaea. Syntrophy of methane-oxidizers, methanogens and sulfate-reducing Bacteria appears to be present within Hospital Hole and Jewfish Sink. Similarities between microbial communities in Hospital Hole and Jewfish sink suggest subsurface connectivity between these sinkholes through the Floridan Aquifer coastal mixing zone. Studying the microbial communities in Hospital Hole and other sinkholes can expand our understanding of microbial processes, aid in the identification of microbial contaminants and predict microbial responses of karst aquifers to eutrophication in the coastal mixing zone.

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CHAPTER 3: TEMPORAL AND SPATIAL MEDIATED CHANGES IN SUBSURFACE MICROBIAL COMMUNITY ASSEMBLAGES AND FUNCTIONS

Abstract

Groundwater ecosystems can host different habitats with unique microbial assemblages and functions. Although groundwater microbes are important to subsurface processes, little is known about the drivers of change in these communities. We used Illumina sequencing and bioinformatic tools to examine whether different groundwater zones could have the same patterns of microbial community change over a two-year period. Five different groundwater zones from Hospital Hole, a stratified sinkhole in west-central Florida, were used in this study since they have been previously shown to host distinct microbial communities. Seasonal patterns of microbial community assemblages and potential metabolic functions were not identified in the sinkhole communities. Different physicochemical parameters correlated to microbial community change within each zone. Local hydrogeology appears to play an important role in subsurface microbial community change since Hurricane Irma and seasonal turnover events did not appear to cause a large perturbation in the microbial communities. Nutrient availability and local hydrogeochemistry appear to be important drivers of microbial community change in the subsurface.

Introduction

Both community interactions and hydrochemistry drive change in microbial communities (e.g. Grubisic et al. 2017, Graham et al. 2016), but physicochemistry plays a greater role in

seasonal changes in microbial community composition (Gilbert et al. 2012). These drivers can cause short-term (Landesman and Dighton 2011, Landesman et al. 2019, Gunnigle et al. 2017) and long-term (Lipson et al. 2004, Siboni et al. 2016, Ward et al. 2017) temporal changes in microbial community structure and function. Temporal variation in microbial assemblages can allow for a new consortium (Rubelmann 2014) or recurring patterns of communal assemblages (Bosshard et al. 2000, Camacho et al. 2000, Dimitriu et al. 2008) when similar physicochemical patterns occur (Bosshard et al. 2000, Ward et al. 2017).

Temporal dynamics of microbial community assemblages vary within and among systems. Some systems have community variation depending on the location of sampling (e.g. Siboni et al. 2016), whereas other systems can have relatively stable microbial communities on a short-term (e.g. Koizumi et al. 2004, Rogozin et al. 2010) or long-term basis (Dimitriu et al. 2008). There are many environmental factors that drive seasonal changes (Landesman et al. 2019), and how these factors manifest appear to be based on the type of ecosystem. For example, seasonal changes in temperature are known to drive microbial community assemblages, but this may manifest as changes in water (Siboni et al. 2016, Ward et al. 2017) or soil temperature (Strickland et al. 2015, Alster et al. 2016).

Perturbation events can cause dramatic changes within microbial communities (Alcocer et al. 1999, Lee et al. 2017, Rubelmann 2014, Menning et al. 2018, Ager et al. 2010), though little is known about how long their impacts may last. Environmental conditions can cause these perturbations, such as seasonal turnover of water columns caused by temperature changes and seasonal weather patterns (Alcocer et al. 1999, Lee et al. 2017, Rubelmann 2014, Menning et al. 2018). While these perturbations can alter the microbial communities, the assemblages may return (Ager et al. 2010) or a new consortium may form with the same functions (Rubelmann 2014). Microbial communities that incur perturbation events may become resistant to other environmental stressors (Bowen et al. 2011, Bressan et al. 2009).

This study sought to determine whether patterns of microbial assemblages and potential metabolic functions are similar in different zones within a submerged sinkhole over a two-year period. We hypothesized that seasonal microbial community changes occurred all zones. This study investigated five different adjacent zones in a stratified sinkhole that had been previously characterized (Davis and Garey 2018, see Chapter 1) to provide a holistic view of microbial community changes within the subsurface.

Materials and Methods

Site description, sampling procedures, and Hurricane Irma

Hospital Hole is a submerged, 40-m deep sinkhole in west-central Florida underneath the Weeki Wachee River, FL about 1.4 km inland (Figure 3.1). Previous analyses (Davis and Garey 2018, see Chapter 1) have indicated four unique hydrochemical zones (Figure 3.1) within this sinkhole: the surface zone (Weeki Wachee River), the hypoxic zone, the chemocline, and the anoxic zone. Subsequent observations demonstrated that a conduit at 22 m deep contributed water with a unique chemistry to the hypoxic zone, thus the fifth zone (Figure 3.1). Other conduits discharging water with similar hydrochemistry were noted in the hypoxic zone.

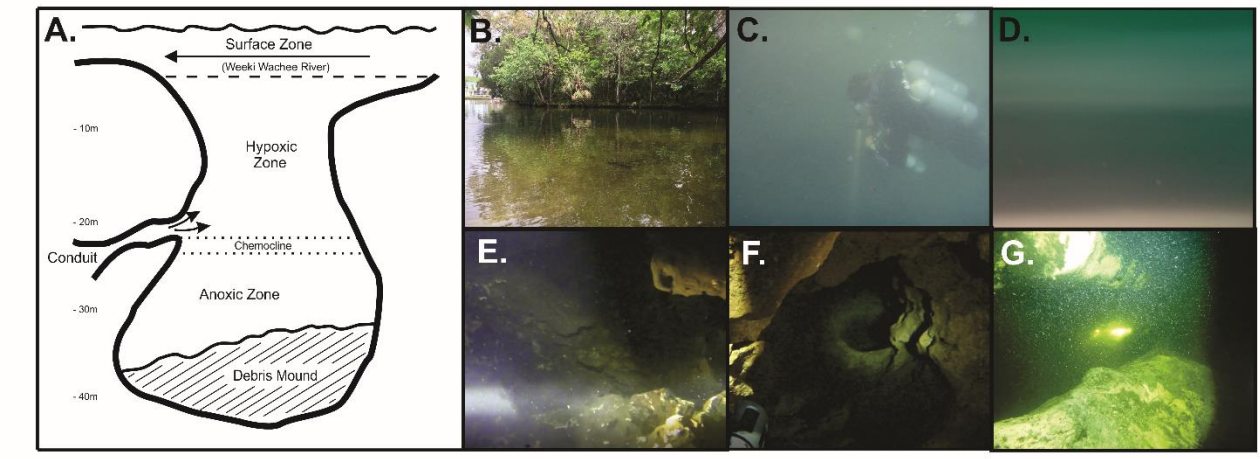

Figure 3.1. (A.) Profile view of Hospital Hole site modified from Davis and Garey (2018), as seen Chapter 1. **(B.)** The surface zone, the Weeki Wachee River, flows over the sinkhole. **(C.)** Diver in the hypoxic zone of the sinkhole, which extends from 3 m depth to roughly 22 m depth. **(D.)** The chemocline is a white, cloudy zone that can range from a couple centimeters to a

couple meters in thickness at roughly 22 m depth, depending on tide. The profile **(E.)** and internal **(F.)** view of the conduit is shown. This conduit is at roughly 21 m depth and flows water into the hypoxic and chemocline zones. **(G.)** The anoxic zone extends from underneath the chemocline at roughly 22 m deep to the bottom of the sinkhole, which is about 40 m depth.

Sampling was conducted by trained scientific cave divers under the auspices of the University of South Florida Scientific Diving Program. Five replicate samples per zone were collected in 1L bottles for biological and three replicate samples were collected in 500 mL glass bottles for hydrochemical analysis (sulfide, sulfate, phosphorus, nitrite, alkalinity, and ammonia). Three separate 50 mL conical vials were used to sample for total organic carbon (TOC) and total nitrogen (TN). A datasonde (OTT Hydromet, Loveland, CO, USA) accompanied divers on each dive to measure pH, salinity, temperature, dissolved oxygen (DO), and depth at 5-s intervals.

The sampling period encompassed two wet seasons and two dry seasons. Hospital Hole was sampled on the following dates: July 11, 2017; September 28, 2017; November 14, 2017; January 16, 2018; April 3, 2018; July 17, 2018; September 18, 2018; December 4, 2018; and April 16, 2019. Hurricane Irma impacted the study region, and the eye passed east of the study region on September 11, 2017 as a Category 1 hurricane (Cangialosi et al. 2018). The conduit at 22 m deep was not sampled prior to Hurricane Irma since it was thought to be inactive until September 28, 2017 when divers felt heavy flow from the conduit.

Hydrochemical analyses of water column samples

Water samples were collected by divers, transported on ice, and analyzed in the lab. Total organic carbon and total nitrogen were analyzed with a Shimadzu TOC-V Analyzer (Shimadzu Scientific Instruments, Kyoto, Japan). Sulfate (method 10248), sulfide (method 8131), alkalinity (method 10239), nitrite (method 10207), phosphorus (method 8190), and ammonia (method 10205) were analyzed using HACH test kits and a HACH DR 3900 spectrophotometer (HACH, Loveland, CO, USA). Protocols from the manufacturer were used. Deoxygenated deionized water was used for all necessary sample dilutions. Monthly rainfall

data for Hernando County for each month sampled was obtained from the Southwest Florida Water Management District (SWFWMD) website.

Statistical analyses of the replicate hydrochemical data were analyzed using Primer v7/Permanova+ statistical software to correlate with community structure data. Hydrochemical data was transformed (log X+1), normalized (subtracted the mean across all samples and divided by the standard deviation of the variable), and clustered using Euclidian distance similarity before correlating with microbial community data (Primer v7/Permanova+).

Microbial community analyses

Water column samples (1 L) were filtered through sterile 0.22-µm filters. Bacterial DNA was extracted aseptically using the Qiagen PowerSoil kit following the manufacturers protocol. The V4-V5 region of the 16S rRNA gene for three replicate DNA samples and was amplified using the Earth Microbiome 515F and 806R primers (Caporaso et al. 2011) adapted for Illumina MiSeq sequencing by Applied and Biological Materials, Inc. (Richmond, BC, Canada). A mock community was constructed from a six-strain mix (ATCC MSA 3000), *Thermococcus gorgonarius* (ATCC 700654D-5), and *Methanococcus maripaludis* (ATCC 43000D-5). Sequences from the mock community samples were analyzed and processed through mothur separately (Schloss et al. 2009) to calculate the sequence error rate. A reference dataset was constructed from the strain information using sequences from Genbank.

Sequencing data for each zone was run separately through mothur software (Schloss et al. 2009) to assemble paired-end reads and to remove ambiguous sequences, sequences greater than 310 base pairs in length, and archaeal, eukaryotic, mitochondrial, chloroplast, and unidentified sequences. Mothur was used to eliminate chimeras using the VSEARCH algorithm (Rognes et al. 2016) and to create operational taxonomic units (OTUs, ≥97% similarity) using the OptiClust algorithm (Westcott and Schloss 2017). Any OTU with less than 20 sequences were removed from subsequent analyses to avoid potential artifacts caused by rare OTUs (Brown et al. 2015). Rarefaction curves were produced using mothur (Schloss et al. 2009). The

average abundance of each OTU from each date was used for community structure analyses with Primer v7/Permanova+ statistical software. Principal coordinate analyses (PCO) were used on data that was clustered using Bray-Curtis similarity. Community similarity was determined using the CLUSTER analyses in Primer v7/Permanova+.

The 200 most abundant bacterial OTUs from each zone were investigated for provisional taxonomic identification and functional analyses. The OTUs that were not identified to the genus level by the Ribosomal Database Project (RDP) version 16 reference file (Cole et al. 2014) using mothur (Schloss et al. 2009) were provisionally identified using the nearest identified sequence match from a GenBank query. Those that were at least 80% similar to taxonomically identified sequences in Genbank or RDP were designated as “unidentified”. Despite a 97% cut-off for OTU clustering, some OTUs have the same provisional identification, which were combined under the same identification.

The potential metabolic function of each OTU was assigned by a review of the literature for each identified prokaryote. These potential functions include the metabolism of sulfur, nitrogen, carbon, manganese, and iron cycling. To reduce the complexity of the function data, some of these functions were categorized together: microbes capable of sulfur disproportionation or dissimilatory sulfate reduction are considered sulfur reducers; sulfur oxidizers include all microbes that can oxidize sulfur compounds; nitrogen reducers include microbes that carry out denitrification, reduction of nitrate to nitrite, reduction of nitrite to ammonia, dissimilatory nitrate reduction to ammonia (DNRA), and nitrogen fixation; and nitrogen oxidizers include microbes that can utilize nitrification and anaerobic ammonia oxidation.

The relative abundance of sequences with metabolic functions (sequence abundance) was calculated independently in each zone by date. For each date, the number of sequences with a provisional function was divided by the total number of sequences in each date and

converted to a percent. The top 200 OTUs were used since they encompass between 60% and 85% of the total sequence abundance.

Results

Hydrochemical variation within the zones of Hospital Hole were evident (Table 3.1). Large ranges of concentrations of sulfide in the anoxic (12,456-54,886 µg/L) and chemocline (11-14,079 µg/L), sulfate in the surface (8-529 mg/L), and nitrite within the hypoxic (0.00-0.16 mg/L) zones occurred during this study period. Rainfall was not abnormally high after Hurricane Irma, and sinkhole hydrochemistry does not reflect a drastic change before and after the hurricane (Table 3.1).

Mothur analyses and OTU clustering of the Illumina sequences revealed a total of 22,487 OTUs representing 5,217,879 bacterial sequences for all five zones analyzed over the two-year study. The sequencing error rate for this dataset was on average 0.09%. Only the surface zone showed clear seasonal patterns when microbial community structure was analyzed by PCO and coded by season (Figure 3.2). Communities within each zone were at 5% - 60% similar over the study period, with the least seasonal similarity in the chemocline (5%) and the greatest seasonal similarities in the surface (60%) and anoxic (40%) zones. All zones showed different patterns of community structure (Figure 3.2). The identity and function of the 200 most abundant bacterial OTUs in each zone, representing a total of 1,000 OTUs, were analyzed in detail and represent 60%-85% of the total sequence abundance in each zone. A condensed, literature-based analysis of the potential microbial community is shown in Table 3.2.

Table 3.1. Hydrochemistry of Hospital Hole.

Region	Phyisochemical parameters	Units	Jul-17	Sep-17	Nov-17	Jan-18	Apr-18	Jul-18	Sep-18	Dec-18	Apr-19
All	*Rainfall*	*cm*	17.6	25.6	6.0	11.2	13.1	27.3	11.2	24.4	8.3
Surface	*Phosphorus*	*mg/L*	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.3
	Ammonia	*mg/L*	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00
	Nitrite	*mg/L*	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Sulfide	*µg/L*	2	1	0	1	1	0	0	0	2
	Sulfate	*mg/L*	14	8	10	16	18	13	529	19	39
	Alkalinity	*mg/L*	120	117	122	182	150	103	123	108	150
	Dissolved oxygen	*mg/L*	6.0	5.2	6.8	7.7	5.8	5.4	5.4	6.3	6.8
	Salinity	*ppt*	0.4	0.3	0.4	1.2	0.9	0.5	0.5	2.5	0.5
	pH		7.7	7.6	7.8	7.8	7.6	7.6	7.5	7.5	8.0
	Temperature	*°C*	24.33	23.98	23.24	22.21	23.19	24.36	25.05	23.24	23.13
	Total organic carbon	*mg/L*	N/A	N/A	N/A	N/A	N/A	2.3	0.2	0.9	1.0
	Total nitrogen	*mg/L*	N/A	N/A	N/A	N/A	N/A	4.1	0.3	1.2	0.6
Hypoxic	*Phosphorus*	*mg/L*	0.3	0.1	0.1	0.1	0.2	0.1	0.2	0.2	0.2
	Ammonia	*mg/L*	0.05	0.06	0.00	0.00	0.01	0.00	0.06	0.02	0.00
	Nitrite	*mg/L*	0.01	0.08	0.00	0.05	0.04	0.16	0.00	0.01	0.00
	Sulfide	*µg/L*	0	1	1	7	1	3	0	0	1
	Sulfate	*mg/L*	724	451	529	357	1 881	607	425	403	482
	Alkalinity	*mg/L*	114	125	125	139	137	136	137	114	118
	DO	*mg/L*	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.4	0.3
	Salinity	*ppt*	9.3	8.0	11.6	10.8	12.4	12.1	8.3	11.1	12.3
	pH		7.3	7.2	7.3	7.3	7.2	7.3	7.3	7.3	7.6
	Temperature	*°C*	24.16	24.17	24.16	24.16	24.16	24.18	24.18	24.18	24.19
	TOC	*mg/L*	N/A	N/A	N/A	N/A	N/A	2.1	0.5	0.8	0.7
	TN	*mg/L*	N/A	N/A	N/A	N/A	N/A	3.9	1.0	2.5	0.6
Conduit	*Phosphorus*	*mg/L*	N/A	0.1	0.1	0.1	0.1	0.1	0.3	0.2	0.2
	Ammonia	*mg/L*	N/A	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
	Nitrite	*mg/L*	N/A	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sulfide	*µg/L*	N/A	3	1	3	1	4	0	1	0
	Sulfate	*mg/L*	N/A	341	486	401	847	598	370	403	402
	Alkalinity	*mg/L*	N/A	N/A	127	141	150	105	136	116	152
	DO	*mg/L*	N/A	0.3	0.6	0.3	0.4	0.5	0.7	0.7	0.3
	Salinity	*ppt*	N/A	7.9	10.7	11.8	12.6	12.1	8.0	10.4	12.3
	pH		N/A	7.3	7.4	7.4	7.4	7.4	7.5	7.5	7.6
	Temperature	*°C*	N/A	24.16	24.16	24.16	24.14	24.17	24.16	24.18	24.19
	TOC	*mg/L*	N/A	N/A	N/A	N/A	N/A	2.0	0.1	0.6	0.9
	TN	*mg/L*	N/A	N/A	N/A	N/A	N/A	4.4	0.1	1.5	0.6
Chemocline	*Phosphorus*	*mg/L*	0.3	0.3	0.2	0.5	0.2	0.3	0.2	0.3	0.4
	Ammonia	*mg/L*	0.12	0.41	0.07	0.34	0.11	0.03	0.06	0.05	0.09
	Nitrite	*mg/L*	0.06	0.02	0.03	0.02	0.04	0.32	0.03	0.05	0.05
	Sulfide	*µg/L*	25	3 870	1 391	14 079	293	30	11	15	475
	Sulfate	*mg/L*	513	421	571	543	1 038	680	380	570	481
	Alkalinity	*mg/L*	133	146	130	190	148	151	127	116	190
	DO	*mg/L*	0.0	0.0	0.2	0.0	0.2	0.0	0.0	0.3	0.1
	Salinity	*ppt*	12.4	8.8	13.1	14.2	13.9	14.0	8.5	13.3	15.8
	pH		7.2	7.2	7.1	7.1	7.0	7.2	7.4	7.2	7.3
	Temperature	*°C*	24.49	24.18	24.22	24.16	24.16	24.19	24.18	24.18	24.16
	TOC	*mg/L*	N/A	N/A	N/A	N/A	N/A	2.3	0.4	1.0	2.3
	TN	*mg/L*	N/A	N/A	N/A	N/A	N/A	3.3	0.9	1.9	1.1
Anoxic	*Phosphorus*	*mg/L*	0.7	0.7	0.6	0.6	0.5	0.7	1.1	0.8	0.4
	Ammonia	*mg/L*	0.06	0.06	0.06	0.13	0.03	0.15	0.10	0.18	0.07
	Nitrite	*mg/L*	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Sulfide	*µg/L*	37 347	43 209	37 685	34 181	23 500	12 456	54 886	44 360	31 490
	Sulfate	*mg/L*	1 311	1 054	1 123	800	1 675	1 067	839	1 094	861
	Alkalinity	*mg/L*	248	276	247	229	204	284	323	256	222
	DO	*mg/L*	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.1	0.0
	Salinity	*ppt*	19.6	18.2	18.8	20.1	20.6	18.6	17.1	17.3	18.1
	pH		7.0	6.8	6.9	7.0	7.0	7.0	7.0	7.1	7.4
	Temperature	*°C*	24.17	24.16	24.17	24.18	24.19	24.20	24.19	24.20	24.21
	TOC	*mg/L*	N/A	N/A	N/A	N/A	N/A	6.8	7.5	2.9	2.4
	TN	*mg/L*	N/A	N/A	N/A	N/A	N/A	7.6	7.9	4.4	1.5

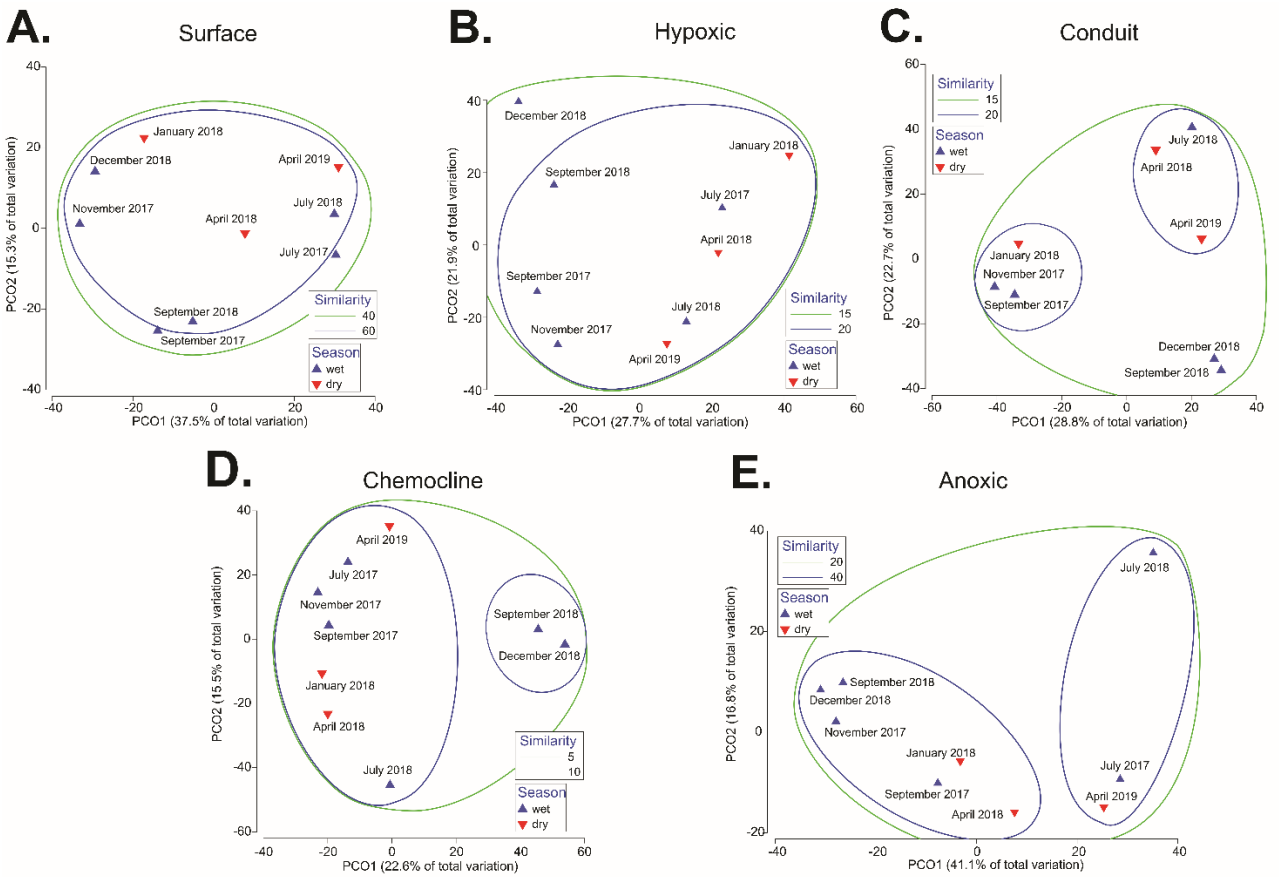

Figure 3.2. Principal coordinate analyses of the (A.) surface, (B.) hypoxic, (C.) conduit, (D.) chemocline, and (E.) anoxic zones are shown. The average of three replicate microbial community samples are shown per date and are color coded by season. Similarities of the communities within each zone are also shown.

Table 3.2. Potential metabolic functions in five zones of Hospital Hole.

Layer	Potential Metabolic Function	Jul-17	Sep-17	Nov-17	Jan-18	Apr-18	Jul-18	Sep-18	Dec-18	Apr-19
Surface	Sulfur oxidizer	3%	4%	3%	27%	5%	2%	2%	3%	3%
	Nitrogen reducer	20%	15%	12%	35%	16%	19%	17%	17%	17%
	Photosynthesis	1%	1%	1%	1%	1%	0%	0%	1%	1%
	Iron reducer	1%	1%	1%	1%	1%	1%	2%	1%	2%
Hypoxic	Sulfur oxidizer	50%	29%	50%	68%	68%	28%	28%	29%	35%
	Sulfur reducer	7%	3%	1%	0%	0%	1%	1%	1%	1%
	Nitrogen oxidizer	0%	0%	0%	0%	0%	0%	0%	8%	0%
	Nitrogen reducer	73%	89%	91%	69%	87%	80%	93%	64%	79%
Conduit	Sulfur oxidizer	N/A	29%	29%	30%	45%	5%	10%	17%	13%
	Sulfur reducer	N/A	1%	1%	0%	0%	0%	0%	0%	0%
	Nitrogen oxidizer	N/A	6%	6%	2%	3%	3%	29%	24%	5%
	Nitrogen reducer	N/A	42%	42%	32%	57%	13%	16%	23%	21%
Chemocline	Sulfur oxidizer	92%	66%	93%	80%	88%	44%	54%	27%	83%
	Sulfur reducer	7%	28%	0%	0%	0%	0%	0%	0%	0%
	Nitrogen reducer	99%	94%	99%	80%	89%	83%	94%	77%	89%
Anoxic	Sulfur oxidizer	7%	25%	37%	52%	56%	11%	34%	48%	8%
	Sulfur reducer	25%	29%	24%	14%	13%	7%	12%	18%	45%
	Nitrogen oxidizer	0%	1%	0%	0%	0%	0%	12%	0%	0%
	Nitrogen reducer	12%	40%	41%	54%	65%	13%	38%	51%	19%

While wet and dry season patterns of hydrochemistry were present in the surface and chemocline zones (Table 3.3), the microbial community structure (Figure 3.2) did not show a clear separation between seasons in the zones. Statistical analyses of the microbial community structure determined that seasonal differences in microbial community assemblages were not significant (Table 3.3). Microbial community function (Table 3.2) did not show seasonal patterns. A BEST test determined the environmental parameters from Table 3.1 that best explained changes in microbial community structure from Figure 3.2 for each of the five zones (Table 3.3). While several physicochemical parameters appear to correlate to microbial community changes within multiple zones, there is not one hydrochemical parameter that correlates to microbial community differences within all zones (Table 3.3).

Table 3.3. Hydrochemical correlates to microbial community structure within each zone.

Statistical analyses		Surface	Hypoxic	Conduit	Chemocline	Anoxic
PERMANOVA p-values	*Seasonal hydrochemistry*	0.044	0.082	0.279	0.044	0.082
	Seasonal microbial communities	0.494	0.25	0.426	0.527	0.275
BEST analysis	p_s	0.343	0.417	0.622	0.307	0.46
	Environmental variables	nitrite, total organic carbon	phosphorus, sulfide	phosphorus	total organic carbon, total nitrogen	sulfide

Rainfall did not correlate to changes in microbial community structure (Table 3.3). Large perturbation events after Hurricane Irma were not identified in the microbial community structure (Figure 3.2). Disturbances may have occurred in the potential metabolic functions after this hurricane, but the percent abundance is comparable to later dates (Table 3.2).

Discussion

Potential causes of microbial community change

Reoccurring physicochemical conditions can cause seasonal patterns of microbial community assemblages (e.g. Bosshard et al. 2000, Strickland et al. 2015, Siboni et al. 2016, Ward et al. 2017). Differences between wet and dry season hydrochemistry occurred in the surface and chemocline zones (Table 3.3), but clear trends in the microbial community structure (Figure 3.2) and estimated metabolic functions (Table 3.2) were not apparent in the two-year period of this study. The spring-fed surface zone did not experience large temperature changes (Table 3.1), which likely prevented seasonal mixing events that have been described in other systems (e.g. Saccà et al. 2008, Rubelmann 2014). Considering perturbation events allow for microbial communities to become resistant to environmental stressors (Bowen et al. 2011, Bressan et al. 2009), the lack of seasonal changes could cause subsurface microbial communities to be more susceptible to anthropogenic stressors.

In subsurface ecosystems, nutrient availability may have an important role in inducing microbial community changes. Sulfide concentrations correlated to microbial community changes in the anoxic zone (Table 3.3). It is likely that the concentrations of sulfide did not elicit changes in the microbial community but was an indicator of changes in the sulfur-reducing community within this zone (Table 3.2). Availability and changes in the concentration of organic carbon (Table 3.1) likely caused changes in the community in this zone. Phosphorus concentrations correlated to microbial community changes in the hypoxic and conduit zones (Table 3.3). While the input of these nutrients is likely from agricultural runoff or leaky septic systems, these communities provide an analogy to what could occur near injection wells for aquifer storage and recovery. Correlations of hydrochemistry to microbial community change varied between zones (Table 3.3), suggesting that different groundwater regions may not have the same drivers of change.

Impacts of Hurricane Irma

Tropical storms and hurricanes may affect subsurface microbial communities (Lakey and Krothe 1996, Pronk et al. 2006, Menning et al. 2018) by concentrating large amounts of rainfall to a small region, which can alter flow and discharge within the surrounding aquifer (Menning et al. 2018). In some cases, weather events may not elicit changes in subsurface communities (e.g. León-Galván et al. 2009) since not all storms produce large amounts of rainfall. Hurricane Irma did not produce a large change in microbial community assemblages in the zones of Hospital Hole (Figure 3.2). The abundance of sulfur oxidizers in the hypoxic zone decreased after Hurricane Irma (29%) and returned to their previous abundance after the storm (50%), but similar abundances were observed in July (28%), September (28%), and December (29%) the following year. It is likely that Hurricane Irma did not have as much impact as other tropical storms and hurricanes in the area (e.g. Menning et al. 2018) since Hurricane Irma did not result in abnormal amounts of rainfall (Table 3.1).

Interactions between distinct hydrochemical zones

There is a general assumption in groundwater management that aquifer microbial communities are homogenous and that communities in different hydrogeochemical regions of aquifers do not interact. The year-round stratification of Hospital Hole allows it to be a model of hydrochemical stratification within the aquifer (Davis and Garey 2018, see Chapter 1). Similar patterns of microbial community assemblages (Figure 3.2B-D) and similar trends in changes of abundance (Table 3.2) in the zones of Hospital Hole suggest interactions between hydrochemically distinct water bodies in the subsurface. It is important for water managers to consider how changes in the microbial community of one region may impact the biogeochemistry of other regions.

Interactions between the different zones in Hospital Hole may have occurred (Figure 3.3). Phosphorus concentrations correlated to microbial community change in the hypoxic and conduit zones (Table 3.3). The hypoxic zone is partially sourced from water discharging from the conduit (Figure 3.1), so changes in flow, water source, and hydrochemistry of the conduit may affect the microbial communities in the hypoxic zone. Species interactions are generally considered on a scale of a few micrometers (Armitridge and Jones 2019), but metabolic byproducts of these communities could impact a larger region. For example, the sulfur reducing community within the anoxic zone (Table 3.2) created high concentrations of hydrogen sulfide (Table 3.1), which the sulfur oxidizers in the chemocline could utilize (Table 3.2). Discharge from Hospital Hole into the Weeki Wachee River (Sinclair 1978) may affect the biogeochemistry of the Weeki Wachee River downstream of the sinkhole but was not analyzed in this study. The relationship of changes in microbial communities among these zones suggests that regional scale features are important to consider for management strategies within karst, which has been noted in other studies (e.g. Scharping et al. 2018).

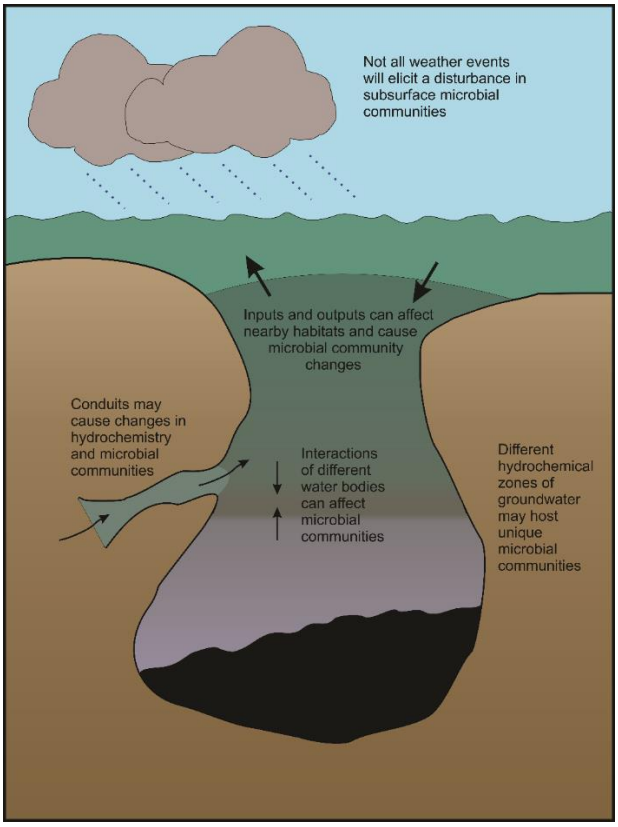

Figure 3.3. Some potential drivers of subsurface microbial community changes are shown in relation to how these drivers could impact Hospital Hole.

Conclusions

The subsurface contains distinct hydrochemical zones with unique microbial community assemblages and functions, and the microbial communities within each of these zones may respond differently to environmental changes. Conduits and the interaction of different water bodies may add to the complexity of understanding drivers of microbial community change in subsurface ecosystems (Figure 3.3). Perturbation events, such as hurricanes, tropical storms, and seasonal turnover, may cause changes in microbial community structure and function if the hydrogeochemistry is altered (Figure 3.3). Water managers should be cautious of the introduction of nutrients to the subsurface and the alteration of groundwater flow. Future studies

should also consider the ecological impacts these microbial communities may have on large ecosystem processes.

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CHAPTER 4: INSIGHTS INTO THE SPATIAL HETEROGENIETY OF SUBSURFACE MICROBIAL COMMUNITIES

Abstract

Groundwater management practices often assume subsurface microbial communities are homogenous. We examined the degree of heterogeneity between microbial communities in sinkholes and wells using 16S rRNA Illumina sequencing and bioinformatic analysis. Hydrochemical analyses demonstrated two distinct water types: sodium-chloride from the Gulf of Mexico and calcium-sulfate from gypsum in groundwater. Water from both the sinkholes and wells was brackish and low in dissolved oxygen. Microbial communities found in the sediments and biofilms of the sinkholes were not significantly different from each other. Microbial communities in sediment and biofilm from one sinkhole to another were significantly different. These communities had similar taxa and similar estimated metabolic functions involved with sulfur cycling. Similarities in microbial community composition and estimated metabolic functions were present between the well and sinkhole water column samples. These findings suggest that wells may become as sulfidic as the sinkholes (14-34 mg/L) if sulfate and organic material are available. Groundwater management strategies should take into account different regions of the aquifer and should investigate local biogeochemistry before implementing managerial practices.

Introduction

Groundwater hydrochemistry has been shown to affect the types of microbial communities that develop (Horton et al. 2019, Muscarella et al. 2019, Aas et al. 2019, Ramette

and Tiedje 2007). Changes in microbial community structure often occur along gradients such as saltwater-freshwater boundaries and oxic-anoxic interfaces (Gray and Engel 2013, Humphreys 1999, Gonzalez et al. 2011, Davis and Garey 2018). Concentrations of nutrients have a greater effect on these communities than the rate of nutrient addition (McTee et al. 2019). It has been suggested that variations in nutrient concentrations below a detectable level have been attributed to variation in microbial community structure in seemingly homogenous environments (Franklin and Mills 2003, Becker et al. 2006, Camacho et al. 2001, Horton et al. 2019).

Microenvironments have been observed within subterranean ecosystems, evidenced by orange, white, and brown patches of biofilms growing on walls and in crevices of sinkholes and caves (Davis and Garey 2018, Garman et al 2011, Menning et al 2018). Environments that appear homogenous may be composed of multiple communities when examined using molecular methods (Franklin and Mills 2003). For example, microbial communities from agricultural soils sampled 1 cm apart (Becker et al 2006) and sediment and water column in meromictic lakes (Camacho et al 2001) have been shown to be heterogeneous. Changes in the environment can have different effects on prokaryotic and eukaryotic microbial community structure (Franklin and Mills 2003, Lehours et al. 2005, Oliviero et al. 2018, Aas et al. 2019), which can be seen in their spatial and organizational patterns (Franklin and Mills 2003). Species abundance and community composition are also affected by the environment, although this only accounts for a small portion of the differences in the microbial community (Ramette and Tiedje 2007).

Blue holes and other submerged sinkholes have been used to study the biogeochemical processes within the subsurface. These karst features may become stratified and develop anaerobic bottom regions (Humphreys 1999, Seymour et al. 2007, Gonzalez et al. 2011, Davis and Garey 2018). Anaerobic regions may be clearly defined under a chemocline (e.g. Davis and Garey 2018) or can gradually become more anoxic with depth (e.g. Garman and Garey 2005,

Garman et al. 2011). Organic matter inputs into these sinkholes occur and can deplete available oxygen, which allows hydrogen sulfide to accumulate. Anaerobic microbes are often present within these bottom layers and have diverse pathways including sulfur reduction, methane cycling, and nitrogen reduction (Garman et al. 2011, Davis and Garey 2018). It is unknown how representative these environments are to the water in the pores of the subsurface bedrock.

The purpose of this study was to test the assumption that subsurface microbial communities in a region are homogenous. Coastal wells and sinkholes were used to compare (Figure 4.1A) microbial communities in the aquifer to the microbial communities in the sinkholes. Biofilms from the bottom regions of these sinkholes had similar appearances (Figure 4.1B-D), so the degree of similarity was examined. Microbial communities from biofilm, sediment, and water column samples from the three sinkholes were investigated to determine whether communities between and among sinkholes were similar.

Figure 4.1. A. Site locations of the three sinkholes and three wells relative to each other. Biofilms in (**B.**) Hospital Hole, (**C.**) Jewfish Sink, (**D.**) and Hudson Grotto.

Materials and Methods

Site Descriptions and sampling

Hospital Hole, Hudson Grotto, and Jewfish Sink are three sinkholes in west-central Florida with saline, anaerobic bottom waters (Figure 4.1). Hospital Hole is a 40 m deep sinkhole underneath the Weeki Wachee River 1.4 km inland (Davis and Garey 2018). The anaerobic bottom water starts at about 22 m. The hydrochemistry and microbial communities of this sinkhole have been described in Davis and Garey (2018). Jewfish Sink is 60 m deep and located 1 km offshore with an opening approximately 2 m beneath the Gulf of Mexico. Two anaerobic regions have been described by Garman et al. (2005). The first is the upper anoxic region, which extends from about 22 m to about 43 m deep. The deeper anaerobic layer, the anaerobic bottom water, was sampled for this study and extends from 43 m to 60 m. Hudson Grotto is 0.5 km inland from the Gulf of Mexico. It is roughly 40 m deep with the bottom water starting about 30 m deep. Brown biofilms have been identified in the anaerobic portions of all three sinkholes. Three wells that are maintained by the Southwest Florida Water Management District (SWFWMD) are located within this region (Figure 4.1) and were used for this study. Well 20891 (total depth: 29.0m; casing depth: 13.1m) and Well 20890 (total depth: 48.8m; casing depth: 41.1m) penetrate the Ocala limestone, and Well 20893 (total depth: 159.4m; casing depth: 146.6m) penetrates the Avon Park formation.

Sampling was conducted by trained scientific cave divers under the auspices of the University of South Florida Scientific Diving Program. Five replicate samples per site were collected in 1L bottles for biological analysis of the water column of each sinkhole and of well water. Five 2 mL microcentrifuge tubes were used to collect sediment and five 20 mL syringes were used to collect biofilms for biological analysis. Three replicate samples were collected in 500 mL glass bottles for hydrochemical analysis (sulfide, sulfate, phosphorus, nitrite, alkalinity, and ammonia). Three 50 mL conical vials were used to sample for total organic carbon (TOC) and total nitrogen (TN) analysis. Water column samples used for major ion analyses were

collected in three 20 mL syringes. A datasonde accompanied divers on each dive to measure pH, specific conductance, temperature, dissolved oxygen (DO), and depth (OTT Hydromet, Loveland, CO, USA). Samples of well water were collected with the assistance of the SWFWMD technicians for analyses. Temperature, pH, specific conductance, and DO were obtained from the published online data by SWFWMD for the sampling on May 30, 2018. Jewfish Sink was sampled on December 19, 2017; Hospital Hole was sampled on January 16, 2018; Hudson Grotto was sampled on February 6, 2018.

Hydrochemical analyses

Depth, pH, temperature, dissolved oxygen, and specific conductance were collected at 5-s intervals using the datasonde for the sinkholes. Water samples were collected by divers, transported on ice, and analyzed in the lab within 12 hours. Total organic carbon and total nitrogen were analyzed with a Shimadzu TOC-V Analyzer (Shimadzu Scientific Instruments, Kyoto, Japan). Sulfate (method 10248), sulfide (method 8131), alkalinity (method 10239), nitrite (method 10207), phosphorus (method 8190), and ammonia (method 10205) were analyzed using HACH test kits and a HACH DR 3900 spectrophotometer (HACH, Loveland, CO, USA). Protocols from the manufacturer were used. Deoxygenated deionized water was used for all necessary sample dilutions. A Perkin Elmer Avio 200 ICP-OES and a Metrohm 881 Compact IC Pro (Metrohm AG, Switzerland) were used respectively for cation and anion analysis to create a Piper diagram to determine water sources of the three sinkholes.

Microbial community analyses

Water samples from the sinkholes and wells (1 L) and 20 mL syringes containing sinkhole biofilm samples were filtered through sterile 0.22-µm filters. Biofilms were transferred to a microcentrifuge tube. Biofilm and sediment samples were dried by vacuum centrifugation before DNA extraction. Bacterial and archaeal DNA was extracted aseptically using the Qiagen PowerSoil kit following the manufacturers protocol and were suspended in molecular grade water. The V4-V5 region of the 16S rRNA gene for three replicate DNA samples was amplified

using the Earth Microbiome 515F and 806R primers (Caporaso et al. 2011) adapted for Illumina MiSeq sequencing by Applied and Biological Materials, Inc. (Richmond, BC, Canada). Well and sinkhole samples were run separately through mothur software (Schloss et al. 2009) for community function analysis and were run together for community structure analysis. Paired-end reads were assembled and sequences that were ambiguous or greater than 310 base pairs were removed in mothur. Chimeras were eliminated using the VSEARCH algorithm (Rognes et al. 2016) in mothur. Operational taxonomic units (OTUs, ≥97% similarity) were clustered using the OptiClust algorithm (Westcott and Schloss 2017) separately for Bacteria and Archaea. For Archaea, the bootstrap cutoff for classification was 50; Bacteria followed Mothur protocol using a cutoff of 80. To avoid potential artifacts caused by rare OTUs (Brown et al. 2015), OTUs represented by fewer than 20 sequences were omitted from subsequent analyses.

The number of sequences across triplicates for each OTU were summed and used for OTU abundance. The sum of triplicates is herein referred to as a sample set (e.g. Hospital Hole biofilm, Jewfish Sink water column, well 20890). The 50 most abundant Bacterial OTUs for each sample set and the top 200 archaeal OTUs for the well and sinkhole communities were provisionally identified taxonomically using the nearest identified sequence match from a GenBank query. Due to overlap of OTUs in each sample set, a total of 543 Bacterial OTUs (394 from sinkholes, 149 from wells) and 400 archaeal OTUs were investigated. Despite a 97% cut-off for OTU clustering, some OTUs have the same provisional identification, which were combined under the same identification. Those that could not be identified to the genus level were identified to the nearest taxonomic level, and those that did not indicate taxonomy (e.g. “16S rRNA amplicon fragment from a soil sample”) were designated as “unidentified”. These provisionally identified taxa for each type of sample (e.g. well, sediment, biofilm, and sinkhole water column) were combined to make tree map plots using the ‘treemap’ package in R software (R Core Team 2017).

Potential metabolic functions for OTUs were assigned by a literature review for each prokaryote. Sulfur reducers included sulfur disproportionation and dissimilatory sulfate reduction, while sulfur oxidizers included microbes that may oxidize any sulfur compounds. Nitrogen reducers included microbes that carry out denitrification, nitrogen fixation, and dissimilatory nitrogen reduction. Nitrogen oxidizers included microbes that can utilize nitrification and anaerobic ammonia oxidation.

The relative abundance of sequences with metabolic functions (sequence abundance) was calculated independently for each sample set by dividing the number of sequences with a provisional function by the total number of sequences in each sample set. The 50 most abundant bacterial sequences for the sinkhole dataset represent 64% of the sinkhole sequences and 54% of the well sequences in our dataset. The top 200 Archaeal OTUs for the sinkhole dataset represent 70% of the sinkhole sequences and 84% of the well sequences in our dataset.

Statistical analyses of the replicate sequence data were analyzed using Primer v7/Permanova+ statistical software for community structure analysis. Biological data were transformed (square-root), clustered using Bray-Curtis similarity were utilized, and analyzed using principal coordinate analyses (PCO). Rarefaction curves were produced using mothur (Schloss et al. 2009).

A mock community was constructed from a six-strain mix (ATCC MSA 3000), *Thermococcus gorgonarius* (ATCC 700654D-5), and *Methanococcus maripaludis* (ATCC 43000D-5). These mock community samples were analyzed and processed through mothur with the environmental DNA samples and used to calculate the sequence error rate with mothur. A reference dataset was constructed from the strain information using sequences from Genbank.

Results

The bottom water of the three sinkholes was characterized by high concentrations of hydrogen sulfide (14-31 mg/L), high sulfate concentrations (500-2000 mg/L), and undetectable dissolved oxygen (Table 4.1). The wells were brackish (1600-3900 µS/cm) with low concentrations of DO (0.3-0.4 mg/L, Table 4.1). Well 20890, Well 20891, and the three sinkholes showed similar sodium-chloride type water (Figure 4.2). A calcium-sulfate signature was evident in Well 20893 and in Hudson Grotto (Figure 4.2). The bottom water in Jewfish Sink was colder (20 °C) and more saline (47,000 µS/cm) than Hospital Hole (24 °C, 30,000 µS/cm) and Hudson Grotto (24 °C, 30,000 µS/cm). Phosphorus, ammonium, and total organic carbon concentrations were higher in Hudson Grotto (0.91 mg/L, 0.79 mg/L, 4.2 mg/L respectively) than Jewfish Sink (0.22, 0.40 mg/L, 1.9 mg/L respectively) and Hospital Hole (0.60 mg/L, 0.13 mg/L, 2.9 mg/L respectively). Total nitrogen was higher in Jewfish Sink (2.1 mg/L) and Hospital Hole (2.2 mg/L) than in Hudson Grotto (1.0 mg/L).

Table 4.1. Hydrochemistry of sinkholes and wells

	Hospital Hole	Hudson Grotto	Jewfish Sink	20891	20890	20893
TP mg/L	0.60 ± 0.01	0.91 ± 0.01	0.22 ± 0.08	0.13 ± 0.02	0.18 ± 0.02	0.12 ± 0.03
NH_3 mg/L	0.13 ± 0.02	0.79 ± 0.08	0.40 ± 0.28	0.29 ± 0.00	0.37 ± 0.00	0.10 ± 0.00
NO_2^- mg/L	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
H_2S µg/L	34000 ± 3000	14000 ± 0	34000 ± 10000	8 ± 2	8 ± 1	300 ± 119
SO^{2-}_4 mg/L	800 ± 0	500 ± 40	2000 ± 100	100 ± 165	16 ± 1	1600 ± 270
$CaCO_3$ mg/L	229 ± 29	239 ± 8	240 ± 14	170 ± 20	275 ± 18	106 ± 5
TOC mg/L	2.9 ± 0.1	4.2 ± 0.1	1.9 ± 0.1	N/A	N/A	N/A
TN mg/L	2.2 ± 0.0	1.0 ± 0.0	2.1 ± 0.0	N/A	N/A	N/A
DO mg/L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Cond. µS/cm	30000 ± 0	32000 ± 0	47000 ± 0	1600 ± 0	2400 ± 0	3900 ± 0
Temp. ℃	24 ± 0	24 ± 0	20 ± 0	23 ± 0	23 ± 0	25 ± 0
pH	7 ± 0	7 ± 0	7 ± 0	8 ± 0	8 ± 0	7 ± 0

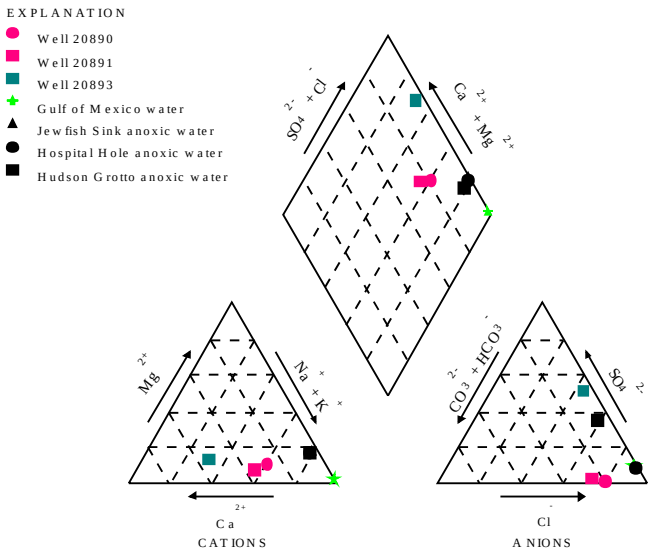

Figure 4.2. Piper diagram of sinkhole and well hydrochemistry. Pink indicates wells in the Ocala limestone and turquoise indicates wells in the Avon Park formation.

Mothur analysis of the combined sinkhole and well data revealed 8,303 distinct bacterial OTUs representing 1,212,741 sequences and 936 distinct archaeal OTUs representing 139,873 sequences. The error rate for the mock community samples was <0.09%. Principal coordinate analyses show a separation of well and sinkhole microbial communities (Figure 4.3A-B), but were not significantly different from each other (PERMANOVA, $p = 0.101$). Microbial communities from the biofilms, water column, and sediments of the three sinkholes were significantly different (PERMANOVA, $p = 0.007$). Replicate samples from sinkhole water column, biofilm, sediment, and well microbial communities were analyzed separately (Figure

4.4), and were significantly different by sample type (PERMANOVA, $p = 0.004$, $p = 0.002$, $p = 0.009$, and $p = 0.003$ respectively).

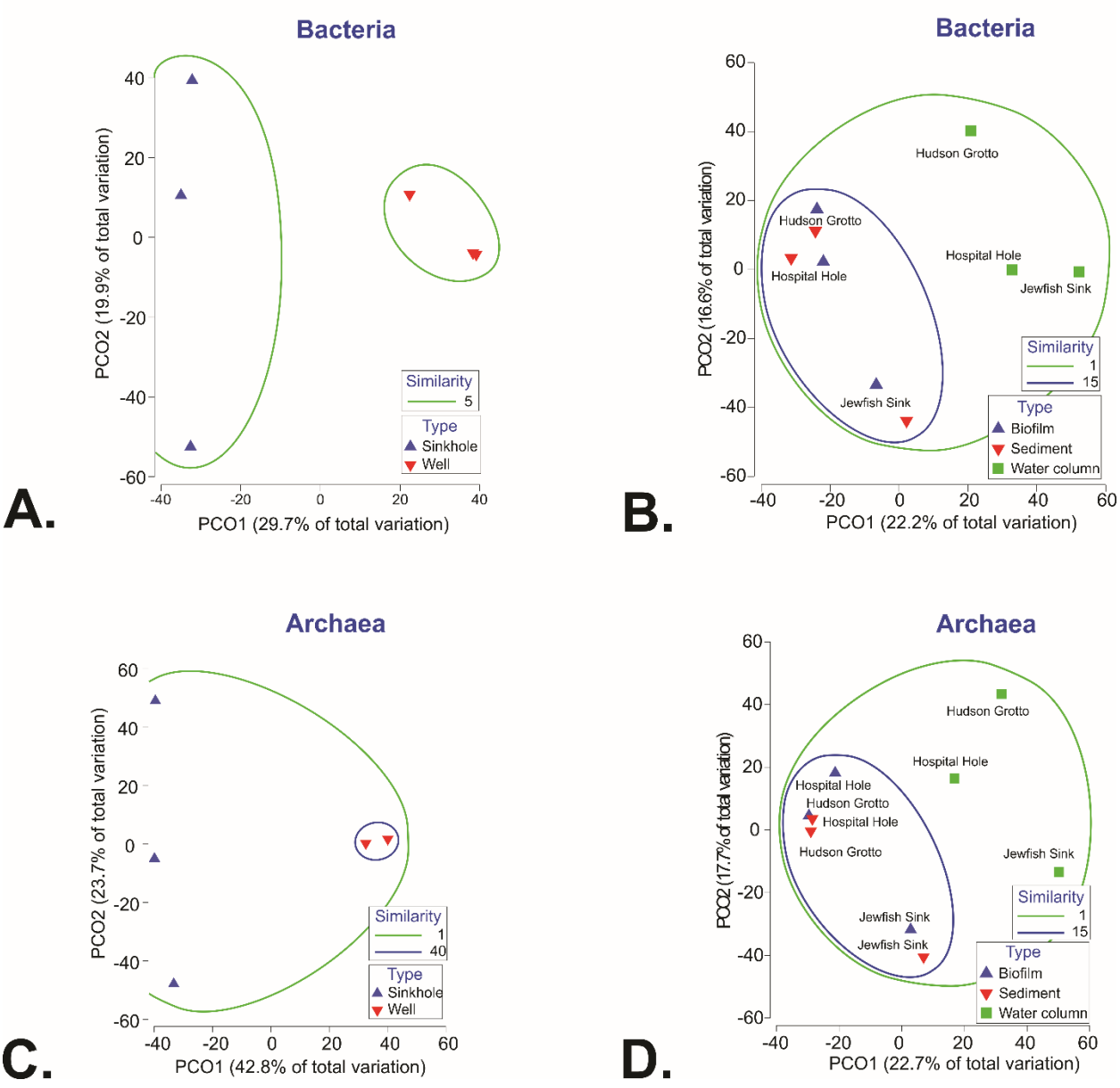

Figure 4.3. Principal coordinate analysis (PCO) of the well and sinkhole microbial community sequences. This PCO shows the Illumina sequencing data with three replicates from each sample for bacteria from the (**A.**) well and sinkhole water column and (**B.**) sinkhole sediment, water column, and biofilm communities, and archaea from the (**C.**) well and sinkhole water column and (**D.**) sediment, water column, and biofilm communities.

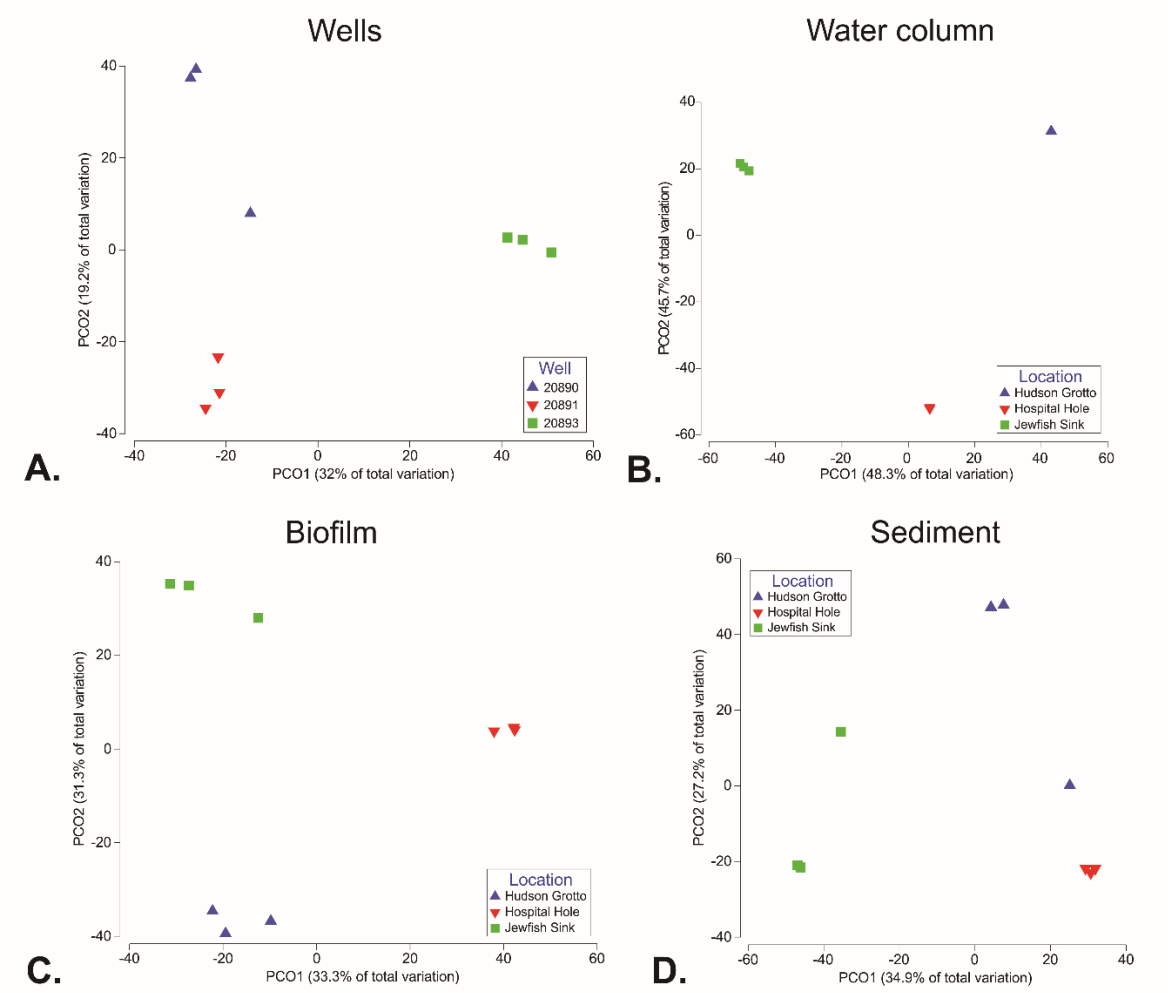

Figure 4.4. Principal coordinate analyses of the bacterial replicates from the (**A.**) three wells, (**B.**) sinkhole water column samples, (**C.**) biofilms, and (**D.**) sediment from the three sinkholes.

Similar taxa were identified in the biofilms, sediments, sinkhole water columns, and wells (Figure 4.5), and *Sulfurimonas* was identified within all sample types. Microbes with the potential for sulfur oxidation and reduction were identified in wells and sinkholes (Table 4.2), and potential sulfur-oxidizers were present in the sediment and biofilm communities (Table 4.3). The abundance of these taxa differed between the different community types with sinkhole communities having greater abundances of microbes involved in sulfur-cycling. Taxa with the

potential for bacterial sulfur disproportionation and archaeal sulfur reduction were only identified in the sinkhole microbial communities (Table 4.2).

Figure 4.5. Taxa present in the well and sinkhole communities. This tree plot shows the most abundant bacterial and archaeal taxa from all replicates of the (**A.**) sinkhole, (**B.**) well, (**C.**) sediment, (**D.**) and biofilm microbial communities.

Table 4.2. Potential metabolic functions of sinkhole and well microbial communities

Potential metabolic function	Hospital Hole	Hudson Grotto	Jewfish Sink	Well 20890	Well 20891	Well 20893
Bacterial S-oxid.	69%	17%	14%	4%	2%	9%
Bacterial S-disprop.	0%	0%	8%	0%	0%	0%
Bacterial S-red.	1%	13%	39%	2%	8%	4%
Archaeal S-red.	8%	7%	5%	0%	0%	0%

Table 4.3. Potential metabolic functions of biofilm and sediment microbial communities

	Potential metabolic function	Hospital Hole Biofilm	Hudson Grotto Biofilm	Jewfish Sink Biofilm	Hospital Hole Sediment	Hudson Grotto Sediment	Jewfish Sink Sediment
Bacteria	S-oxid.	2%	96%	7%	11%	31%	3%
	S-disprop.	22%	0%	6%	35%	0%	2%
	S-red.	3%	1%	23%	13%	0%	6%
	N-red.	43%	81%	66%	33%	27%	50%
	CH_4-oxid.	0%	0%	50%	0%	0%	1%
Archaea	Methanogenesis	78%	70%	76%	60%	60%	90%
	S-red.	3%	4%	8%	0%	1%	3%
	Inorg. C fix.	3%	4%	3%	0%	1%	3%
	Fe-red.	5%	7%	2%	0%	1%	1%
	Lignin degr.	0%	8%	9%	10%	20%	42%

Discussion

Homogeneity of regional groundwater

Water from sinkholes and wells were brackish with low concentrations of DO (Table 4.1). While hydrogen sulfide was higher in the sinkholes, the presence of ammonia suggests reducing conditions in both environments (Table 4.1). Significant differences between microbial community assemblages were not detected between the wells and sinkholes (PERMANOVA, $p = 0.101$). The separate clustering of these community types on the PCO analyses (Figure 4.3) was likely due to different abundances of taxa (Figure 4.5). Potential community function was similar between the sinkholes and the wells despite differences in abundances (Table 4.2). These analyses suggest homogeneity between the sinkholes and the wells.

Similarities in the microbial community assemblages (p = 0.101) and functions (Table 4.2) between the wells and the bottom regions of these sinkholes suggests that the wells have the potential to become sulfidic. Sulfide is common in residential wells in Florida and was detected in Well 20893 (300 µg/L, Table 4.1), but is not typically as high in concentration as the sinkholes (14,000-34,000 µg/L). Higher concentrations of sulfate (Table 4.1) appear to be related to higher salinities in the sinkholes (30,000-47,000 µS/cm) than the wells (1600-3900 µS/cm). Inputs of organic matter, which have been identified at the bottom of these sinkholes (Garman et al. 2011, Davis and Garey 2018) could be used for sulfur reduction by the microbial community. Sulfate sources, such as saltwater or gypsum, combined with organic matter inputs into the aquifer could allow for low-abundant anaerobic and sulfur-reducing microbes to increase in abundance and thus displace existing aquifer microbial communities. Well injections (e.g. Nordbotten et al. 2005), septic systems, and other sources may input organic materials and saltwater intrusion could increase sulfate concentrations available for sulfur reduction in the aquifer.

Evidence for subsurface heterogeneity

Sediment, water column, and biofilm microbial communities from the three sinkholes had common taxa (Figure 4.5) but were significantly different from each other when analyzed together with PCO (PERMANOVA, p = 0.007). Community clustering was based on sinkhole location (Figure 4.3), and microbial communities in sediment and biofilm from one sinkhole to another were significantly different (PERMANOVA, p = 0.009). The estimated metabolic functions identified had large differences in the abundance of function between communities (Table 4.3). For example, sulfur oxidizers comprised 96% of the Hudson Grotto biofilm and 2% of the Hospital Hole biofilm. While sediment and biofilm microbial communities were similar within a sinkhole, these communities appear to be different based on the sinkhole location.

When examining wells, biofilms, sediment, and water columns separately (Figure 4.4), the microbial communities were significantly different from each other (PERMANOVA, p =

0.003, p = 0.002, p = 0.009, p = 0.004 respectively). These differences could be the result of varying abundances of taxa or the limitations of marker gene analysis (e.g. Kunin et al. 2010, Schloss et al. 2011, Zhou et al. 2013, Brown et al. 2015). The potential microbial community function also showed differences between the communities within each type of sample (Tables 2.2, 2.3). For example, estimated abundance of sulfur reducers ranged from 1% to 39% in sinkhole water column samples (Table 4.2). Analyses of the sinkhole and well microbial communities did not show heterogeneity between samples, but each biofilm, well, and water column sample is a unique microbial community.

Other ecosystems have reported heterogeneity within a study area (e.g. Martínez García et al. 2009, Becker et al. 2006, Koizumi et al. 2004, Lehours et al. 2005, Ramette and Tiedje 2007). Variation in microbial communities across a transect may be the result of undetectable changes in the environment (Ramette and Tiedje 2007). Investigating metacommunities may elucidate community diversity and species dynamics within a study region (Becker et al. 2006). Analyzing transects in the subsurface may not accurately portray microbial community structures unless investigating hydrogeochemical gradients (e.g. Seymour et al. 2007, Davis and Garey 2018).

Groundwater management implications

Subsurface microbial communities can have similar consortia (PERMANOVA, p = 0.101) and potential metabolic functions (Table 4.2). The presence of organic matter and a sulfate source may cause the accumulation of hydrogen sulfide (Humphreys 1999, Seymour et al. 2007, Garman et al. 2011, Gonzalez et al. 2011, Davis and Garey 2018). If sulfate and organic carbon are supplied to the aquifer, these microbial communities may become anoxic and highly sulfidic. Management practices that involve injecting organic material into the aquifer, including aquifer storage and recovery programs, should investigate the long-term ecological impact to these ecosystems.

Microbes have been traced through the aquifer (Paul et al. 1995, Lipp et al. 2001, Celico et al. 2004) since they can be transported through highly porous bedrock such as karst (Celico et al. 2004, Blaschke et al. 2016). These organisms may travel long distances by conduit or diffuse flow (Celico et al. 2004). It is unclear whether the similarity between microbial communities in the wells and sinkholes (PERMANOVA, $p = 0.101$) is due to dispersal. More studies on the dispersal and colonization of microbes within the subsurface may elucidate community dynamics within groundwater.

It should be noted that the homogeneity present within this study is representative of a region no larger than 10 km (Figure 4.1). The sinkhole and well microbial communities also have similar water sources (Figure 4.2) and reducing conditions (Table 4.1). Different groundwater hydrochemistry with unique microbial community assemblages and functions can occur in a small region (e.g. Davis and Garey 2018). While microbial communities may be similar in respective hydrochemical regions, management strategies should be cautious of assuming homogeneity of different groundwater regions. Similar microbial communities were present within the Edward's Aquifer in Texas (Gray and Engel 2013) and in the Floridan Aquifer (Table 4.2), but the variability between aquifer microbial communities is poorly understood.

The degree of homogeneity between subsurface microbial communities appears to depend on whether comparing among (Figure 4.4, PERMANOVA, $p < 0.05$) or between (Figure 4.3, PERMANOVA, $p > 0.05$) microbial community types. Groundwater management practices should consider the scope of the project before assuming aquifer homogeneity. For example, aquifer storage and recovery programs in karst landscapes such as Florida can be used to prevent saltwater intrusion and protect the freshwater aquifer. There are many biogeochemical challenges for these programs due to the variability of local hydrogeology and the effects of mixing two different water bodies (see Malira et al. 2006, Ojha et al. 2015). The different microbial communities present in the three sinkholes (Figure 4.3B, Figure 4.3D, Figure 4.4) shows variability in the microbial community structure in the coastal subsurface. Taxa with the

potential for bioremediation may not be present throughout the aquifer, but common potential metabolic functions such as sulfur oxidation (see Table 2.2, Table 2.3, Davis and Garey 2018, and Chapter 2) may be part of the aquifer flora in different subsurface regions. Microbial community analysis of intended injection sites is therefore recommended before well injections for such programs.

Conclusions: can we assume homogeneity?

The findings of this study demonstrate the complexity of subsurface microbial community heterogeneity (Figure 4.6). Sinkhole and well microbial communities were not significantly different from each other. Similarities in microbial community composition and estimated metabolic functions were present between the well and sinkhole water column samples, which suggests that wells have the potential to become highly sulfidic if sulfate and carbon become available. Sediment, water column, and biofilm microbial communities were significantly different by location and within a sinkhole (Figure 4.6). These communities had similar potential metabolic functions, but the abundance of these functions differed. Within a sample type, microbial communities were significantly different from each other (Figure 4.6). Groundwater managers should be cautious of assuming homogeneity within the aquifer.

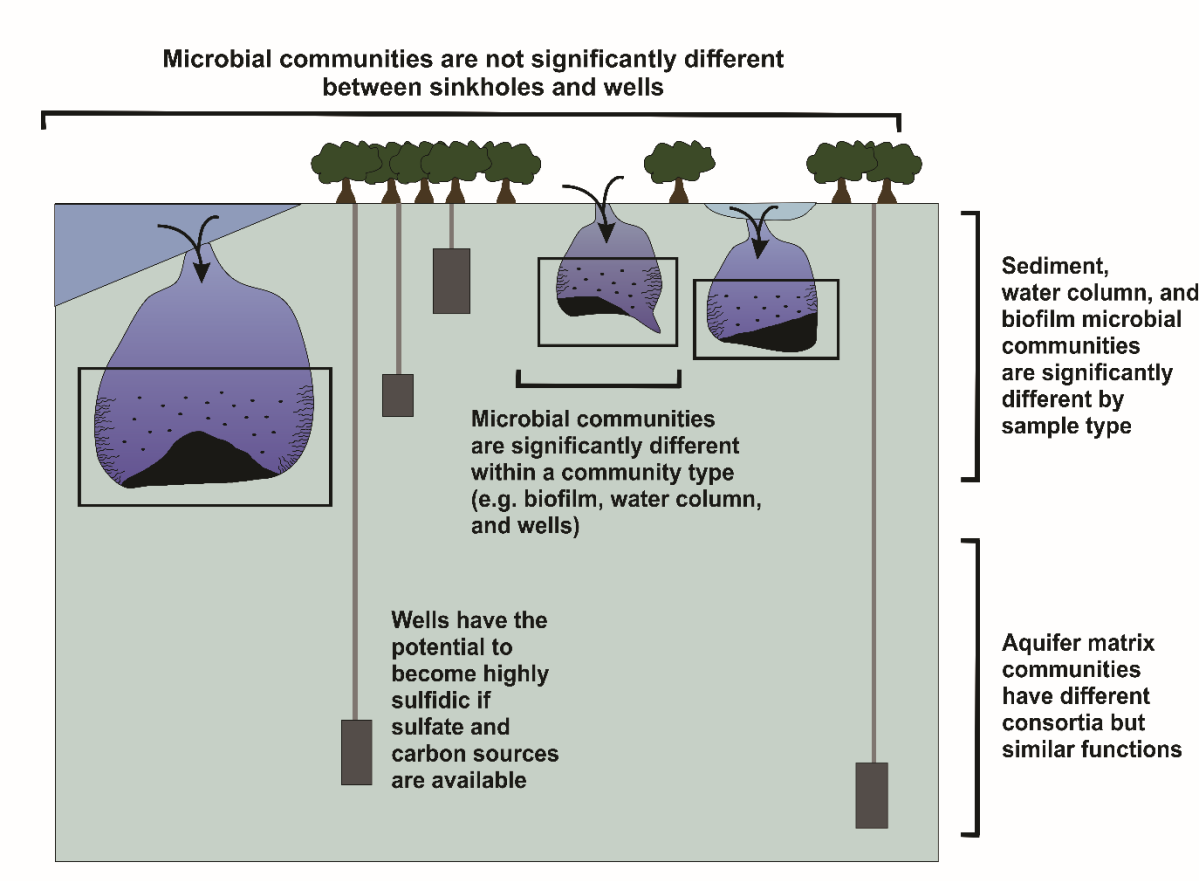

Figure 4.6. Microbial community heterogeneity of subsurface microbial communities.

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CHAPTER 5: SURFACE RUNOFF ALTERS CAVE MICROBIAL COMMUNITY STRUCTURE AND FUNCTION

Note to reader: This chapter has been accepted for publication (Davis, M.C.; Messina, M.A.; Nicolosi, G.; Petralia, S.; Baker, M.D.; Mayne, C.K.S.; Dinon, C.M.; Moss, C.J.; Onac, B.P.; Garey, J.R. Surface runoff alters cave microbial community structure and function. PLoS ONE. 2020.) with permission from the publisher. All supplemental material will be available online.

Abstract

Caves formed by sulfuric acid dissolution have been identified worldwide. These caves can host diverse microbial communities that are responsible for speleogenesis and speleothem formation. It is not well understood how microbial communities change in response to surface water entering caves. Illumina 16S rRNA sequencing and bioinformatic tools were used to determine the impact of surface water on the microbial community diversity and function within a spring pool found deep in the Monte Conca Cave system in Sicily, Italy. Sulfur oxidizers comprised more than 90% of the microbial community during the dry season and were replaced by potential anthropogenic contaminants such as *Escherichia* and *Lysinibacillus* species after heavy rains. One sampling date appeared to show a transition between the wet and dry seasons when potential anthropogenic contaminants (67.3%), sulfur-oxidizing bacteria (13.6%), and nitrogen-fixing bacteria (6.5%) were all present within the spring pool.

Introduction

A number of caves worldwide are now recognized to be the result of a process often identified as sulfuric acid speleogenesis (SAS) (see Klimchouk et al. 2017 and references therein). This process was first proposed by Principi (1931) and is used to describe the formation of caves via dissolution of limestone by sulfidic groundwaters. Among the most well-known and investigated SAS caves are those from Guadalupe Mountains, USA (Hill et al. 1987, DuChene et al. 2017), Movile, Romania (Sarbu et al. 1996), Frasassi, Italy (Galdenzi et al. 1995, 2017), Cueva de Villa Luz, Mexico (Hose et al. 2000, 2017), and Lower Kane Cave, USA (Egemeier et al. 1973, Engel et al. 2004).

Microbial communities in caves play a role in precipitation of speleothems, such as pool fingers (Northup et al. 2000, Melim et al. 2001), moonmilk (Northup et al. 2000, Maciejewska et al. 2017), snottites (Hose et al. 2000, Macalady et al. 2007), and appear to contribute to sulfuric acid speleogenesis (Hose t al. 2000, Engel et al. 2001, 2004, Macalady et al. 2006). Cave microbial communities are often diverse and influenced by the cave environment (Barton et al. 2007, Banks et al. 2010). Soil bacteria may be brought into caves from surface water inputs (Lavoie et al. 2017), whereas the presence of fecal coliforms may be caused by anthropogenic contamination and/or bat guano deposits (D'Angeli et al. 2017, Modra et al. 2017).

Cave microbes can have diverse metabolic functions (Barton et al. 2007). Those involved in sulfur cycling have been identified in several caves, including Cesspool (Engel et al. 2001), Frasassi (Macalady et al. 2006, Vlasceanu et al. 2000, Jones et al. 2008), Movile (Sarbu et al. 1996, Chen et al. 2009), and Lower Kane (Engel et al. 2003). Carbon fixation (Engel et al. 2003), nitrogen cycling, and methane cycling (Jones et al. 2008, Chen et al. 2009) appear to be important microbial processes within caves. Iron and

manganese deposits found in caves have been attributed to the presence of microbial iron and manganese cycling (Northup et al. 2003, Spilde et al. 2005).

Monte Conca is a karst cave in Sicily, Italy that has a sulfidic spring within the inner part of the lower gallery. The hydrochemistry of this spring was investigated by Messina et al. (Messina et al. 2015), who suggested there could be an active sulfur-cycling microbial community. During heavy rains, large volumes of water (3-15 L/s) enters the cave and reach the spring pool. The large volumes of water entering the cave make it difficult for cavers to safely access the cave due to flooded passages. The present study focuses on identifying the impact of surface water entering the cave on the microbial community diversity and function of the Monte Conca spring pool. We hypothesize that surficial inputs are the primary drivers of seasonal change within the microbial community function and diversity within the spring pool.

Materials and Methods

Site description

Monte Conca is the first reported gypsum cave with an active sulfidic spring (Messina et al. 2015). The cave develops in upper Miocene (Messinian) evaporites and is the longest and deepest gypsum karst system in Sicily (Figure 5.1). Madonia and Vattano (2011) provide the most recent description of the cave's genesis. A sulfidic spring is present within the inner part of the lower gallery year-around (Figure 5.1). This spring creates a small pool, which changes depth according to seasons. After heavy rains, surface water can reach the spring pool.

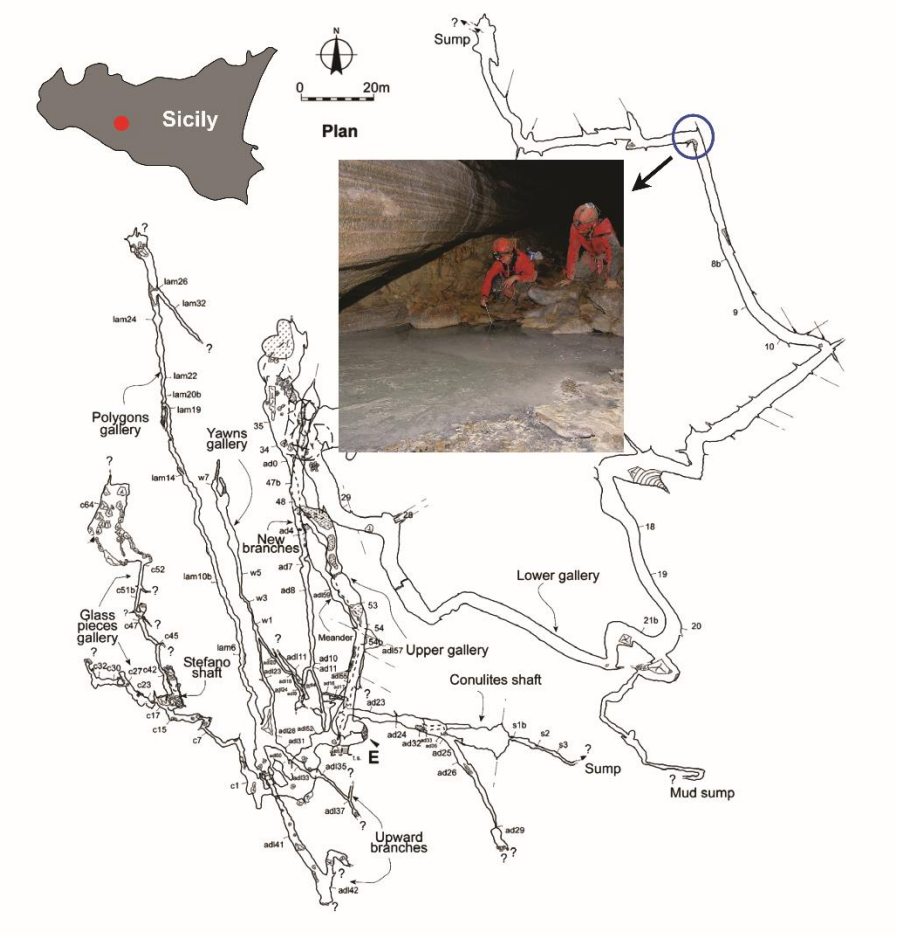

Figure 5.1. Map of the Monte Conca Cave system in Sicily, Italy. Map showing the location of Monte Conca (red circle) modified from Messina et al. 2015 and used with permission. The entrance coordinates are 37°29´23´´N - 13°42´49´´E. Plan view map of the Monte Conca Cave. The "E" indicates the entrance in the cave, whereas the blue circle denotes the location of the sulfidic spring. Team sampling the water at the sulfidic spring pool. Note the stream exiting the pool in the foreground.

Sampling strategies

Water samples were collected from the sulfidic spring pool for biological and hydrochemical analyses on the following days: July 11, 2015; August 29, 2015; February 6, 2016; and December 10, 2016. July and August samples reflect the dry season, whereas the sample from February is typical for the wet season. Sampling was not possible during much of the wet season due to dangerous conditions resulting from high water levels within the cave.

The December sampling is considered as a transition period between the wet and dry seasons because the rainfall amount could not be classified as wet or dry season.

Five replicate water samples were collected using gloves and stored at 5°C for each of the following: sulfide, total organic carbon (TOC), sulfate, and biological analysis. Replicates for sulfide were stabilized with 1.5 mL of zinc acetate (9 g/30 mL) and stored at room temperature until UV-Vis spectrophotometric analysis. TOC samples were acidified at pH<2 with phosphoric acid and stored in dark glass bottles. All bottles filtration apparatuses, and filters were sterilized by UV treatment for 1 hour at 254 nm.

Hydrochemical analyses

The following measurements were performed *in situ*: pH (Carlo ERBA pH-meter), air temperature (HOBOware sensor), water conductivity, and temperature (CM-35 Crison conductometer). Sulfate and TOC were measured using Thermo Scientific Dionex ion chromatography and Hach instruments, following the UNI EN ISO 10304-1:2009 and UNI EN 1484:1999 procedures, respectively. Sulfide concentrations were measured by Cline's (1969) methylene blue method.

Statistical analyses of the replicate hydrochemical data were performed using Primer v7/Permanova+ statistical software (Primer-E Ltd., Albany, New Zealand). Hydrochemical data were transformed (log X+1), normalized (subtracted the mean across all samples and divided by the standard deviation of the variable), and clustered using Euclidean distance before analyzing with principal coordinate analysis (PCoA Primer v7/Permanova+).

Biological analyses

Water samples (500 mL) were filtered through sterile 0.22-µm filters (Isopore, Ireland). Filters were shipped frozen and on ice to the lab in sterile 6 cm Petri dishes for DNA extraction. Environmental DNA was extracted aseptically from the filters using the PowerSoil kit (Qiagen, USA). Preliminary length heterogeneity polymerase chain reaction (LH-PCR) analyses were carried out as described by Menning et al. (2017), who profiled microbial communities utilizing

the V4 region of the 16S rRNA gene in bacteria. These preliminary measurements (Moss et al. 2017) were used to determine the variability of the microbial communities at different sampling locations within the spring.

A year-long study using 16S Illumina 300-bp paired end sequencing on three replicate DNA samples from each date. Gene sequencing was carried out by PCR amplification of the V4 region with pro341f and pro851r primers (Takahashi et al. 2014) adapted for Illumina MiSeq sequencing by Applied Biological Materials, Inc (Richmond, BC and subsequent purification with AmPure XP beads (Illumina, San Diego, California, USA). Mothur software (Schloss et al. 2009) was used to assemble paired-end reads and to remove sequences that were ambiguous or greater than the expected length. Chimeras were eliminated using the VSEARCH algorithm (Rognes et al. 2016) in mothur. Sequences were aligned in mothur using the Silva Version 128 database. Operational taxonomic units (OTUs, ≥97% similarity) were clustered using the OptiClust algorithm (Westcott and Schloss 2017).

Microbial community structure and statistical analyses of the replicate sequence data were analyzed using Primer v7/Permanova+ statistical software. Square-root transformation and clustering using Bray-Curtis similarity were utilized for the top 2000 OTUs for Bacteria before analyzing with PCoA (Primer v7/Permanova+). A Bio-Env (BEST) analysis was performed to determine the relationships between the biological and abiotic data. Rarefaction curves were produced using Mothur (Schloss et al. 2009). Diversity indices were calculated for each replicate separately for all sequences excluding singletons, using EstimateS software (EstimateS 9.1.0). Evenness was calculated by dividing the mean Shanon diversity by the natural log of the total number of OTUs of each replicate.

The 100 most abundant Bacterial OTUs (referred herein as the top 100) were used in our functional analysis to ensure over 80% of the sequence abundance for each date was analyzed. A total of 342 Bacterial OTUs were investigated due to overlap of OTUs between dates. A representative sequence from each OTU was used as a Genbank query for provisional

identification, and those that could not be identified were called “unidentified”. OTUs with the same provisional identification were combined for subsequent analyses.

The potential metabolic function of each OTU was assigned by a review of the literature for each identified prokaryote. Predictive functional profiling may not accurately characterize the extremophiles within caves due to high variability of some gene families (Langille et al. 2013) that may be present within the cave microbiome. Obligate anaerobes and obligate or microoxic aerobes were classified separately than facultative bacteria. Halotolerant and halophilic microbes were categorized together. Sulfur reducers included sulfur disproportionation and dissimilatory sulfate reduction, whereas sulfur oxidizers comprised microbes that may oxidize any sulfur compounds. Nitrogen reducers included microbes that carry out denitrification, nitrogen fixation, and dissimilatory nitrogen reduction. Nitrogen oxidizers consisted of microbes that can utilize nitrification and anaerobic ammonia oxidation. Though not technically a function, the term “anthropogenic microbes” was used to describe microbes from potential contaminants that may entered the cave system and could therefore affect the endemic community’s function.

The relative abundance of sequences with metabolic functions (referred to herein as sequence abundance) was calculated independently by each date. The number of sequences with a provisional function was divided by the total number of sequences in each date and converted to a percent. The 342 Bacterial OTUs that represent the 100 most abundant Bacterial sequences for each sampling date were analyzed for function and represent over 92% of the sequences in our dataset.

Results

Conductivity, sulfate, hydrogen sulfide, and rainfall measurements were significantly different between samples collected in the wet and dry seasons (Table 5.1). Conductivity was higher (3.81 mS/cm) at the transition between wet and dry seasons, whereas sulfate was lowest (1831 mg/L) during this time. TOC values for the transition

period (4 mg/L) were between the wet season (2 mg/L) and the dry season (5-7 mg/L). Hydrogen sulfide concentrations during the dry season (10-14 ppm) are more similar to the transition period (8.5 ppm) compared to the wet season (3 ppm). The lowest temperature of the spring water was recorded during the wet season (13°C).

Table 5.1. Meteorological, physical, and hydro-chemical data for Monte Conca Spring.

Date / **Parameters**	**July 11, 2015** **Dry**	**August 29, 2015** **Dry**	**February 6, 2016** **Wet**	**December 10, 2016** **Transition**
Rainfall (mm) **(previous month)**	37	18	117	56
Number of rainy days **(previous month)**	11	5	21	19
Conductivity (mS/cm)	3.37	3.21	2.83	3.81*
TOC (mg/L)	5	7	2	4*
Sulfate (mg/L)	2210	2114	1886	1831*
Hydrogen sulfide (ppm)	10	14	3	9*
Cave water **temperature (ºC)**	15	15	11	14*
Spring pool water **temperature (ºC)**	17	17	13	15*
Cave air **temperature (ºC)**	16	16	11	16*
Air temperature (ºC) **at the spring site**	12	17	15	17*

** denotes average of five replicate measurements*

Mothur analysis of Illumina sequencing revealed a total of 353,008 sequences represented by 16,381 Bacterial OTUs. Illumina sequences from the 2000 most abundant bacterial OTUs encompassed over 97% of the sequence abundance and were analyzed with PCoA and coded by season (Figure 5.2). Each point on this PCoA represents a replicate that contains thousands of sequences from the top 2000 OTUs from the entire dataset. Both axes together account for 48.7% of the total variation within these samples. The lines on Figure 5.2 correspond to a correlational analysis of the hydrochemical results to the sequence data. Sequences from the wet season clustered separately from the dry season. The transition is different than the other two seasons (p=0.001) but appears more similar to the wet one.

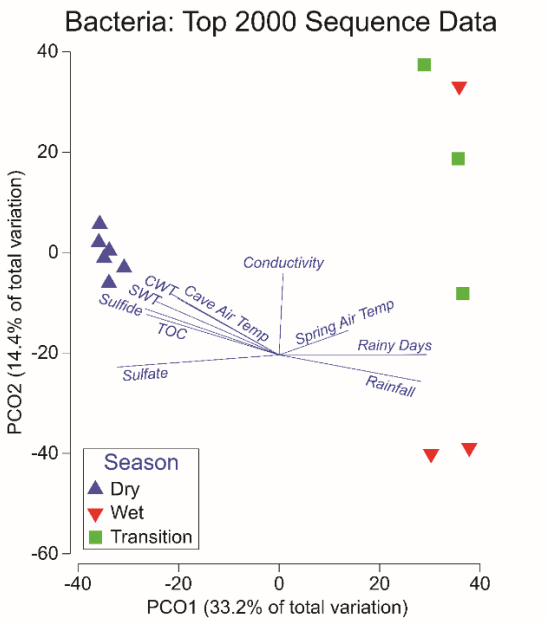

Figure 5.2. Principal coordinate analysis (PCoA) of the top 2000 OTUs. Each data point represents a replicate sample of bacterial communities, each representing thousands of 16S rRNA sequences from the data in S2 Table. The three replicates from each date are shown, and are labeled by season. The hydrochemical information from Table 5.1 is incorporated in this PCoA plot.

Bioinformatic results are shown in S1 Table. These include the percent match to closest identified genus and the known metabolic functions of the provisionally identified genus. The relative abundance of each OUT from the triplicate samples are shown in S2 Table. The rarefaction curves (Figure 5.3) indicate the completeness of the microbiome sequencing. This Targeted Locus Study project has been deposited at DDBJ/EMBL/GenBank under the accession KDAC00000000. The version described in this paper is the first version, KDAC01000000.

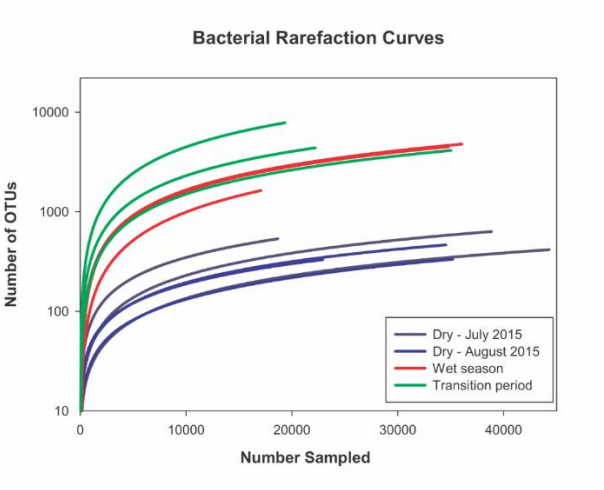

Figure 5.3. Microbial diversity of the Monte Conca spring pool. Bacterial rarefaction curves of the triplicates from each season/period.

Identity and function of the top 100 Bacterial OTUs in each date were analyzed in detail and represent 326,479 sequences. Excluding singletons, the 100 most abundant OTUs account for ~95% of the sequences within the dataset. Despite a 97% cut-off for OTU clustering, some OTUs have the same provisional identification, which were combined together under the same provisional identification. The 10 most abundant provisionally-identified bacterial taxa from each date are shown in Table 5.2.

Table 5.2. Top 10 most abundant bacterial taxa for each sampling date and the abundance (abund.) and relative abundance (relative abund.) of each. Relative abundance was calculated for each taxa by dividing the sequence abundance of the taxa by the total sequence abundance for the sample date.

Dry season

July 11, 2015				August 29, 2015			
Provisional identification	**Num. OTUs**	**Abund.**	**Relative abund.**	**Provisional identification**	**Num. OTUs**	**Abund.**	**Relative abund.**
Thiovirga	12	72157	92.91%	*Sulfurovum*	17	52013	41.26%
Sulfurimonas	5	13188	16.98%	*Thiovirga*	18	22775	18.07%
Sulfurovum	9	5086	6.55%	*Sulfurimonas*	5	4512	3.58%
Arcobacter	3	3605	4.64%	*Thiomicrospira*	4	4496	3.57%
Unidentified	7	1526	1.96%	*Arcobacter*	1	3168	2.51%
Escherichia	1	1175	1.51%	*Escherichia*	2	2197	1.74%
Sulfurospirillum	4	712	0.92%	*Sulfurospirillum*	4	613	0.49%
Sulfuricurvum	2	648	0.83%	Unidentified	13	386	0.31%
Bacillus	5	249	0.32%	*Lysinibacillus*	1	329	0.26%
Thiomicrospira	2	201	0.26%	*Paludibacter*	4	251	0.20%

Wet season **Transition**

February 6, 2016				December 10, 2016			
Provisional identification	**Num. OTUs**	**Abund.**	**Relative abund.**	**Provisional identification**	**Num. OTUs**	**Abund.**	**Relative abund.**
Escherichia	71	64501	81.79%	*Escherichia*	23	34595	54.66%
Lysinibacillus	18	7358	9.33%	Unidentified	18	5826	9.20%
Phyllobacterium	3	85	0.11%	*Lysinibacillus*	11	3551	5.61%
Mycoplasma	2	43	0.05%	*Sulfurimonas*	16	2941	4.65%
Afipia	1	28	0.04%	*Thiovirga*	13	2671	4.22%
Sphingopyxis	1	21	0.03%	*Sulfurovum*	4	886	1.40%
Unidentified	1	16	0.02%	*Thiothrix*	4	316	0.50%
Bdellovibrio	1	16	0.02%	*Phaeocystidibacter*	2	179	0.28%
Pseudomonas	1	16	0.02%	*Arcobacter*	3	155	0.24%
Shigella	1	16	0.02%	*Sulfurospirillum*	1	76	0.12%

An abbreviated potential metabolic function of the microbial communities is illustrated in Table 5.3. Roughly 90% of the bacteria sequence abundance in the wet season was identified as anthropogenic microbes (Table 5.3). Sulfur oxidizers were present in the dry season (90.5-94.9%) and in the transition period (11.4%). Denitrification (6.8%) appears in the transition period. Nitrogen fixers were identified in the dry season (3.8%) and in the transition period (6.5%), but not in the wet season (0.0%). The percentage of microbial community function does not equal 100% due to overlap in taxa that may perform more than one function. The diversity indices for each date are shown in Table 5.4.

Table 5.3. The percent abundance of the potential metabolic functions from the 100 most abundant bacterial OTUs.

Potential Bacterial Function (%)

date	7/11/2015	8/29/2015	2/6/2016	12/10/2016
season	dry	dry	wet	transition
anthropogenic	3.6	3.7	89.5	67.3
sulfur oxidizer	94.9	90.5	0.0	13.6
denitrifier	0.2	0.1	0.0	6.8
nitrogen fixation	3.8	3.8	0.0	6.5

Table 5.4. The average microbial diversity of each sampling date.

Microbial diversity indices				
Date	7/11/2015	8/29/2015	2/6/2016	12/10/2016
Average of total OTUs	289	228	626	1017
Shannon Mean	1.14 ± 0.43	1.70 ± 0.22	1.82 ± 1.08	3.77 ± 0.17
Evenness	0.20 ± 0.07	0.31 ± 0.03	0.28 ± 0.14	0.54 ± 0.17

Discussion

Wet season

During the wet season, lasting from January until May, water runoff from the surface enters the Monte Conca Cave (Messina et al. 2015, Figure 5.4). Spring water temperature, conductivity, TOC, sulfate, and hydrogen sulfide concentrations were all lower in the wet season compared to the dry season, whereas the microbial diversity was similar to the dry season (Table 5.1, Table 5.4). Microbes identified as potential anthropogenic contaminants, such as *Escherichia* and *Lysinibacillus* comprise 89.5% and 3.7% of the sequences within the wet and dry season, respectively (Table 5.2, Figure 5.5). The abundance of these microbes during the wet season suggests that surface runoff is introducing them into the cave, and their dominance of over 90% of the community may explain the low evenness values found during this season (Table 5.4).

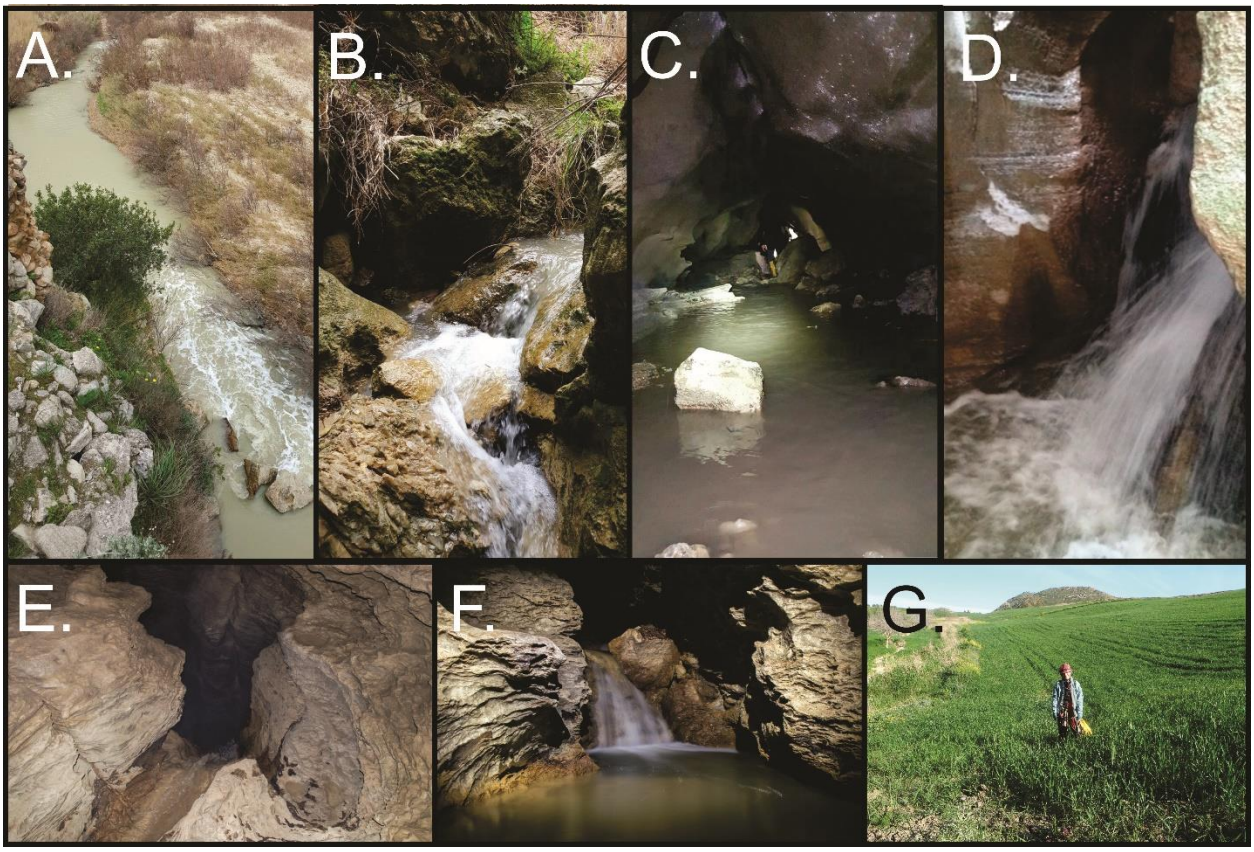

Figure 5.4. Surface runoff in Monte Conca Cave after heavy rainfall. (A) Resurgence flooding in the Gallo d'Oro River during the wet season. (B) Water to flowing into the Monte Conca cave. This water may cause flooding in the (C) entrance galley and may produce (D) large volumes of water flowing through the cave system, which may cause flooding and dangerous conditions to cavers. Water can flow into the (E) entrance shaft and into the (F) second shaft during the wet season, which will then flow to the Monte Conca Spring. (G) The area surrounding Monte Conca contains a little stream that flows into the cave (as shown in B), vineyards, and fields with legumes and vegetables.

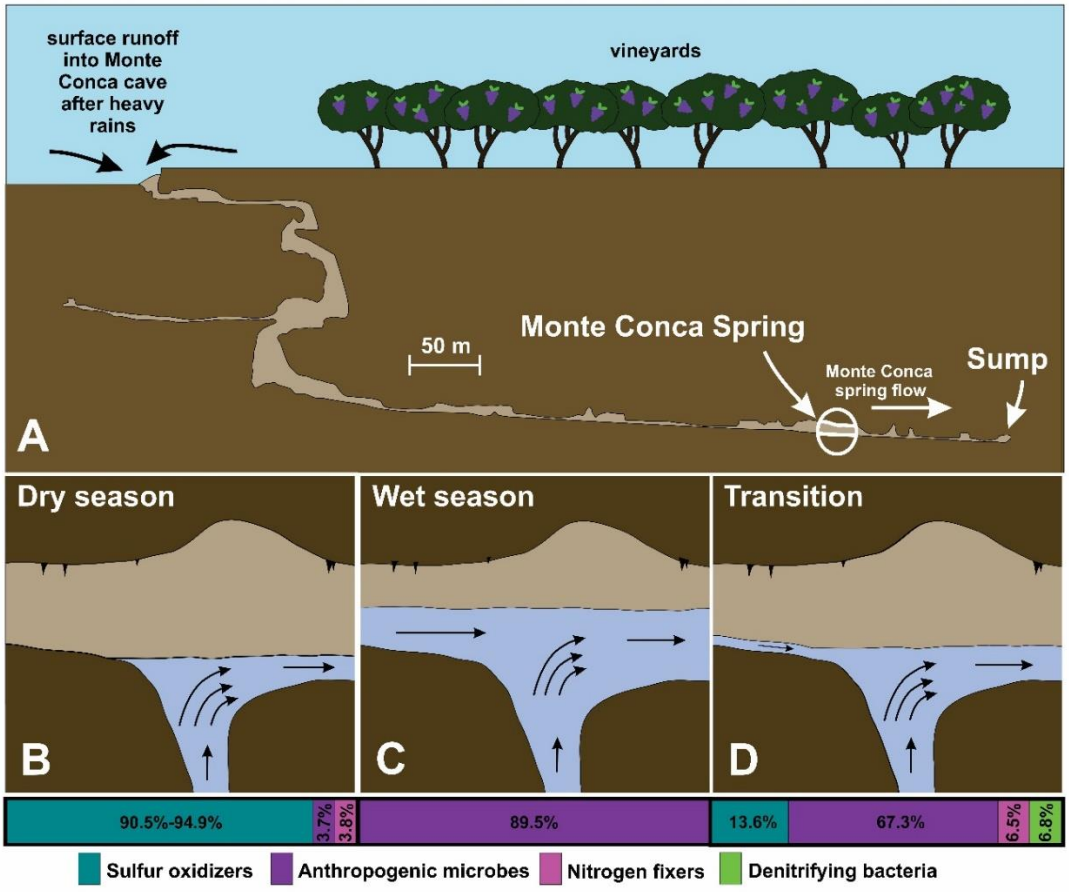

Figure 5.5. The seasonal relationship between surface runoff and sulfidic spring in Monte Conca Cave. (A) Profile view of the Monte Conca Cave. Hydrogen sulfide is produced in deeper parts of the gypsum karst and is discharged into the spring pool (circled), which generates a small stream that flows northward towards the sump (see Figure 5.1). After heavy rains, surface water enters the cave and reaches the lower gallery flowing northward towards the sump. (B) The hydrological settings at the sulfidic spring during different periods. (C) The relative microbial abundance is shown in bar graphs for each of the three seasons to demonstrate community changes over time with the abundances from Table 5.3.

The presence of *Escherichia* has been documented outside cave entrances (Fernandez-Cortes et al. 2011) and in caves (e.g. Bastian et al. 2009, Carmichael et al. 2013). Surface water can be contaminated by a number of mechanisms (Mulec et al. 2012, Seman et al. 2015) and can support *Escherichia* for several days (McFeters et al. 1974). Once inside the cave, contaminated water may flow along karst conduits for several kilometers allowing for large portions of the cave to become contaminated with fecal microbes (Green et al. 1990). Sources

of contamination, the storage capacity of bacteria in soil and water, and the bacterial survival rate in groundwater are responsible for seasonal variations of bacterial contaminants in caves (Pasquarell and Boyer 1995). Since enterobacteria are known to survive in soils (McFeters et al. 1974), the *Escherichia* in Monte Conca are likely derived from the surrounding soils. The Monte Conca cave is surrounded by a nature preserve, which is often used for outdoor recreational activities, and is within 3 km of Campofranco and Milena. The Gallo d'Oro river crosses from east to west through the nature preserve near the cave (Figure 5.4A). Vineyards, fields, and other agricultural activities are present in the region (Figure 5.4G). The presence of *Lysinibacillus*, a common soil microbe (Kong et al. 2014, Cheng et al. 2015), also supports the hypothesis of anthropogenic contaminants entering the cave. Some *Lysinibacillus* species are pathogenic and/or can be found in farming soil (see S1 Table), so this genus has been classified as "anthropogenic" for the purposes of this study. The presence of the soil and enteric bacteria identified in Monte Conca are consistent with other subsurface studies (e.g. Bastian et al. 2009, Carmichael et al. 2013, Green et al. 1990, Davis and Garey 2018). Molecular-grade water processed through each step in the biological analyses process and did not yield these genera, so it is unlikely that these communities are the result of process contamination.

Dry season

During the dry season, which lasts from June through December, the Monte Conca spring pool has a different microbial community compared to the wet season (Figure 5.5). Communities in the dry season had low diversity and evenness (Table 5.4), likely because few taxa dominate during this period. Although Messina et al. (2015) suggested that *Acidithiobacillus* and *Beggiatoa* could be responsible for sulfur oxidation within this cave, this study identified *Sulfurovum, Sulfurimonas, Thiovirga*, and *Arcobacter* in the Monte Conca spring pool (Table 5.3). These genera have been found in Movile Cave and the Frasassi cave system (Engel et al. 2001, Macalady et al. 2006), but other sulfur-oxidizers have been documented from many other cave environments (Engel et al. 2001, Vlasceanu et al. 2000, Jones et al. 2008,

Davis and Garey 2018). Decreases in pH of the sulfidic spring during the dry season (Messina et al. 2015) may be attributed to these microbes. Similar sulfidic environments with high concentrations of carbon (Burgin and Hamilton 2007) and low levels of oxygen (Macalady et al. 2008) have been shown to host sulfur oxidizers. Denitrifiers are known to be inhibited by high concentrations of hydrogen sulfide (Sorensen et al. 1980, Beristain-Cardoso et al. 2006), which may explain why they are not abundant in the dry season community.

Transition between wet and dry seasons

Large volumes of water entering the Monte Conca Cave (3-5 L/s) can create dangerous situations that limit access to the spring pool during the wet season. The December 2016 sampling was carried out after heavy rainfall, originally to be included as a wet season sample. Rainfall during this period (56 mm) was approximately half of the typical wet season (117 mm) and greater than the dry season (18-37 mm), so these samples were designated as representing a transition between the wet and dry seasons.

The microbial analysis identified microbes that are common to both the wet and dry seasons (Table 5.2). Sulfur oxidizers and anthropogenic microbes identified in the dry and wet seasons, respectively, were present within this sampling date. The identification of microbes from both wet and dry seasons explains why this period had the greatest diversity (Table 5.4). Anthropogenic microbes at this sampling date (67.3%) were less abundant than the wet season (89.5%), but richer than the dry season (3.6-3.7%). Microbes with the ability to fix nitrogen were higher during the transition period (6.5%) compare to the dry season (3.8%), and absent in the wet season. Microbes with the potential for denitrification were only identified within the December 2016 sample (6.8%).

The ecosystem

Surface water inputs greatly affect the Monte Conca Cave environment. Examination of the bacterial PCoA analysis in Monte Conca Cave demonstrates different wet/dry season microbial communities (Figure 5.2). A chemolithoautotrophic community is present during the

dry season months until it is replaced by anthropogenic microbes likely derived from surface-runoff during the wet season. The transition period between the seasons had the greatest microbial diversity (Table 5.3). According to the BEST analysis, rainfall (p_s =0.578) accounted for the greatest variance within the microbial community, demonstrating the seasonal impact of surficial inputs into the cave system. The flooding events prior to the transition period and wet season could explain the diversity during these respective intervals (Table 5.3).

Hydrogen sulfide (Table 5.1) is likely produced underneath the Monte Conca Cave by gypsum reduction and is discharged into the spring pool (Figure 5.5). Although hydrogen sulfide is present within the cave year-round (Table 5.1), its highest concentration occurs in the dry season (10-14 ppm). Dry season microbial communities are dominated by sulfur-oxidizing bacteria (Table 5.3) due to the sulfidic spring conditions and from access to oxygen in the cave. Surface runoff into the cave disrupts these communities and may dilute the hydrogen sulfide (Table 5.1) in the spring pool.

Surface runoff can affect cave microbial communities such as those found in the Monte Conca spring pool. Anthropogenic microbial contaminants originating from outside of the cave environment can replace endemic cave communities. We identified one sampling date that appears to show a transition between the dry and wet seasons, which was corroborated by an increase in bacterial diversity. The microbial community during this transition period was the most diverse and consisted of potential anthropogenic contaminants from the surface in addition to the sulfur oxidizers that were identified in the dry season. This study demonstrates the impact of surface runoff on the microbial community structure and function of endemic cave communities.

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CHAPTER 6: SUMMARY AND CONCLUSIONS

Drivers of subsurface microbial community change

The studies presented in this dissertation provide insight into the causes of subsurface microbial community change. The groundwater zones in Hospital Hole demonstrated that different hydrochemical regions may host unique microbial community assemblages and functions. Different groundwater zones in Hospital Hole do not appear to have similar patterns of microbial community change. Well and sinkhole microbial communities were not significantly different from each other, but microbial communities were significantly different between sinkholes. Rainfall and surficial inputs into cave systems, such as Monte Conca, can cause changes in the microbial communities, but hurricanes and tropical storms, such as Hurricane Irma, may not induce microbial communities change. Subsurface ecosystems appear to be vulnerable to anthropogenic inputs, which may contain *Escherichia* and other biological contaminants.

Subsurface microbial community changes: spatial

Spatial heterogeneity may occur in the subsurface (Figure 6.1). Coastal karst systems may have regions where different bodies of water may interact and form chemoclines, as seen in Hospital Hole. This sinkhole provides an example of different groundwater zones that host unique microbial community assemblages and functions. Chemoclines can have a unique microbial community that is comprised of members from microbial communities in adjacent groundwater. The microbial communities in karst features, including Hospital Hole, Hudson Grotto, and Jewfish Sink, appear to be representative of the microbes in the surrounding aquifer. Sinkhole and well microbial communities have similar taxa and estimated metabolic

functions. Microbial communities in sediment and biofilm from Hospital Hole, Hudson Grotto, and Jewfish Sink were significantly different.

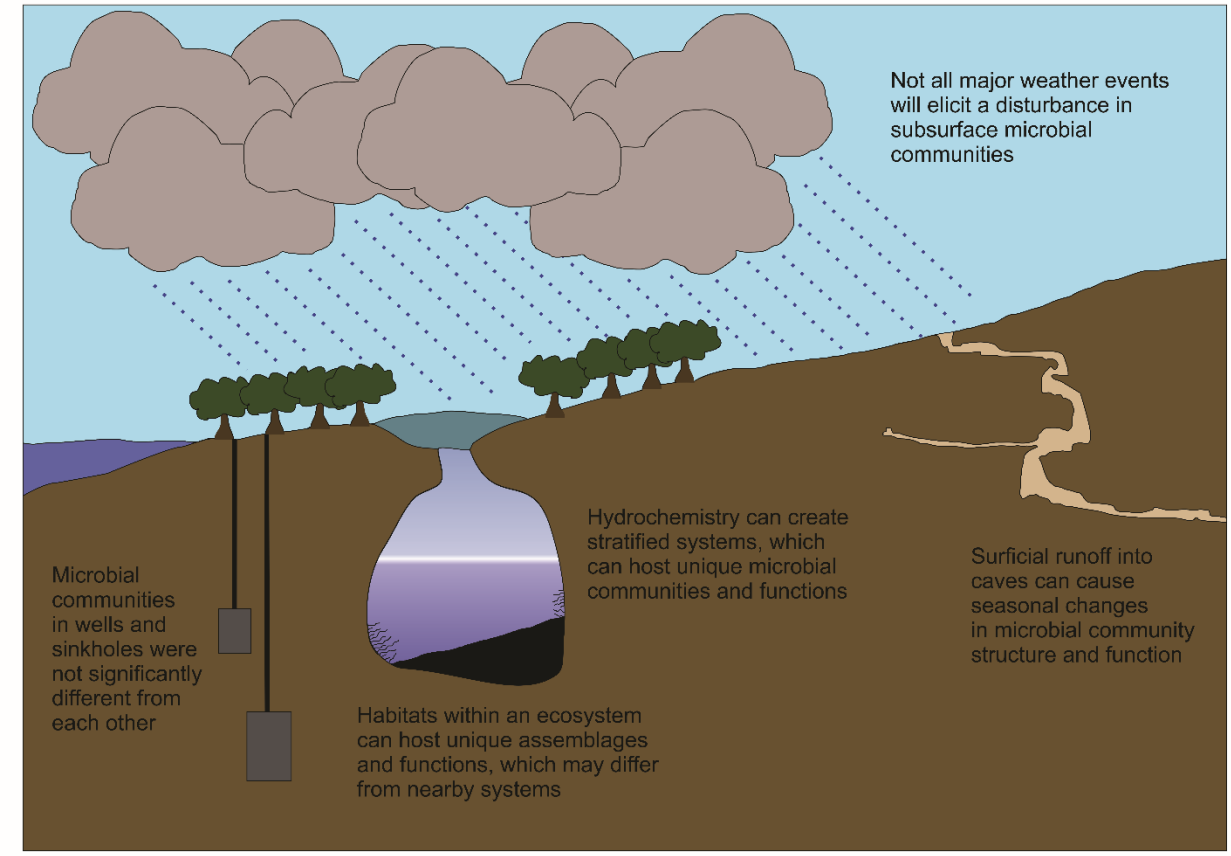

Figure 6.1. Potential spatiotemporal causes of microbial community change in the subsurface.

Subsurface microbial community changes: temporal

Seasonal patterns of microbial communities were evident in the Monte Conca spring pool, but not in the five zones of Hospital Hole. A seasonal turnover event was not evident in Hospital Hole likely due to the Weeki Wachee River preventing the mixing of water layers. The drivers of change in each region of Hospital Hole varied due to physicochemical differences between each region. Interactions between groundwater regions can occur, which may cause changes in microbial consortia and function (Figure 6.1). Seasonal microbial community changes may occur if the local hydrogeology is altered. Surface runoff from seasonal rainfall caused changes in microbial community structure and function in the Monte Conca spring pool

(Figure 6.1). Hurricane Irma did not appear to induce large changes in the zones of Hospital Hole likely because it did not produce abnormal amounts of rainfall in the study region. Further investigation of how hydrogeology, such as discharge and confining units, affects microbial community interactions may elucidate the spatiotemporal drivers of microbial community change.

Connectivity appears to play an important role in subsurface microbial community assemblages and potential metabolic functions (Figure 6.1). Surface runoff into caves can impact the microbial communities by bringing surface-derived microbes, nutrients, and/or contaminants as seen in the Monte Conca Spring. It is unknown how long the impacts of these surface-derived contaminants may persist in the subsurface. Even in groundwater, different regions may indirectly or directly impact each other and cause microbial community changes. Further studies are needed to characterize the transmissivity of microbes in aquifers and how these microbes colonize different regions of the aquifer.

Subsurface microbial community changes: spatial and/or temporal

Other factors may cause spatial and/or temporal changes in microbial community assemblages depending on the duration of these events. Tidal patterns in Hospital Hole could alter conduit flow into the hypoxic zone. Increased or decreased conduit flow may impact local hydrogeology and impact microbial communities. Surface runoff into subsurface ecosystems could have a greater impact on recharge areas if contaminants accumulate in a small region. Wastewater from septic tanks or leaky sewage systems can introduce nutrients, cause eutrophication, or introduce enteric bacteria. The detection of *Escherichia* in Hospital Hole and in the wells suggest that untreated wastewater could be discharging into the local aquifer.

Implications for groundwater management

Groundwater managers should be cautious of the inputs into subsurface ecosystems. For example, phosphorus concentrations correlated to microbial community changes in the

hypoxic and conduit zones of Hospital Hole. Nutrient inputs to this system from nearby septic systems or local agriculture could cause changes in phosphorus, which may impact subsurface microbial communities. In Monte Conca, surface runoff into the cave introduced *Escherichia* into the cave system and altered the microbial communities in a spring pool, which was not close to the cave entrance. Alterations to recharge areas of subsurface ecosystems could impact microbial communities in the subsurface. The input of water or nutrients to aquifers could allow for low-abundant microbes to increase in abundance and thus displace existing aquifer microbial communities. Well injections, septic systems, and other anthropogenic sources could supply nutrients available for sulfur reduction in the aquifer, which may allow for sulfide to accumulate.

Subsurface heterogeneity may complicate groundwater management strategies. In coastal regions, mixing processes may allow for different groundwater regions with varying salinities to interact. These different groundwater zones may host unique microbial community structures and functions, as seen in Hospital Hole. Interactions between groundwater zones allow for a unique microbial community to develop at chemoclines. Conduits may cause changes in microbial communities by introducing groundwater with different hydrochemistry or by altering groundwater flow. Microbial communities within different hydrochemical regions of the aquifer can respond differently to hydrogeochemical changes. It is therefore important to investigate local biogeochemistry before practices are implemented.

APPENDIX I

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Marco Vattano
to me

Hi Madison,

of course! You can use the map, for all your study, for your PhD thesis and also for the dissertation!
I'm very glad about this.
If you need other information about the cave, just let me know.
All the best and finger crossed for your dissertation.

Marco

Madison Davis <[redacted]> ha scritto: