PHYLOGENETIC AND FUNCTIONAL DIVERSITY OF MICROBIAL COMMUNITIES ASSOCIATED WITH SUBMARINE GROUNDWATER DISCHARGE

By

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To my parents and grandparents
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<td>Banana River Lagoon</td>
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<td>C7B</td>
<td>Cenotes Siete Bocas</td>
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<td>IRL</td>
<td>Indian River Lagoon</td>
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<td>SGD</td>
<td>Submarine groundwater discharge</td>
</tr>
<tr>
<td>SI</td>
<td>Saltwater intrusion</td>
</tr>
<tr>
<td>STEs</td>
<td>Subterranean estuaries</td>
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<td>Tg</td>
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The activities of groundwater microbial communities control much of the geochemical cycling that impacts water quality associated with submarine groundwater discharge. An understanding of those communities should yield insight into the controls on biogeochemical cycles in these environments. The objectives of this study were to characterize microbial communities, particularly those groups associated with nitrogen cycling and sulfur cycling in samples collected from two locations: (1) A channelized and hydrologically connected system in the Yucatan Peninsula, Mexico, which includes a stratified cenote (C7B) and a nearshore spring (Pargos); and (2) Diffuse seepage faces in the Indian River Lagoon (IRL) basin, FL, USA. Total microbial community structures were determined by 16S rRNA amplicon sequencing and concentrations of a set of nitrogen cycling genes and one sulfate reduction gene were determined via quantitative PCR (qPCR).

In the Yucatan samples, the microbial communities were dominated by the phylum *Proteobacteria*; *Firmutes* exhibited a clear seasonal variation in groundwater, while *Cyanobacteria* exhibited shifts during tidal reversals in Pargos (offshore spring).
All water samples collected from groundwater and offshore spring clustered together, and showed a greater spatial variation from groundwater to offshore spring when compared with temporal variation, and the interactions between the microbial community changed along the environmental gradients with a greater number of negative connections in high salinity zones than in low salinity. Sulfate reducing bacteria predominately fell within the Deltaproteobacteria, and the bottom of C7B exhibited favorable thermodynamic conditions for supporting sulfate reduction.

Seasonal variations in DIN and N functional genes were observed, with archaeal ammonia oxidizers (AOA) as the dominant nitrifiers, which were weakly coupled with denitrifiers in the groundwater C7B. Tidal variations in flow were observed with bacterial ammonia oxidizers (AOB) as the dominant nitrifiers, which were strongly coupled with denitrifiers at spring Pargos.

In the IRL Basin, the physiochemical properties varied with distance along the seepage face from the shoreline to offshore. A consistent pattern of N functional genes was observed, with higher copy numbers of NH$_4^+$ producers (nifH and nrfA), lower copies of nitrifiers (AOA and AOB), and higher copy numbers of denitrifiers (nirK, nirS and nosZ) at these sites. This explained the accumulation of NH$_4^+$ and was consistent with the prediction that denitrification would proceed to N$_2$ due to high DOC/NO$_3^-$ ratios in the basin area.

For the lab water flow through column experiments, we confirmed that saltwater intrusion has no significant effects on the top layer of sandy sediments, but a significant effect on the second layer of silty sediments was observed with a significant suppression of denitrification.
CHAPTER 1
INTRODUCTION

Research Progress Related to Submarine Groundwater Discharge in Subterranean Estuaries

Subterranean estuaries (STEs) are the freshwater-saltwater interfaces (or transition zones) between hypoxic fresh groundwater and oxic seawater in coastal areas where fresh groundwater discharges to coastal regions via submarine groundwater discharge (SGD). Microbiological activities within this zone may modify the composition of the water (Moore, 1999 and 2010; Roy et al., 2010). STEs are recognized as globally important sites of diagenesis and sources of nutrients in coastal waters (Moore, 1999 and 2010). There have been many reports of the hydrological interactions between terrestrial and marine waters; however, SGD hasn’t drawn much attention until recently due to difficulty in quantification, even though it has been recognized as being potentially important for a long time (Taniguchi et al., 2002; Burnett et al., 2003; Sawyer et al., 2016). The discrepancy in definitions for SGD has sometimes lead to conflicting results in studies comparing SGD between hydrologic models and oceanographic mass balances (Taniguchi et al., 2002).

Burnett et al. (2003) defined SGD as “any and all flow of water on continental margins from the seabed to the coastal ocean, regardless of fluid composition or driving force.” They summarized the components of SGD (meteoric water, recirculated seawater, and connate water); driving force (hydraulic gradients, oceanic processes, such as wave/tidal pumping, endogenic drivers-thermal gradients, and osmotic pressures); measurement (modeling, physical measurement, and tracer techniques) of SGD, which makes an important step forward in the systematic study for SGD. Later, Moore (2010) provided more information about SGD at all aspects that mentioned by
Burnett et al. (2003), and revised the definition of SGD as “the flow of water through continental margins from the seabed to the coastal ocean, with scale lengths of meters to kilometers, regardless of fluid composition or driving force.” They emphasized the importance of mixing and chemical reaction of fluids between land and sea, and proposed the importance of bacterial mediation for chemical reactions and diagenesis process in this area. Knee and Paytan (2011) expanded SGD studies to a larger scale that included the ecological significance of SGD. They summarized studies related to the SGD all around the world, which included the fluxes of nutrients, trace metals, microbes, pharmaceuticals, and their ecological importance to coastal areas, as well as the relationship to climate change.

Although much progress has been made, precise measurements in the flux of SGD are still to be developed. Unlike river flow, which is fairly straightforward to gauge, SGD in STEs is difficult to measure, because it may present as diffuse seepage along the shoreline, offshore seepage, or spring discharge through the sea floor (Slomp and Cappellen, 2004; Moore et al., 2010; Knee and Paytan, 2011). Moore (2010) called offshore seepage or springs as one example of deep pore water upwelling (DPU). Seepages are regarded as more important volumetrically than discrete spring discharge. Diffuse seepage is the groundwater that slowly flows out the sediments with generally decreased magnitude with increasing water depth and distance from the shoreline in shallow unconsolidated aquifers. Submarine springs are the groundwaters that quickly flow out through caves in the seafloor and are connected with underground conduits that extend to the groundwater system. This is common in karst aquifer systems (Taniguchi et al., 2002; Burnett et al., 2003; Slomp and Cappellen, 2004; Chu,
Knee and Paytan (2011) indicated that reducing the uncertainty in SGD flux estimates (2.0-4.8 M³/min; Burnett et al., 2006) to compare different locations and different times is a major challenge. Sawyer et al. (2016) pointed out that different scales were used for SGD measurements in different sites, which left critical gaps in understanding of SGD. They employed a high-resolution continental scale approach to study SGD across the contiguous United States based on a simple water budget analysis and continental scale hydrography and climate datasets, and found that field measurements may overestimate fresh SGD. Their approach likely underestimates SGD, such that new approaches should be developed to combine the advantages of these two sets of measurements.

In addition to the definition and measurements of SGD, another topic related to the biogeochemical cycling affected by the coastal hydrological dynamics is also drawn much attention. SGD is driven by hydraulic forcing between the terrestrial aquifers and ocean, and functions as one of the most important connections between these two aquifers (Castro et al., 2003; Santos et al., 2008). The different hydraulic gradients between the groundwater and seawater control the direction of water flow and the interface position, where the position of interface is argued a lot, but it is generally viewed as a boundary and transition zone formed by difference water density between freshwater and seawater (Smith et al., 2008; Moore, 2010; Gonneea, 2014; Shishaye, 2016). SGD or saltwater intrusion (SI) are viewed as an opposite, however, complementary hydrological processes to balance the hydraulic and density gradients between the end-members of two aquifers at the interface (Figure 1-1; Taniguchi et al., 2002). Gonneea (2014) stated that the mixing zone moved seaward when hydraulic
gradient of freshwater was larger than that of saltwater, resulting in SGD with low salinity conditions and reduced mixing, while the mixing zone moved landward when the hydraulic gradient of freshwater was smaller than that of saltwater, resulting in SI with high salinity groundwater and enhanced mixing. Therefore, the biogeochemical reactions and fluxes in STEs will be highly sensitive to the switch of these two processes due to mixing alkaline seawater with relatively more acidic groundwater, which relates directly to alteration of nutrient, metals, and carbon inputs to the ocean (Chu, 2006).

**The Effects of SGD on Coastal Area**

SGD is recognized as another important source of continental freshwater to the oceans besides river discharge after being neglected for many years due to difficulty in assessment. It is volumetrically and chemically significant in supporting geochemical cycling in coastal area (Taniguchi et al., 2002; Burnett et al., 2003; Chu, 2006; Moore, 2010; Taylor et al., 2013; Beusen et al., 2013; Rodellas et al., 2015; Szymczycha and Pempkowiak, 2016). Moore (2010) summarized that the studies of SGD had recently grown rapidly, which not only focus on reporting measurements and its related techniques, SGD modeling, the importance of SGD, and topic reviews, but also include the transport of nutrients, metals, carbon, and bacteria. Gonneea (2014) demonstrated that SGD from karst subterranean estuaries may play an important role in local and global element budgets.

**The effects of SGD on nutrient fluxes:** The transportation of nutrient fluxes to coastal area linked with SGD are studied earlier from the 1980s (Marsh, 1977; Johannes, 1980; D’Elia et al., 1981), and Boehm et al. (2006) speculated that the source of nutrients in SGD in California beach aquifer may be supplied by microbial re-
mineralization of particulate matter. Brandes et al. (2007) reported that nitrogen (N) and phosphorous (P) input from SGD to coastal systems has the potential to significant effect nutrient cycling of coastal aquifers at the global scale. Coastal ecosystems are generally N-limited as a consequence of N/P ratio below Redfield ratio in water column, and the ratios of N/P delivered by SGD to the coastal area are significantly higher than the Redfield ratio, which will drive coastal systems from N-limitation towards P-limitation (Slomp and Van Capellen, 2004; Moore, 2010). Some studies found that an increasing nitrate concentration is associated with decreasing salinity with increasing depth of the sediments and/or decreasing distances from the coast shoreline, which indicates the possibility of N-nutrient derived from SGD (Chu, 2006; Costa et al., 2006). Szymczycha et al. (2012) reported that the levels of NH$_4^+$ and PO$_4^{3-}$ were significantly higher in samples from SGD sites than in seawater. Niencheski et al. (2007) found that the total dissolved inorganic nitrogen in SGD may represent as much as 55% (total N) nutrient fluxes to the adjacent shelf environment in Patos–Mirim Lagoon system along the southern coast of Brazil, Beusen et al. (2013) indicated that nitrate (NO$_3^-$) transported from SGD to coastal waters increased from 1.0 to 1.4 Tg per year between 1950 and 2000 at the global scale. Knee and Paytan (2011) summarized the variation of nutrient fluxes including N, P to the coastal area via SGD all around the world and emphasized the ecological importance of the nutrients to coastal area. They pointed out that excessive input of SGD-borne nutrients to coastal area will cause eutrophication problems, harmful blooms and damage of coral reefs in the coastal waters.

The effects of SGD on metal and carbon fluxes were summarized by Moore (2010). For metal fluxes, he pointed out that many metals showed non-conservative
behavior along the flow path from groundwater to coastal water, and iron was recognized as an essential micronutrient for plankton in the coastal water. Roy et al. (2010, 2012, and 2013) also studied iron in the IRL, USA, and described the diagenesis and variation of iron and its effects on primary productivity in this area, they found that dissolve Fe would be precipitated from SGD when it carried to the sulfate reduction zone, and then affected the growth of phytoplankton. Knee and Paytan (2011) showed the geological variation of metal fluxes around different locations and their transformation processes and related influence factors. In addition, they presented the ecological effects (e.g. micronutrients, toxic effects, paleoceanographic records) of metals on coastal ecosystems, and they found Fe, Mn and Zn would limit phytoplankton growth, and Cu would interfere in nitrogen fixation in cyanobacteria.

For carbon fluxes, Moore (2010) and Szymczycha and Pempkowiak (2016) reported that only a few papers had been published that link carbon fluxes to SGD, and the carbon fluxes estimated in those studies were greater than riverine inputs, such that they agreed that SGD must be considered in land–sea budgets of carbon cycles. Szymczycha et al. (2014) reported the dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) loads from groundwater into the southern Baltic Sea, and found they were an important component of the carbon budget and gave the sea a firmly heterotrophic status. Smith and Cave (2012) found freshwater-derived DOC had a low C/N ratio, while marine-derived DOC had high C/N ratios in west estuary of Ireland. For the coupling metal and carbon cycling, Knee and Paytan (2011) pointed out that DOC can affect metal mobility and increase their concentration. Young (2013) summarized that iron is related to carbon and phosphorus cycling, where desorption of phosphate
from iron oxyhydroxides and subsequent release to surface water, and sequester carbon by iron sulfide precipitation in the solid phase of aquifers.

SGDs serve as an important pathway for pollutants such as sewage, pathogens, pesticides, and industrial waste to enter coastal aquifers. These contaminant inputs and their environmental impacts on coastal area are drawn much attention for coastal management (Burnett et al., 2003; Boehm et al., 2004; Knee and Paytan, 2011; Pavlidou et al., 2014; Sawyer et al., 2016). Meanwhile, SGDs serve as an important pathway to reduce dissolved components such as H₂S, DOC, NH₄⁺, CH₄ (Moore, 2010; Knee and Paytan, 2011). SGD can influence physical properties, such as estuarine temperature and alkalinity partially, which impact ecological and biogeochemical processes in coastal areas (Frederick and Buffett, 2015; Sawyer et al., 2016). SGD can also affect the distribution and assemblages of marine fauna and flora, as well as microbes (Taniguchi et al., 2002; Chu, 2006; Knee and Paytan, 2011). Kotwicki et al. (2014) provided evidence for the effects of SGD on meiofaunal assemblages, and they found that SGD significantly declined some meiofaunal taxa, and changed the patterns of temporal distribution and vertical zonation of meiofaunal assemblages.

The Effects of Sea Level Changes on the Coastal Area

Sea level changes will affect many coastal ecosystems (FitzGerald et al., 2008; Barlow and Reichard, 2010), and sea level rise will cause saltwater intrusion into estuaries and freshwater aquifers in coastal areas (Williams, 2009). Concern about seawater intrusion into groundwater aquifers in coastal regions were reported as early as the 1890s (Taniguchi et al., 2002). Ferguson and Gleeson (2012) stated that some freshwater aquifers were vulnerable to saltwater intrusion before sea level rise due to low hydraulic gradients, and Barlow and Reichard (2010) found that saltwater had
intruded into many of the coastal aquifers of the United States, Mexico, and Canada, and intrusion varied widely among localities and hydrogeological conditions. Meanwhile, coastal springs in the karstic aquifer are open to the sea and the groundwater is frequently affected by saltwater intrusion (Pavlidou et al., 2014). Larsen et al. (2010) and Passeri et al. (2015) summarized the hydrodynamic, morphologic, or ecologic effects of SLR on the coastal area, which included problems in the erosion, groundwater quality, ecosystem services.

Saltwater intrusion (SI) to the coastal freshwater aquifers will become a big problem caused by sea level rise, and the coastal aquifers responding to sea level rise will become a hot issue in the future and should be considered in future studies (Moore, 2010; Passeri et al., 2015). Burnett et al. (2003) said that saltwater intrusion would not only render the groundwater impotable, but also cause ion exchange and other reactions, which alters the components in the water. Gonneea (2014) found that sea level and precipitation controlled by climate fluctuations may ultimately contribute to the magnitude and timing of chemical and water fluxes via SGD, and reported that the fluxes of sedimentary bounded cations to the ocean increased coincidently with sea level rise due to desorption. And thereupon, the concentrations of nutrients, metals and carbon source will be changed due to the changes in salinity, oxidation and reduction potential (ORP), and other physicochemical properties in the fluids derived by sea level changes in STEs (Burnett et al., 2003; et al., 2008; Roy et al., 2010 and 2013).

The effects of sea level changes on nutrients are summarized below:

1. Salinity gradient will be changed due to sea level changes, Santos et al. (2008) found that the higher the sea level rise, the further onshore the salinity plume extended, and Baldwin et al. (2006) summarized that increasing ionic strength due
to increasing salinity can cause flocculation of particles and shifts the nutrient availability.

2. Salinity also can cause changes in processes related to N transformation. Gardner et al. (1991) and Seitzinger et al. (1991) found that salinity affects the nitrification and denitrification processes and the N form released from the sediments, and higher salinity would suppress the N removal. Santoro (2010) also said that salinization would increase N mineralization and decrease nitrification in coastal areas.

3. The redox conditions change with the sea level change in the subsurface will strongly affect the transformation processes and mobilities of N and P (Slomp and Van Capellen, 2004; Wallmann et al., 2016). One classical scenario for the influence of sea level rise on the N and P cycling is presented in Figure 1-2. Sulfate reducing bacteria will produce sulfides, when the sulfate-abundant sea water meets the low ORP freshwater, which will result in iron becoming sequestered as iron sulfides and P would be released back to the water from the mineral, subsequently changing the N:P ratio in the coastal area (Gunnars and Blomqvist, 1997; Lein, 1983; Bianchi et al., 2000). Long et al. (2013) also found that saltwater intrusion had a significant effect on nutrient structure, and may alleviate phosphorus-limitation due to release of P via desorption, while iron and the ratio of N:P is very important for N cycling, especially for N fixation (Ward et al., 2013).

4. Changes in residence time and strong particle-water interactions due to the hydraulic gradient changes between two aquifers when sea level changes will affect nutrient cycling in this special zone (Slomp and Van Capellen, 2004; Santos et al., 2008; Gonneea, 2014). Stalnacke et al. (2003) reported that the longer time for interaction between particle and water, the more nutrients would be removed. Young (2013) studied the nitrate attenuation mechanisms in SGD area and found that more nitrate was denitrified during SGD to the mud cap sediments than during SGD through sand sediments. Gonneea (2014) found that nitrogen attenuation was enhanced during winter due to longer groundwater residence times, while greater nutrient was delivered to coastal waters during the spring and summer.

The effects of sea level changes on the metals and carbon may be summarized as follows:

1. For metal fluxes, saltwater intrusion will enhance the cation exchange between Ca$^{2+}$ (freshwater origin), Na$^+$, Mg$^{2+}$ (Slomp and Van Capellen, 2004), and trace metals (Fe, Zn, Ni and Mn). Protons adsorbed to sediments will be desorbed to the water column due to increase in the ionic strength of the seawater, which causes an initial decrease in pH and increase mobilisation of trace metals (Wong et al., 2010 and 2013b), and subsequent decrease in the solubility of the trace metals due to high pH in seawater (Johnston et al., 2010). Meanwhile, saltwater intrusion will shift oxidation and reduction reactions, which mainly includes sulfate reduction instead of
methanogenesis, and reduction of Fe-oxides with sulfide (Slomp and Van Capellen, 2004; Edmond et al., 2009; Roy et al., 2010).

2. For carbon flux, numerous studies reported that salt-water intrusion will reduce carbon sequestration attributed to increasing oxidation rate carbon. Because, salt enhances hydrolysis of solid organic matter in coastal ecosystem (Slomp and Van Capellen, 2004; DeLaune, and White, 2012; Whittle and Gallego-Sala, 2016). However, Neubauer et al. (2013) found that a short-term saltwater intrusion would reduce carbon availability and increase organic matter recalcitrance, which was contrasted with long-term effects. All those studies indicate that long-term saltwater intrusion will enhance the decomposition of organic carbon; meanwhile, saltwater will suppress the production of CH₄ (Pattnaik et al., 2000; Slomp and Van Capellen, 2004; Baldwin et al., 2006).

Rationale and Significance

The biogeochemical reactions and fluxes affected by SGD or SI in STEs region are mostly mediated by microbial communities inhabiting the sediments and aquifers (Boehm et al. 2006; Falkowski et al., 2008; Moore, 2010; Algar and Vallin, 2014). The structures and activities of microbial communities and their functions are controlled by many factors (Luo et al., 2007; Hansel et al., 2008; Mosher et al., 2012) including: availabilities of electron donors, electron acceptors, and nutrients; C source (quality and quantity); and redox potential. In turn, these factors will change the activities and functions of the microbial communities related to biogeochemical processes due to the switch between SGD and saltwater intrusion (SI) (Carrero-Colón et al., 2006; Bernhard et al. 2007, Mosher et al., 2012; Unno et al., 2015; Lee et al., 2016). The roles of microbes, and these feedbacks between chemical composition of water in STEs and microbial community functioning in regulating biogeochemical cycling is an important area for active research direction in STEs (Knee and Paytan, 2011).

Nitrogen (N), as one of the most critical nutrients, is closely intertwined with other biologically important elements (e.g. sulfur, phosphorous and carbon), and plays a pivotal role in ecosystem function and services (Zehr and Kudela, 2011; Voss et al.,
2013; Jones et al., 2014; Tsiknia et al., 2015). Furthermore, sulfur (S) is one of the most abundant elements on the earth, which is also closely linked to carbon and N cycling, and provides an important energy source for microbial activities in sulfuric aquifers such as coastal systems (Muyzer and Stams, 2008; Baker et al., 2015). Therefore, it is very important to study how microbial communities relate to N and S cycling changes due to the switch from SGD to SI in STEs.

**Response of Microbial Community and Their Related Function to the SGD**

There have been numerous studies about the effects of SGD on coastal areas with respect to delivery of nutrients; however, SGD also brings fresh water microbial components to the coastal waters (Moore, 2010). First, some human related microbes (e.g., fecal indicator bacteria) will enter coastal water via SGD (Boehm et al. 2004; Knee et al., 2008; Knee and Paytan, 2011; Brown and Boehm, 2016). Second, the nutrients and carbon source input with SGD could alter the proportion and structure of the microbial community in coastal areas. SGD with low anthropogenic impact can stimulate autotrophic plankton growth, thereby shifting the microbial structure. Conversely, SGD with excess nutrient delivery will increase the possibility of outbreaks of opportunistic species and result in harmful algal blooms, and thereby decrease species diversity (Burkholder, 2001; Garcés et al., 2011). The redox potentials in SGD will also control the composition and structure of the microbial community. Moore (2010) said that groundwater flow through permeable sediments make the sediment more reduced and are inhabited by organisms that do not need oxygen in some area. Lee et al. (2016) also found that SGD can detach and transport facultative anaerobes to the coastal water body. Finally, the recharge rate of SGD will affect bacterial growth and abundance as well as the community structure. Carlson and Wiegner (2016) found that SGD effects
on bacterial dynamics were greatest with highest discharge rates of SGD, and SGD reduced the bacteria growth efficiency and diluted marine bacterial cells. Lee et al. (2016) found that the microbial communities presented a periodic shift as a result of SGD rate, and were markedly different between flood and ebb tide even under similar water chemistry.

There have been several reports on the effects of SGD on N nutrients status in STEs (Niencheski et al., 2007; Beusen et al., 2013; Bernard et al., 2014; Lecher et al., 2016), while the concomitant changes in microbial activity involved in N cycling related to the increased N nutrients is not well documented. Spiteri et al. (2008a) built four models with redox-dependent N transformations and removal processes to study the fate of dissolved inorganic N (DIN) under different types of mixing SGD with seawater in STEs, and they found significant decreases in NO$_3^-$ via denitrification when anoxic groundwater met oxic seawater. Bernard et al. (2014) discussed the N cycling genes along the sediment in the SGD area, but didn’t discuss any effects of SGD on the concentrations of those genes, only mentioning how the ratio of DIN to dissolved inorganic phosphorus (DIP) affected N fixation and denitrification. Stoliker et al. (2016) was the first study to report the SGD effects on microbial N cycling in lake systems by investigating N cycling genes. They found that the direction of water flow across the groundwater and surface water interface and the chemical composition were two keys for regulating the N processes, and that organic carbon played a key role in controlling the co-occurrence of denitrification and anammox processes.
Response of Microbial Community and Their Related Function to the Saltwater Intrusion (SI)

The effects of sea level rise on microbial ecology is an emerging area of research with broad implications (Chambers et al., 2015). Microorganisms must be capable of osmoregulation for survival due to increases in the ionic strength and osmotic pressure at high salt concentrations. This process depends on the energy generated and osmotic adaptation. Therefore, SI would directly alter the structures and activities of microbial communities, however, different processes may function differently under the same salt conditions (Oren, 2011), and there have been contradictory reports regarding the effects of salinity on the activities and compositions of microbial communities. These are summarized below:

1. Jackson and Vallaire (2009) found that salinity decreased the enzyme activity related to P and N mineralization, and carbon decomposition, while Morrissey et al. (2014) summarized the effects of salinity on microbial activity, and found that salinity would stimulate extracellular enzyme activity and increase carbon decomposition rate in freshwater tidal marshes. Chambers et al. (2011) found that seawater addition stimulated CO₂ production in the short term, but continuously suppressed CH₄ production. Oren (2011) summarized that high energy microbial processes such as aerobic respiration, denitrification, and photosynthesis can occur at high salinities, while autotrophic nitrification and methanogenesis were not found at salt concentrations >100-200 g/L. Fermentation and incomplete sulfur oxidization function poorly in salt-saturated environments. Chambers et al. (2016) summarized the reasons for effects of salt on microbial activity, which included interference with enzyme synthesis by direct osmotic stress, changes in availability of substrate.

2. For the microbial community structure, Pattnaik et al. (2000) conducted the sulfate saline salt treatment study and showed that microbial biomass C and soil microbial population sizes decreased, including that of methanogens, while Jackson and Vallaire (2009) and Morrissey et al. (2014) both found that salinity was positively related to bacterial abundance. Edmonds et al. (2009) found no significant differences in microbial community composition between control and seawater amendment experiments for several weeks, and they speculated that the variation in microbial activities were due to changes in gene expression and regulation rather than changes in microbial community composition. Héry et al. (2014) also found that microbial diversity pattern did not follow the salinity gradient, even though salinity was one of the major drivers of bacterial community composition. Baldwin et al., (2006) found that the overall microbial diversity and microbial community structure
changed only at the highest NaCl concentration (100 mmol/L), and the bacteria 16S rRNA gene showed little response to increasing NaCl concentration, while archaeal 16S rRNA gene showed significant changes with increasing NaCl concentration in sediment collected from freshwater wetlands. Gennari et al. (2007) found an activation for the α-proteobacteria and no-detectable response for the β-proteobacteria and actinomyces with the addition of Na$_2$SO$_4$. Chambers et al. (2016) pointed out that no major shifts in microbial community composition maybe due to sulfate limitation threshold, and changes in small groups may not affect the whole composition and structure, and Shade et al. (2014) also said that rare taxa greatly contribute to microbial community dynamics even with low proportional abundances and are essential for understanding community changes.

There have been some reports on the saltwater effects on the exchangeable ammonium concentrations, and the consequent effects on nitrification in sediments. Some studies found that most of the N nutrients are released as ammonium in estuarine systems due to low nitrification under saltwater conditions, while N nutrients are often released as nitrogen gas in freshwater systems due to coupling of nitrification and denitrification (Gardner et al., 1991; Seitzinger et al., 1991). Some studies report that nitrifiers would be suppressed by saltwater intrusion in water flow-through column experiments (Weston et al., 2006; Spiteri et al., 2008b), such that SGD will become the source of NH$_4^+$, while denitrification was not significantly affected (Laverman et al., 2007). Meanwhile, seawater intrusion may also decouple nitrification-denitrification, but it will enhance other N removal process like DNRA-Anammox, so that it may not substantially change the N removal capacity of microbial processes in STEs (Hines et al., 2015).

**Research Gaps**

Complex microbial communities are the foundation of key biogeochemical cycling in aquifer and sedimentary ecosystems, and little is known about the partitioning involved ecological and metabolic processes (Héry et al., 2014; Baker et al., 2015).
Meanwhile, microbial related studies in subterranean estuaries (STEs) are greatly understudied.

Few reports, if any (Ye et al., 2015), try to put the groundwater and its related SGD together to conduct their study on microbial communities and their functions, to reveal the difference and similarities between these two systems on a large scale environmental gradient (e.g. salinity and nutrient gradients from groundwater to SGD). Meanwhile, even if there are some studies focus on spring discharge (Chaudhary et al., 2009; Häusler et al., 2014), few studies have been conducted on the effects of tidal periodic variations on the structures of microbial communities. Lee et al. (2016) also pointed out that those previous microbial studies had focused on nutrient cycles in SGD area at coastal aquifers, and very few studies investigated the effects of periodic variations in SGD on the dynamic changes in the microbial communities in coastal systems.

Microbially mediated N processes and the complete microbial N cycle are not well documented in marine sediments along the seepage face in STEs. In addition, there are still few reports, if any, conducted on how the microbial N cycling genes respond differently and how the whole distribution pattern of the N cycling genes respond to the hydrological shifting between SGD and saltwater intrusion in STEs at both DNA and cDNA level. Thus, the primary focus of my research is to quantify the microbial community composition and structure, and their related N cycling to understand:

1. The roles that environmental gradients (e.g. salinity and nutrient gradients from groundwater to SGD) play in governing microbial community structure and composition, as well as the whole N cycling process in a karst aquifer with a spring discharge at different tides. This work was conducted in the Yucatan, Mexico.
2. How microbial N cycling respond to the shifts between SGD and saltwater intrusion via flow-through experiments by combing field freshwater and seawater together for different types of sediments. The materials for these experiments were collected from the saltwater-freshwater interface in a diffuse seepage area in IRL Basin, FL, USA.

The Objectives of the Dissertation

The Study in the Yucatan, Mexico

My work in the Yucatan aquifer was to Characterize microbial communities by 16S rRNA amplicon sequencing and concentrations of microbial N functional genes by quantitative PCR (qPCR) in water samples collected from groundwater in a stratified water-filled sinkhole (Cenote Siete Bocas, C7B) and a submarine spring (Pargos) in the Yucatan Peninsula, and build a model to interpret the N cycling process in the two systems.

I hypothesize that the fundamental assembly of communities differs between the different environments indicate niche partitioning with depth and salinity, and saltwater intrusion would establish mutually exclusive interactions among microbial communities, and the N functional genes and their coupled processes are different from groundwater to coastal SGD.

The Study in Indian River Basin, FL, USA

My work in IRL was to Characterize N functional genes by qPCR and Reverse Transcriptase qPCR (RT-qPCR) in sediment samples collected from IRL in field and in water flow-through column experiments in the laboratory.

I hypothesize that Both DNA and cDNA of N functional genes (DNA and mRNA) show higher concentrations of NH$_4^+$ producers ($nifH$ and $nrfA$), significantly lower concentrations of nitrifiers (AOA and AOB), and higher concentrations of denitrifiers
(nirK, nirS and nosZ) in Indian River Basin, which accounts for the accumulation of NH$_4^+$ in the sampling area. The microbial activities related to N cycling differently respond to the changes in shifts of hydrological processes in different layers.
Figure 1-1. The interface between the end-members in STEs
Figure 1-2. Potential N cycling associate to P and S cycling affected by sea level changes.
CHAPTER 2
THE EFFECTS OF HYDRODYNAMICS ON MICROBIAL COMMUNITIES FROM CENOTE TO SUBMARINE GROUNDWATER DISCHARGE

Background

Subterranean estuaries (STEs) are the transition zones between hypoxic fresh groundwater and oxic seawater with a freshwater-saltwater interface in coastal areas, where fresh groundwater discharges to the coastal regions by submarine groundwater discharge (SGD) (Moore, 1999 and 2010; Roy et al., 2010). SGD plays an important role in delivering nutrients and microorganisms to coastal areas (Dang et al., 2008; Santoro et al., 2008; Moore, 2010; Walker, 2012; Lee et al., 2016), such that it shapes the composition of nutrients and microbial communities in STEs. This is particularly true in karst systems, such as those found in the Yucatan Peninsula, where groundwater is connected with coastal waters by massive networks of underground caves and conduits, and discharges to the sea via SGD (Parra et al., 2015 and 2016). Microbial communities mediate the biogeochemical cycles that control the composition of much of the solute that is transferred between terrestrial and marine waters via SGD, such that information related to hydrological transfers on the controls on community structures along the flow pathways is critical to understanding this connection, and of the potential impacts of future sea level rise.

There have been studies conducted on how the hydrological regimes affect the microbial communities in river and reservoir aquifers; for instance, Foulquier et al. (2013) illustrate that the hydrological regimes rather than biogeochemical processes affected the structural change in bacterial taxa in riverine wetland, and Le et al. (2016) found that the allochthonous DOC coincident with water migration exerted a greater effect on the microbial communities in tropical reservoir aquatic system than that of
autochthonous DOC due to biogeochemical processes related to carbon metabolism, and Stegen et al. (2016) find a higher abundance of microbial taxa related to mixing processes in groundwater-surface water mixing zone in riverine area. Little information is available regarding the effects on microbial communities in karst systems where groundwater and surface waters mix.

Groundwaters systems are complex and heterogeneous, and microbial communities in groundwaters are structured by a variety of factors, including redox potential, terrestrial input of nutrients, and limitations in electron donors and acceptors (Socki et al., 2002; Griebler and Lueders, 2009; Sahl et al., 2010; Bougon et al., 2012; Ben Maamar et al., 2015). Cenotes that are characteristic of the karst systems of Yucatan are open to inputs of light, nutrients, and organic carbon from runoff from adjacent surface soils, all of which may have significant impacts on the compositions and structures of groundwater microbial communities (Haack et al., 2004; Bougon et al. 2012). The composition of water, and consequently the structures of the microbial communities, may also be affected by tidal pumping via the intricate conduits that connect marine systems with the bottom of cenotes (Fleury et al., 2007; Carey et al., 2009; Martin et al., 2012; Parra et al., 2015 and 2016). Meanwhile, most previous reports focussed on sulfur and nitrogen cycling in the spring discharge (Chaudhary et al., 2009; Häusler et al., 2014; Sylvan et al., 2017), with very few reports on the effects of tidal variations on the composition and structures of microbial communities released during discharge and reversal, and Lee et al. (2016) also pointed out that most existing microbial studies had focus on nutrient cycles in SGD area at coastal aquifers, and very
few studies investigated the effects of periodic variations in SGD on the dynamic changes in the microbial community in coastal systems.

Studies on the diversity and distribution of microbial communities are still in the early stages for groundwater ecosystems (Griebler and Lueders, 2009), and exploration of relationships between groundwater microbial communities and SGD is understudied (Garcés et al., 2011; Walker, 2012; Kotwicki et al., 2013; Ye et al., 2015; Lee et al., 2016). Some research put groundwater and SGD together, but they study diffuse seepage face and don’t give detail information about the hydrological effects on microbial communities (Ye et al., 2015), and some just study the Fe and sulfur cycling in the mixing zone before SGD discharge (Roy et al., 2010, 2011 and 2012; McAllister et al., 2015). Few reports, if any, try to put the groundwater and its related SGD together to conduct their study about the hydrologic characteristics (water stratification, mixing, and tidal cycling) impacts on the distribution of microbial communities, or elaborate salinity effects on the interaction between microbial communities by sampling different water members from groundwater, mixing zone within flow pathway, and offshore SGD area along a large scale environmental gradient (Kan et al., 2016; Lee et al., 2016; Stegen et al., 2016).

Therefore, a large stratified and open-mouth cenote (Cenote Siete Bocas; C7B) and a nearshore spring (Pargos) were chosen for our research. We assumed that the chemical and microbial compositions in C7B and Pargos represent groundwater and its related SGD, respectively, even though the two sites are not known to be directly connected. We hypothesize microbial community structures will show a greater spatial
variation in comparison with temporal variation, and community composition will reflect the nutrients, DOC and ORP. The primary objectives of this study were to:

1. Study the similarities and differences between microbial communities along the steep environmental gradient from the cenote to the spring;

2. Evaluate and clarify the potential effects of hydrological conditions on microbial communities;

3. Evaluate the effects of various environmental parameters on the structures of microbial communities across the environmental gradient from the cenote to the SGD.

**Materials and Methods**

**Site Description**

The field area is in Quintana Roo, Mexico (20.8536° - 20.87643° N, 86.8986° - 87.04377° W) (Figure 2-1). The region is tropical, with seasonal precipitation (Fig. S2). A dry season extends from December to April with an average of around 40 to 60 mm precipitation per month. The wet season is from June to October with an average precipitation of between 160 to 180 mm per month for the period between 2000 and 2012 (Parra et al., 2015 and 2016; Figure 2-2).

The Yucatan Peninsula is characterized by a highly permeable karst aquifer system, which is host to numerous deep, water-filled, karstic sinkholes (cenotes) (Back and Hanshaw 1970; Schmitter-Soto et al., 2002). This study focused on a single cenote, Cenote Siete Bocas (C7B, Figure 2-1(A)), which is characterized by a halocline at a depth of 29 m, similar to many deep cenotes in the region. Cenote water is connected to aquifers through both porous matrix and conduit systems. Although groundwater ultimately discharges at the coast, connections, flow paths, and water residence times between the cenotes and the coast are poorly constrained. Aquifer flow is driven toward
the ocean by a small hydraulic gradient of around (~10 mm/km, Perry et al., 1995) that reflects the elevated permeability of the aquifer (Fleury et al., 2007; Smith et al., 2008).

In addition to C7B, this study also focuses on a single submarine spring (Pargos; in Figure 2-1(B)) to represent characteristics of the aquifer discharge to the coast. Spring discharge varies depending on tidal stage and wind and wave setup with maximum discharge rates of 0.4 m³/s during low tide (Parra et al., 2015). At elevated lagoon levels during high tide, the spring reverses flow, allowing lagoon water to flow into the spring (Parra et al., 2015). The spring vent has an open mouth approximately 1.2 m in diameter at a depth of 5 m below the ocean surface. The conduit bifurcates into two small conduits at around 2 m beyond the mouth (Parra et al., 2015; Figure 2-1).

Sample Collection

**C7B:** Water samples were collected above the halocline (at 20 and 28 m below cenote openings), at the halocline (29 m), and below the halocline (30 and 32 m below cenote openings) during the dry (March 22 to April 3, 2014) and wet seasons (September 6 to 17, 2014). Ten samples were collected and designated according to their sampling time and location: codes reflect season (dry, D; wet, W), and depths (28 to 32m): D-C7B20, D-C7B28, D-C7B29, D-C7B30, D-C7B32 and W-C7B20, W-C7B28, W-C7B29, W-C7B30, W-C7B32.

**Pargos:** Water samples were collected at the surface (1m below surface water level), mouth (7m), right channel (10m) and left channel (11m) over a low-high tidal cycle during the wet season only (September 6 to 17, 2014). Seven samples were collected and designated according to the collection time and place: sample nomenclature includes S (spring), and the following descriptors: wet season (W); Dry season (D); surface (s); mouth (m); right channel (rc) or left channel (lc); and low tide
(LT) or high tide (HT): D-SPs, D-SPm1, D-SPm2, W-SPs, W-SPm-LT, W-SPrc-LT, W-SPlc-LT; W-SPm-HT, W-SPrc-HT, W-SPlc-HT.

**Sampling:** Water was pumped from each sampling depth using a Geotech peristaltic pump (Geotech, USA) at flow rates of approximately 1 L/min. The water was initially pumped into an overflow cup instrumented with a YSI Professional Plus Multiparameter Meter (calibrated daily) (YSI, USA) that measured temperature, salinity, pH, and oxidation-reduction potential (ORP). Water was pumped until all values stabilized, typically within a few minutes. For microbial samples, five to ten L of water were filtered through an in-line 3 µm filter (Capsule Pleated Versapor Membrane; Pall Life Sciences, Ann Arbor, MI, USA), and then through an in-line 0.22 µm filter (Supor polyethersulfone; Pall Life Sciences, NY, USA) (Hollibaugh et al., 2014). After filtration, water was removed from the 0.22 µm filters, and the filters were placed on ice for transport back to the lab.

All samples for geochemical analyses were filtered through 0.45µm trace metal grade GEOtech in-line canister filters (Simsii, Inc, CA, USA) into various vial types and preserved according to the solute to be measured. Nutrient samples were filtered directly into 15 ml centrifuge tubes frozen until analysis. Dissolved organic carbon (DOC) samples were filtered into pre-combusted amber vials and acidified in the field with HCl to a pH<2 and stored at 4°C.

**Samples Processing**

**DNA extraction, quantitative PCR (qPCR) and sequencing:** DNA was extracted from filters using Powerwater Sterivex DNA Isolation Kit (Mobio, Carlsbad, CA, USA) (Hollibaugh et al., 2014), and stored at -20 °C. Prokaryotic abundance was
estimated via qPCR using published primers targeting the 16S rRNA gene of Bacteria (Haems et al., 2003). The V3 and V4 regions of approximately ~460 bp of 16S rRNA was amplified by PCR and sequenced by Illumina MiSeq System (Klindworth et al., 2013) according to Illumina protocols.

**Chemical analysis:** The YSI ProPlus sonde (calibrated daily) recorded temperature, Salinity, pH, Oxidation-reduction potential (ORP). Ammonium (NH$_4^+$), nitrate (NO$_3^-$) and phosphate (PO$_4^{3-}$) were analyzed on a Seal AA3 Autoanalyzer continuous flow system following the methods of Strickland and Parsons (1978), Detection limits of NO$_3^-$, NH$_4^+$ and PO$_4^{3-}$ were 0.1, 0.5 and 0.03 µmol L$^{-1}$ respectively. Anion concentration of SO$_4^{2-}$, was analyzed with an automated Dionex DX500 Ion Chromatograph.

**Data Analysis**

**DNA sequence analysis:** The pipeline for 16S rRNA analysis in QIIME (Caporaso et al., 2010; Rideout et al., 2014) was initially employed.

$\alpha$ diversity (Chao1, Shannon, PD whole tree, observed otus, goods coverage) measurements were calculated based on sampling depth with 16,600 reads to cover most of comparable samples (Wang et al., 2012; Gotelli and Chao, 2013) in QIIME by using script “core_diversity_analyses.py -i otu_table.biom -o diversity -e 16600 -t repset.tre -p qiime_parameters.txt”, which contains code “alpha_diversity: metrics chao1, shannon, PD whole tree” in qiime_parameters.txt. (a) Chao 1 index is commonly used for species richness based upon the number of rare classes (i.e. OTUs) found in a sample, the Chao 1 index will estimate greater species richness in samples with more undetected OTUs than a sample without rare OTUs. (b) Shannon diversity: accounts for both abundance and evenness of the species present, the more unequal
the abundances of the types, the smaller the corresponding Shannon entropy. (c) The metric PD\_whole\_tree is Faith's Phylogenetic Diversity, and it is based on the phylogenetic tree. Basically, it adds up all the branch lengths as a measure of diversity. So, if you find a new OTU and it's closely related to another OTU in the sample, it will be a small increase in diversity. However, if you find a new OTU and it comes from a totally different lineage than anything else in the sample, it will contribute a lot to increasing the diversity (Wang et al., 2012; Gotelli and Chao, 2013). Shared and unshared microbial communities were analyzed using Venn diagrams at genus level (Oliveros, (2007-2015)).

Weighted UniFrac distance-based PcoA analysis ($\beta$ diversity) was applied to the spatial and temporal variation of microbial communities, and the analysis of similarity (ANOSIM) was used to test the differences between different groups in QIIME based on the results from PcoA (Kittelmann et al., 2015). The shared and unshared microbial communities were analyzed using Venn diagrams at genus level (Oliveros, (2007-2015)). The distribution of microbial communities along the environmental gradients was analyzed by using ordination analysis in CANOCO 4.5 program (ter Braak and Šmilauer, 2002); non-parametric randomization test-permutation tests were used to detect the important environmental factor contributing to the distribution pattern (Knijnenburg et al., 2009; Phipson and Smyth, 2010). Phylogenetic networks were analyzed via a weighted UniFrac distance-based network, computed by package bioconductor in R (McMurdie and Holmes 2013), which is an abundance-weighted phylogenetic differences used for phylogenetic deviation analysis (Lozupone and Knight, 2005; Staley et al., 2013). The relationships between microbial communities
were analyzed by package Bioconductor and igraph network analysis in R using Spearman correlation analysis (McMurdie and Holmes 2013). Pearson correlation and residual tests were conducted in R (Gardener, 2012).

**Mixing Model**

A physical mixing model (Equations 2-1) was built to study how the hydrologically physical mixing process affect microbial community base on the conservative mixing in coastal area (Fry, 2002; Stegen et al., 2016).

Mixing model for physical process:  \[ M_{cl}=F_f \times C_{f\_cl} + F_s \times C_{s\_cl} \]  
\[ \ldots \text{eq (2-1)} \]

Where \( M_{cl} \) represents the concentrations of Cl in mixing zone (low tide samples from Pargos); \( C_{f\_cl}, \ C_{s\_cl}, \) are the concentrations of Cl in freshwater endmembers (W-C7B29) and saltwater endmembers (W-SPs), respectively; \( F_f \) and \( F_s \) are the fractions of freshwater and saltwater endmembers, respectively, and \( F_f + F_s = 1 \).

**Nucleotide Sequence Accession Numbers**

Paired end Illumina 16S rRNA sequence data for all the water samples in this study were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRR5136460.

**Results**

**Microbial Communities in C7B**

**Sequence reads and copy numbers of 16S rRNA genes:** A total of 1,002,908 raw sequence reads were obtained from 9 samples (out of a total of 10 samples; no sequences were obtained from the dry season 20m sample at depth; (D-C7B20). A total of 9,843 Operational Taxonomic Units (OTUs) based on 97% sequence identity cutoff were obtained from the sequences, with greater numbers of OTUs observed in the in wet season than the dry season, and greater numbers of OTUs below the halocline than
above halocline in both seasons (Table 2-1). The numbers of copies of 16S rRNA genes were between $10^8$ and $10^{10}$ per liter water, with higher numbers observed in the dry season than in the wet season (Table 2-1). Bacteria amounted to more than 95.8% (in average) of the total OTUs, with higher values observed in the dry season than in the wet season, while archaea amounted less than 0.02% (on average) of total OTUs with higher values in the wet season than the dry season except at the 30 m depth, the unassigned sequence was around 4.1% (in average) (Table 2-1).

Composition and structure: Excluding the sample at 32 m depth from dry season, samples from C7B predominantly consisted of Proteobacteria (48.98 % in average), followed by Firmicutes (15.65% in average), Actinobacteria (7.96% in average), Cyanobacteria (4.55% in average), Bacteroidetes (4.23% in average), Planctomycetes (2.44% in average), Chlorobi (1.89%), Verrucomicrobia (1.69%), Chloroflexi (1.17%), and other phyla accounted for a mean relative abundance of less than 1% (Figure 2-3(A) and Table 2-2). The relative abundances of Firmicutes and Cyanobacteria were higher, while Chlorobi was lower in the dry season than that in wet season, and relative the abundance of Actinobacteria was higher above the halocline relative to below the halocline in both seasons (Figure 2-3(A) and Table 2-2). Deltaproteobacteria was dominant among the Proteobacteria, and the relative abundances of Alpha- Beta- and deltaproteobacteria were greater in the dry season, while Gammaproteobacteria were less numerous in wet season. Higher proportions of Beta- and Gammaproteobacteria, and lower percentages of Epsilon- and Deltaproteobacteria were observed above the halocline relative to below the halocline in both seasons (Figure 2-3(B)). In general, samples collected from the dry season were
dominated by *Acinetobacter*, *Exiguobacterium*, and *Synechococcus*, in contrast to those samples collected from wet season (Figure 2-3(C)).

**Diversity:** β diversity (PcoA) based on Unifrac distances showed the seasonal and spatial variations in microbial communities in C7B (Figure 2-4(A)). The samples collected during the dry season and wet season separated along axis 2 (31% variation), and the samples collected above the halocline and below the halocline were also well separated along the axis 1 (40.2% variation) based on PcoA analysis, and the analysis of similarities (ANOSIM) indicated that the four clusters were significantly different (*P*=0.01; *R*=0.77). The variation indicates that the microbial communities differed according to position relative to the halocline, and that the depths above the halocline were more sensitive to seasonal changes than the depths below the halocline, which was likely due to the greater seasonal impacts on microbial communities in shallower depths (Lin et al., 2012). Based on the separation of β diversity, the Venn diagram in Figure S3B was applied to investigate the shared and unshared genera between each cluster in Figure S3A, total 338 and 499 genera were found in dry and wet seasons, respectively, and 119 genera (21.6%) were shared between above and below halocline during the two seasons (Figure 2-4(B)). More genera were shared between above and below halocline in wet season (314; 57%) than in the dry season (157; 28.5%) (Figure 2-4(B)), which suggested a greater exchange of water between the areas above and below the halocline during the wet season. Goods_coverage for the taxonomic α diversity was higher than 96% for all samples, other measures of α diversity were higher in the wet season than in the dry season at all depths, with the exception of 30 m in C7B. Samples below the halocline (from 30 and 32m) exhibited higher values than
those above the halocline in both seasons (Figure 2-4 (C1, C2 and C3). With the exception of pH, environmental parameters (salinity, and NH$_4^+$, PO$_4^{3-}$, and SO$_4^{2-}$ concentrations) were positively correlated with two measures of α diversity (Chao1 and PD_whole_tree) (Figure 2-5).

**Microbial Communities in Pargos**

**Sequence reads and copy numbers of 16S rRNA genes:** A total of 688,169 raw sequence reads were obtained from 9 samples (out of a total of 10 samples; no sequences were obtained from one high tide sample at the mouth: SPm-HT) and passed the quality filtering process. The sequences were defined as 19,713 OTUs (more than twice that of C7B) based on 97% sequence identity cutoff, with a higher number of OTUs observed at low tide than at high tide. Greater numbers of OTUs were observed at the mouth and right channel in comparison with other depths in both seasons (Figure 2-6(A) and Table 2-4). The copy numbers of 16S rRNA genes were similar to those observed for C7B (between $10^8$ and $10^{10}$ copies/L), except in low tide sample at left channel (106). Greater numbers were observed at high tide than at low tide. Bacteria took up more than 95.2% (on average) of total OTUs with somewhat higher values in dry season than in wet season at the mouth, and higher values at high tide than at low tide in channels. Archaea comprised 0.02% (on average) of total OTUs with no OTUs assigned to archaea at the mouth in wet season (W-SPm-LT), and at channels during high tide (W-SPr-HT and W-SPL-HT; Table S3), the unassigned sequences amounted to approximately 4.7% (in average) (Table 2-3).

**Composition and structure:** Samples from Pargos predominantly consisted of Proteobacteria (62.89% in average) followed by Bacteroidetes (15.42% average), Cyanobacteria (5.75%), and Firmicutes (4.55%); other phyla accounted for a mean
relative abundance < 1% (Figure 2A and Table S4). *Cyanobacteria* were more numerous at the surface of Pargos during both seasons, and other groups were higher during low tide both at both the mouth and the channels. Higher relative abundances of *Cyanobacteria* and *Bacteroidetes* and lower relative abundance of *Firmicutes* were observed during high tide than during low tide at channel. *Deltaproteobacteria* constituted a minor group within the Proteobacteria, and the relative abundance of *Betaproteobacteria* was higher in the dry season than in the wet season, and a higher proportion of *Alphaproteobacteria* than *Epsilon*- or *Deltaproteobacteria* was observed during high tide than low tide (Figure 2-6(B)). Generally, the dominant genus for samples collected from the surface and mouth were more dominant by some groups during wet season than that during dry season, and higher relative abundance of *Synechococcus* and *Flavobacterium* were observed in samples collected from high tide than low tide during the wet season (Figure 2-6(C)).

**Diversity:** β Diveristy (PcoA) measures based on Unifrac distances showed the seasonal and spatial variation in microbial communities in Pargos (Figure 2-7(A)). The samples collected from surface and mouth, as well as high tide and low tide, were well-separated along axis 1 (59% variation), and a greater variation was observed for samples collected from different tides than from different seasons, and all four clusters were significantly different ($P=0.007$, $R=0.89$). Based on the differences in β diversity, the Venn diagram in Figure 2-7(B) was applied to explain the shared and unshared genera between each cluster in Figure 2-7(A). The largest number of shared genera were between the Spr+L_HT (High tide samples at right and left channel in wet season) and SPm+r_D+LT (Low tide samples at right channel and mouth in wet season, as well
as samples at mouth from dry season) clusters (259; 40.6%), and the smallest number of shared genera were between Spr+L_HT and SPL +LT (Low tide samples at left channel in wet season) clusters (8.3%), with a total of 46 genera (7.2%) shared among all clusters during two seasons (Figure 2-7(B)). Goods_coverage for the taxonomic α diversity was higher than 90% for most samples, and the measured diversity indices at the mouth were higher in the wet season than in dry season, and were higher during low tide than high tide at the right channel (Figure 2-7(C1, C2 and C3)). No measured environmental parameters with the exception of PO_{4}^{3-} concentrations showed a significant positive correlation with two measures of α diversity (Chao1 and PD_whole_tree), (Figure 2-8).

The Ordination and Connection Pattern of Microbial Communities and Sites along the Environmental Gradients in C7B and Pargos

The original community structure data were tested by a single plot of DCA (Detrended Correspondence Analysis), and the lengths of the gradient for axis 1 were 2.579 from C7B to Pargos (Table 2-5). In cases where the DCA gradient lengths are less than 3, RDA (Redundancy discriminant analysis) is appropriate for interpreting the ordination pattern (Legendre et al., 2011; Sun et al., 2014). The percentage of variation explained and the significant levels are presented in Table 2-6. The angles between the arrows represents the relationships between the variables (angle<90°: positively correlated; 90°<angle<180°: negatively correlated; angle=90°: no correlation; Figure 2-9(A)). The linear distance between each data point represents the similarity of each sampling site; closer data points are more similar (ter Braak and Šmilauer, 2002).

The first and second axes of RDA explained 44.7% and 15.8 % of cumulative variance of the relationship, respectively (Figure 2-9(A)). The first ordination (axis 1)
was mostly affected by salinity, \( SO_4^{2-} \), ORP, and pH, and the second ordination (axis 2) was dominated by nutrients (\( NO_3^- \), \( NH_4^+ \) and \( PO_4^{3-} \)). pH, salinity, and \( NO_3^- \) contributed to the ordination pattern, explaining 49%, 36% and 59% of the variation, respectively (\( P=0.001 \) for pH and salinity, \( P=0.014 \) for \( NO_3^- \) (Table 2-6)).

Four distinct regions are observed on the RDA (Figure 2-9(A)): (i) composed of samples from the dry season of C7B, with dominant phyla of \textit{Planctomycetes}, \textit{Synergistetes}, \textit{Spirochaetes}, \textit{Firmicutes}, \textit{Delta}- and \textit{Epsilon}-proteobacteria, \textit{Lentisphaerae}, \textit{Chloroflexi}, and \textit{Verrucomicrobia}; (ii) samples most from the wet season of C7B with dominant phyla of \textit{Tenericutes}, \textit{Nitrospirae}, \textit{Acidobacteria}, \textit{Beta}-proteobacteria, \textit{Chlorobi}, \textit{Crenarchaeota}, \textit{Actinobacteria}; (iii) composed of samples from surface, right channel, left channel at high tide during wet season together with samples from Pargos, with dominant phyla of \textit{Alpha}- and \textit{Gamma}-proteobacteria, \textit{Bacteroidetes}; (iv) composed of samples from low tide at right channel, left channel, and mouth of Pargos with dominant phyla of \textit{Fusobacteria}, \textit{Chlamydiae}, and \textit{Deferribiaeteres}, \textit{Cyanobacteria}. Generally, the samples from above the halocline in C7B are freshwater end members, from below the halocline in C7B, low tide in Pargos are mixing members, and from high tide are salt end members (Figure 2-9(A)).

Samples from C7B and Pargos were clearly separated along axis 1, with 44.7% of total variation due to salinity, \( SO_4^{2-} \), ORP and pH (physiochemical parameters); However, samples from above and below the halocline in C7B during two seasons, as well as samples at low tide and high tide in Pargos, were separated along axis 2 with 15.8 % of total variation explained by the gradients of \( NO_3^- \), \( NH_4^+ \) and \( PO_4^{3-} \) (nutrient parameters), which indicated the spatial variation was greater than seasonal and tidal
variation from groundwater to marine spring system, and Fortunato et al. (2012) also found that spatial variability overwhelmed seasonal patterns in bacterioplankton across a river to ocean gradient. *Alpha- and Gamma-Proteobacteria* clustered with higher salinity than that did *Delta-, Epsilon-, and Beta-Proteobacteria*; *Epsilon-, Delta-, and Gamma-Proteobacteria* clustered with higher NH$_4^+$ and PO$_4^{3-}$ concentrations than did *Beta- and Alpha-Proteobacteria* (Figure 2-9(A)).

**Connection pattern:** A phylogenetic network was used to study the similarities in community structures between sites based on phylogenetic differences (weighted UniFrac distance) (Figure 2-9(B)). The separation of microbial communities was much greater according to spatial variation than to temporal variation. Samples from C7B clustered separately from Pargos, and the two clusters showed a significantly different from each other ($P=0.001$, $R=0.81$; ANOSIM). The exceptions were the samples taken from the right channel at low tide, which appeared between the Pargos and C7B clusters, and the left channel during low tide in Pargos, which appeared apart from either major cluster. Compared with the left channel, the sample from the right channel at low tide showed many more connections (i.e., greater similarity) with other samples from both C7B and Pargos, suggesting that the right channel rather then left channel may be representative of a hydrological connection between groundwater with a composition and microbial community similar to the the groundwater sampled at C7B and Pargos.

**The Interactions between Microbial Communities along the Salinity Gradient from C7B to Pargos**

To determine the changes in the interactions between microbial communities (exclude archaea from this analysis because they are missing at some depths) along
the salinity gradient from C7B to Pargos, three co-occurrence networks were constructed based on spearman correlations at the genus level for all water samples collected from above the halocline (salinity=0.74±0.10, samples from 20m to 29m), below the halocline (salinity=4.05±4.29, samples from 29m to 32m) in C7B, and high tide (salinity=32.04±1.92, samples from surface, mouth, and channels at high tide) in Pargos. Correlation coefficients with values of r>0.8 at significant level (P<0.05) were chosen for constructing the network (Figure 2-10(A)). More phylotypes with higher connection densities and more closely connected subgroups were observed in the lower salinity zone than that in the higher salinity zone (Figure 2-10(B) and Table 2-7). The proportion of positive connections and the connections between different phyla decreased, while the proportion of negative together with the connections within same phyla increased, along the salinity gradient from C7B to Pargos (Figure 2-10(B) and Table 2-7). The numbers of phyla involved in the network decreased from 23 to 15 with increased salinity, and the proportion of connections with Proteobacteria dominated all networks independent of salinity. Alphaproteobacteria was the dominant class in low salinity zone, Gammaproteobacteria was the dominant connection in the high salinity zone. Actinobacteria, Betaproteobacteria, Firmicutes, and Planctomycetes showed a higher proportion of connections in C7B than that in high salinity zones of Pargos. A higher proportion of connections with Cyanobacteria was found above the halocline in C7B and in Pargos in our study than below halocline in C7B, and the connections with Nitrospirae just presented above the halocline in C7B (Figure 2-10(B)).
Discussion

Microbial Community Variation in the Stratified Cenote-C7B

The effects of seasonal hydrological dynamics on microbial community in the stratified cenote-C7B

The hydrological variation that accompanied recharge during different seasons has considerable effects on the dynamics of microbial communities suspended in groundwater in the estuary area (Haack et al., 2004; Bougon et al., 2010; Zhou et al., 2012; Ben Maamar et al., 2015). A greater seasonal variation was observed in microbial communities above the halocline relative to below halocline (Figure 2-6 (B)). Generally, Proteobacteria was the dominant phylum in the cenote during the two seasons (Figure 2-3 (A)), which was consistent with other groundwater studies (Lin et al., 2012; Zhou et al., 2012; Ben Maamar et al., 2015; Ye et al., 2015), while the contribution of Firmicutes was highly variable between the two seasons, which may be due to changes in organic carbon and dissolved oxygen (Tian et al., 2014). A higher diversity with low bacterial numbers (16S rRNA gene data in Table 2-1) was observed after recharge in the wet season in comparison with the dry season, which was consistent with Bougon et al. (2010). This is in contrast with Ginige et al. (2013), who found that bacterial numbers increased and microbial diversity was reduced after recharging. It should be noted that lower copies of 16S rRNA genes with higher diversity was observed in the wet season, even though some studies showed the reverse results between the number of microbes and diversity (Zhou et al., 2012; Ginige et al., 2013). We speculate that the decreased copy numbers of 16S rRNA genes can be explained by the dilution of the indigenous microbial communities during recharge process (Pronk et al., 2009), and the higher diversity was attributed to either the external microorganisms input by recharge.
including runoff or percolation from surrounding soil after rain (Allton et al., 2007; Bougon et al., 2010; Zhou et al., 2014; Ben Maamar et al., 2015), or growth of new microorganisms supported by fresh electrons and/or nutrients, carbon source inputs during recharge (Haack et al., 2004; Le et al., 2016).

**Depth related effects on microbial community in the stratified cenote-C7B**

Depth was an important factor to differentiate the composition of microbial communities in water system (Fortunato et al., 2012; Lin et al., 2012), especially in deep sinkhole with physicochemical gradients (Seymour et al., 2007). The separation of microbial community structures above and below the halocline in C7B was correlated with NO$_3^-$ concentrations at a significant level ($P=0.014$), with NH$_4^+$, PO$_4^{3-}$ at non-significant levels along the second axis of the RDA plot (Figure 2-9 (A)). The higher connection with *Cyanobacteria* above the halocline relative to below the halocline in C7B was likely due to light intensity (Sahl et al., 2010), which may be one possible reason contributing higher ORP at top of C7B.

The higher ORP, and/ or lack of organic carbon and dissolved Fe may not be in favor of complete denitrification process occurring (Ben Maamar et al., 2015) even though a higher representative of *Betaproteobacteria* (Figure 2-3 (A) and 2-9(A)) related to some potential denitrifiers at the top of groundwater system (Hemme et al., 2010; Li et al., 2014). Conversely, those conditions are favorable for nitrification synchronized with a slightly higher contribution by the potential ammonia oxidizing *Nitrospirae* at the top of C7B (Daims et al., 2015). A potentially strong nitrification process coupled with a weak denitrification process would result in accumulation of NO$_3^-$ at the top of C7B (Huang, et al., N cycling paper, in prep).
Larger proportions of *Beta- and Gamma-proteobacteria*, as well as *Actinobacteria* were observed above the halocline than below the halocline during both seasons (Fig 2-3(A)) which might be due to water transfer from surrounding soil layer (Bougon et al., 2010; Ginige et al., 2013; Unno et al., 2015), and/or their preference in fresh water conditions (Fortunato and Crump, 2015; Ye et al., 2015). Lower diversity was observed at the top of C7B during two seasons (Table 2-1), which may be due to either higher disturbance level (Torsvik et al., 2002), or lower nutrients (Haack et al., 2004; Bougon et al., 2010) at the top than the bottom of C7B, which was supported by the significant positive correlation between nutrients (NH$_4^+$, PO$_4^{3-}$) and $\alpha$ diversity in C7B (Figure 2-5).

**Microbial Community Variation in the SGD Spring- Pargos**

Yucatan current, tides, and wind were the three major factors controlled the submarine groundwater discharge (SGD) and saltwater intrusion into the spring (Pargos) during dry and wet periods (Parra et al., 2016). SGD during low tide would bring brackish components (e.g. N-nutrients, metals) together with the microorganisms inhabiting the brackish water (e.g. *Firmicutes, Epsilonproteobacteria*, in Figure 2-6 (A)) to the ocean, while saltwater intrusion during high tide will push saltwater components (e.g. SO$_4^{2-}$) together with the saltwater representative microorganisms (e.g. *Bacteroidetes, Cyanobacteria*, in Figure 2-6 (A)) into the spring. The prevailing conditions may change dynamically over a short time period of tidal cycle (< 1day) due to SGD and sea level fluctuation (Lee et al., 2014; Santoro, 2010), which may explain the greater variation in structures of the microbial communities between different tides than that between different seasons and depths (Figure 2-7 (A)).
A large proportion of common microorganisms (44.5%) between the high and low tides at the channels of Pargos (Figure 2-7 (B)) may be because of the development of diverse functional and broadly adapted communities over the long-term course of alternating discharge and intrusion processes (Bougon et al., 2010; Santoro, 2010; Zhou et al., 2012). The similarity in microbial community structures between the right channel in Pargos and C7B (Figure 2-9(B)) was likely controlled much more by SGD than by saltwater intrusion. Higher microbial diversity was observed in low tide when compared with high tide (Figure 2-7 (C)), which indicated that SGD acted as a seed bank for diversity rather than that microorganisms proliferation due to higher nutrient from SGD (Mason et al., 2016), a contention that is supported by the non-significant correlation between nutrients (NH$_4^+$, PO$_4^{3-}$) and $\alpha$ diversity in Pargos (Fig 2-8).

**The Effects of Hydrologic Mixing on Microbial Community in Mixing Zone between C7B and Pargos**

Three end members were identified by RDA analysis in Figure 2-11(A). If the microbial communities in the mixing zone can be described by mixing freshwater and saltwater endmembers, then we can develop a mixing model by nonreactive tracer chloride in (Equation 2-1; Table 2-8) to estimate the abundance of microbial communities and to study how the physical mixing process might be related to the composition of the microbial community in the mixing zone. After we compared the prediction value from model with in situ value, we found that the majority of genera did not fit the model with around 147 genus falling out as the outliers by using residual test (Figure 2-11(B)). Among of 147 outliers, genus belonging to *Epsilonproteobacteria*, *Firmicutes* with higher positive residual values had higher relative abundance in the
mixing zone, while genus belonging to *Bacteroidetes, Alphaproteobacteria, Betaproteobacteria, Cyanobacteria* with higher negative residual values had higher relative abundance in the two endmember zones (Figure 2-11(A and C)). which indicated those genus were much more sensitive to the environmental changes, and the composition and structure of microbial communities were more controlled by environmental factors cause by mixing processes other than the single physical mixing processes and in agreement with the study from Le et al. (2016). The white filaments flowing out of the spring mouth during the low tide possible attributed to the filament producer, sulfide-oxidizing bacteria *Arcobacter*, who was enriched in mixing zone with highest positive residual value and produced filamentous sulfur (Wirsen et al., 2002).

The values of all α diversities were higher than could be explained strictly by the mixing model (Table 2-8), which may explain the higher microbial diversity in the mixing zone in comparison with two endmembers and affect the microbial diversity in coastal area when groundwater discharged during the low tides. Higher DOC and nutrient concentrations may greatly contribute to growth of diverse microbial groups in mixing zone (Le et al., 2016; Bougon et al., 2012). Stegen et al. (2016) said that the mixing of complementary electron donors and acceptors in the transition zones usually had higher diversity, which suggested SGD also acted as a seed bank for microbial diversity to marine system as similar as riverine area did (Mason et al., 2016).

**Response of Microbial Communities to Steep Salinity Gradients from Cenote C7B to Spring Pargos**

Many studies showed that salinity were the primary drivers structuring microbial communities across a river to ocean gradient (Fortunato et al., 2012; Fortunato and Crump, 2015; Mason et al., 2016), even though Edmonds et al. (2009) didn’t find any
significant differences between microbial community composition in their short term of control and seawater amendment experiments, except for microbial activities. In our field study across cenote to SGD spring, results also displayed that samples were separated with salinity along the first coordinate at significant level ($P=0.001$) by RDA analysis (Fig 2-9(A), and Table 2-6). *Proteobacteria* presented a clear distribution pattern along salinity gradient from cenote to spring, with a dominance of *Betaproteobacteria* in the freshwater regions (top of C7B), and *Alphaproteobacteria* in the saltwater regions (Pargos), and a prominent peak of *Verrucomicrobia* phylum at the brackish water regions (bottom of C7B), which was in accord with previous studies (Bouvier and del Giorgio 2002; Herlemann et al., 2011; Fortunato and Crump, 2015).

Lots of studies summarized the adverse effect of salinity on the microbial diversity (Fortunato et al., 2012; Severin et al., 2012; Mason et al., 2016), but still some other studies found that salinity was positive related to bacterial abundance (Jackson and Vallaire, 2009; Morrissey et al., 2014). Positive effects were found in cenote (C7B) along the water profile, while negative effects in spring (Pargos) during tidal cycle in our study. So the composition and structure of microbial community changes were possible partially attributed to the salinity changes (Severin et al., 2012), and partially attributed to nutrients, depth and the hydrological physical process in our study, which was already reported in many other researches (Haack et al., 2004; Fortunato et al., 2012; Unno et al., 2015; Mason et al., 2016). Higher microbial diversity was shown during low salinity periods at low tide in Pargos may due to the extra microorganisms input from SGD (Moore, 2010; Mason et al., 2016).
Also the salinity would affect the interaction between different microbes, the negative correlations in network (more than 25% of the total connections in Table 2-7) might be caused by antagonistic associations between microorganisms, which was consistent with other studies (Lima-MendezG et al., 2015; Milici et al., 2016). Meanwhile the negative correlations between different phyla and the proportion of connections within the same phylum were increased with increased salinity gradient (Fig 2-10 (B)), which indicates that salinity may shape community antagonistic interaction and establish of mutual exclusion processes between different phyla, yet enhance the cooperative interaction within the same phylum (Milici et al., 2016). Whereas *Proteobacteria was* dominant in the network connections and was independent of salinity gradient, which was possible due to their high abundance and diverse function in both groundwater and saltwater regions (Griebler and Lueders, 2009; Ginige et al., 2013; Li et al., 2014).

For the subgroup of *Proteobacteria, a higher proportion of connections between Alphaproteobacteria, Betaproteobacteria and other communities in anoxic groundwater system-C7B was possible because of their versatile function as same. Some related to sulfur cycle (e.g. *Rhodobacter: Alpha-47; İnceoğlu et al. (2015)), and linked sulfur cycle together with carbon and nitrogen cycle (e.g. *Methylophilaceae: Beta-23 in Figure 2-10(A)) (Friedrich et al., 2001; Kalyuzhnaya et al., 2009; Eyice et al., 2015), and some involving in methane oxidation process (*Methylosinus:Alpha-27 in Figure 2-10(A)) (Chistoserdova, 2011). While a higher number of connections with *Epsilonproteobacteria in oxic marine system-Pargos compared to groundwater system was greatly attributed to the sulphidic habitats in marine system (Campbell et al., 2006).
Summary

We studied the hydrological dynamics on microbial communities along physicochemical gradient by systematically combining the terrestrial stratified groundwater with its related SGD. The study also provide insights into the potential challenge for indigenous groundwater microbial communities caused by environmental fluctuations, such as the recharge process and its related changes in electrons and nutrients during different seasons, depth related factors including light accessibility and disturbance level, as well as saltwater intrusion during tidal cycles. We found that salinity, however was one of the complex reasons that contributed to the changes in the composition and structure of microbial community, the spacial variation in microbial communities was simultaneously attributed to the changes in other physiochemical gradient and hydrological process, and was greater than seasonal changes from cenote to spring. Fortunato et al. (2012) also found that spatial variability overwhelmed seasonal patterns in bacterioplankton across river to ocean gradient. Specifically, we realized that lower diversity may attribute to higher disturbance level, and/or low nutrient as well as dilution effects at the top of groundwater system. Lights accessibility would also affects some oxidation and reduction processes in the deep groundwater system. While in the spring, SGD served as an important role in contributing the microbial diversity during the tidal cycles at current sea level in marine system. Once the sea level rise perennially in the future, the microbial community would be shifted to high salinity dominant groups due to single saltwater intrusion process and the sensitivity of microbial communities to salinity changes in groundwater (Unno et al., 2015), and meanwhile more mutual exclusive interactions among microbial communities would be built.
Table 2-1. 16S rRNA copy number, sequence reads, OTUs and the percentage of bacteria and archaea in water samples collected from C7B in April (dry season) and September (wet season), 2014

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<th>Samples</th>
<th>16S rRNA (Copy/L)</th>
<th>raw reads</th>
<th>paired_end reads</th>
<th>non_chimera filtered reads</th>
<th>Chimeras filtered Reads</th>
<th>OTUs</th>
<th>Unassigned (%)</th>
<th>Bacteria (%)</th>
<th>Archaea (%)</th>
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</tr>
<tr>
<td>W-C7B20</td>
<td>5.08E+08</td>
<td>182202</td>
<td>98051</td>
<td>97316</td>
<td>97101</td>
<td>1032</td>
<td>2.158%</td>
<td>97.813%</td>
<td>0.030%</td>
</tr>
<tr>
<td>W-C7B28</td>
<td>1.51E+09</td>
<td>111847</td>
<td>63134</td>
<td>62518</td>
<td>62400</td>
<td>942</td>
<td>15.627%</td>
<td>84.309%</td>
<td>0.064%</td>
</tr>
<tr>
<td>W-C7B29</td>
<td>6.34E+08</td>
<td>117391</td>
<td>68381</td>
<td>67860</td>
<td>67681</td>
<td>1079</td>
<td>6.356%</td>
<td>93.633%</td>
<td>0.010%</td>
</tr>
<tr>
<td>W-C7B30</td>
<td>2.04E+09</td>
<td>139607</td>
<td>85172</td>
<td>84496</td>
<td>84360</td>
<td>1068</td>
<td>1.290%</td>
<td>98.710%</td>
<td>0.000%</td>
</tr>
<tr>
<td>W-C7B32</td>
<td>6.16E+08</td>
<td>101354</td>
<td>61639</td>
<td>60715</td>
<td>60611</td>
<td>2182</td>
<td>6.042%</td>
<td>93.942%</td>
<td>0.016%</td>
</tr>
<tr>
<td>Total</td>
<td>1002908</td>
<td>530606</td>
<td>524610</td>
<td>517231</td>
<td>9843</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>7.10E+09</td>
<td>111434.2</td>
<td>58956.2</td>
<td>58290.0</td>
<td>57470.1</td>
<td>1093.7</td>
<td>4.137%</td>
<td>95.847%</td>
<td>0.016%</td>
</tr>
</tbody>
</table>
Table 2-2. Phyla with the mean relative abundance > 1% for samples collected from C7B in April (dry season) and September (wet season), 2014

<table>
<thead>
<tr>
<th></th>
<th>Proteobacteria</th>
<th>Firmicutes</th>
<th>Actinobacteria</th>
<th>Cyanobacteria</th>
<th>Bacteroidetes</th>
<th>Planctomycetes</th>
<th>Chlorobi</th>
<th>Verrucomicrobia</th>
<th>Chloroflexi</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-C7B28</td>
<td>63.06%</td>
<td>18.21%</td>
<td>12.02%</td>
<td>2.59%</td>
<td>0.77%</td>
<td>1.82%</td>
<td>0.19%</td>
<td>0.47%</td>
<td>0.06%</td>
</tr>
<tr>
<td>D-C7B29</td>
<td>57.48%</td>
<td>28.38%</td>
<td>9.43%</td>
<td>2.56%</td>
<td>0.21%</td>
<td>0.92%</td>
<td>0.06%</td>
<td>0.35%</td>
<td>0.04%</td>
</tr>
<tr>
<td>D-C7B30</td>
<td>48.27%</td>
<td>32.49%</td>
<td>0.77%</td>
<td>6.45%</td>
<td>1.02%</td>
<td>0.49%</td>
<td>0.04%</td>
<td>2.02%</td>
<td>0.72%</td>
</tr>
<tr>
<td>D-C7B32</td>
<td>17.37%</td>
<td>27.74%</td>
<td>3.83%</td>
<td>23.94%</td>
<td>3.65%</td>
<td>1.92%</td>
<td>0.06%</td>
<td>10.58%</td>
<td>3.41%</td>
</tr>
<tr>
<td>W-C7B20</td>
<td>49.82%</td>
<td>8.56%</td>
<td>18.75%</td>
<td>1.18%</td>
<td>1.37%</td>
<td>9.02%</td>
<td>0.95%</td>
<td>0.30%</td>
<td>0.39%</td>
</tr>
<tr>
<td>W-C7B28</td>
<td>38.05%</td>
<td>0.67%</td>
<td>14.38%</td>
<td>1.89%</td>
<td>1.31%</td>
<td>3.43%</td>
<td>11.53%</td>
<td>0.87%</td>
<td>0.22%</td>
</tr>
<tr>
<td>W-C7B29</td>
<td>56.01%</td>
<td>7.83%</td>
<td>9.00%</td>
<td>2.18%</td>
<td>4.77%</td>
<td>2.94%</td>
<td>2.94%</td>
<td>0.45%</td>
<td>0.60%</td>
</tr>
<tr>
<td>W-C7B30</td>
<td>46.33%</td>
<td>6.85%</td>
<td>1.77%</td>
<td>0.02%</td>
<td>26.09%</td>
<td>0.34%</td>
<td>0.97%</td>
<td>0.12%</td>
<td>0.42%</td>
</tr>
<tr>
<td>W-C7B32</td>
<td>64.43%</td>
<td>10.15%</td>
<td>1.68%</td>
<td>0.18%</td>
<td>1.58%</td>
<td>1.09%</td>
<td>0.29%</td>
<td>0.08%</td>
<td>4.69%</td>
</tr>
<tr>
<td>Average</td>
<td>48.98%</td>
<td>15.65%</td>
<td>7.96%</td>
<td>4.55%</td>
<td>4.53%</td>
<td>2.44%</td>
<td>1.89%</td>
<td>1.69%</td>
<td>1.17%</td>
</tr>
<tr>
<td>Std</td>
<td>12.98%</td>
<td>10.21%</td>
<td>5.69%</td>
<td>6.72%</td>
<td>7.35%</td>
<td>2.40%</td>
<td>3.34%</td>
<td>3.03%</td>
<td>1.50%</td>
</tr>
</tbody>
</table>

Std: Standard deviation
Table 2-3. 16S rRNA copy number, sequence reads, OTUs and the percentage of bacteria and archaea in water samples collected from Pargos in April (dry season) and September (wet season), 2014

<table>
<thead>
<tr>
<th>Samples</th>
<th>16S rRNA (Copy/L)</th>
<th>raw reads</th>
<th>paired_end joined reads</th>
<th>non_chimeras filtered reads</th>
<th>Chimeras filtered Reads</th>
<th>OTUs</th>
<th>Unassigned (%)</th>
<th>Bacteria (%)</th>
<th>Archaea (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-SPs</td>
<td>1.50E+09</td>
<td>47740</td>
<td>27273</td>
<td>26859</td>
<td>26789</td>
<td>980</td>
<td>0.433%</td>
<td>99.518%</td>
<td>0.049%</td>
</tr>
<tr>
<td>D-SPm1</td>
<td>8.24E+09</td>
<td>196077</td>
<td>105278</td>
<td>102306</td>
<td>102032</td>
<td>3914</td>
<td>2.673%</td>
<td>97.317%</td>
<td>0.011%</td>
</tr>
<tr>
<td>D-SPm2</td>
<td>6.21E+09</td>
<td>91377</td>
<td>51236</td>
<td>49852</td>
<td>49762</td>
<td>2706</td>
<td>2.964%</td>
<td>97.030%</td>
<td>0.006%</td>
</tr>
<tr>
<td>W-SPs-LT</td>
<td>3.72E+09</td>
<td>25118</td>
<td>25,118</td>
<td>16996</td>
<td>16976</td>
<td>477</td>
<td>0.271%</td>
<td>99.652%</td>
<td>0.077%</td>
</tr>
<tr>
<td>W-SPm-LT</td>
<td>3.60E+08</td>
<td>29608</td>
<td>29,608</td>
<td>16655</td>
<td>16699</td>
<td>3225</td>
<td>12.600%</td>
<td>87.400%</td>
<td>0.000%</td>
</tr>
<tr>
<td>W-SPr-LT</td>
<td>5.92E+08</td>
<td>118708</td>
<td>64526</td>
<td>60473</td>
<td>60443</td>
<td>5803</td>
<td>10.855%</td>
<td>89.107%</td>
<td>0.038%</td>
</tr>
<tr>
<td>W-SPL-LT</td>
<td>2.73E+06</td>
<td>2349</td>
<td>724</td>
<td>702</td>
<td>701</td>
<td>134</td>
<td>8.845%</td>
<td>91.155%</td>
<td>0.000%</td>
</tr>
<tr>
<td>W-SPr-HT</td>
<td>4.05E+09</td>
<td>71426</td>
<td>35985</td>
<td>35085</td>
<td>34822</td>
<td>1218</td>
<td>2.217%</td>
<td>97.783%</td>
<td>0.000%</td>
</tr>
<tr>
<td>W-SPL-HT</td>
<td>3.77E+09</td>
<td>105766</td>
<td>49994</td>
<td>48948</td>
<td>48576</td>
<td>1256</td>
<td>2.055%</td>
<td>97.945%</td>
<td>0.000%</td>
</tr>
<tr>
<td>Total</td>
<td>688169</td>
<td>389742</td>
<td>357876</td>
<td>356800</td>
<td>19713</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>3.56E+09</td>
<td>76463.2</td>
<td>43304.7</td>
<td>39764.0</td>
<td>39644.4</td>
<td>2190.3</td>
<td>4.705%</td>
<td>95.276%</td>
<td>0.020%</td>
</tr>
</tbody>
</table>
Table 2-4. Phyla with the mean relative abundance > 1% for samples collected from Pargos in April (dry season) and September (wet season), 2014

<table>
<thead>
<tr>
<th></th>
<th>Proteobacteria</th>
<th>Bacteroidetes</th>
<th>Cyanobacteria</th>
<th>Firmicutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-SPs</td>
<td>66.67%</td>
<td>20.53%</td>
<td>9.69%</td>
<td>0.12%</td>
</tr>
<tr>
<td>D-SPm1</td>
<td>76.52%</td>
<td>8.96%</td>
<td>7.50%</td>
<td>0.07%</td>
</tr>
<tr>
<td>D-SPm2</td>
<td>77.07%</td>
<td>8.98%</td>
<td>7.14%</td>
<td>0.07%</td>
</tr>
<tr>
<td>W-SPs-LT</td>
<td>44.87%</td>
<td>39.00%</td>
<td>8.62%</td>
<td>6.02%</td>
</tr>
<tr>
<td>W-SPm-LT</td>
<td>64.41%</td>
<td>2.40%</td>
<td>3.08%</td>
<td>0.30%</td>
</tr>
<tr>
<td>W-SPr-LT</td>
<td>38.62%</td>
<td>4.54%</td>
<td>2.38%</td>
<td>31.70%</td>
</tr>
<tr>
<td>W-SPL-LT</td>
<td>67.76%</td>
<td>1.43%</td>
<td>2.14%</td>
<td>2.28%</td>
</tr>
<tr>
<td>W-SPr-HT</td>
<td>62.58%</td>
<td>29.61%</td>
<td>4.72%</td>
<td>0.26%</td>
</tr>
<tr>
<td>W-SPL-HT</td>
<td>67.56%</td>
<td>23.37%</td>
<td>6.46%</td>
<td>0.08%</td>
</tr>
<tr>
<td>Average</td>
<td>62.89%</td>
<td>15.42%</td>
<td>5.75%</td>
<td>4.55%</td>
</tr>
<tr>
<td>Std</td>
<td>11.67%</td>
<td>11.89%</td>
<td>2.49%</td>
<td>9.28%</td>
</tr>
</tbody>
</table>

Std: Standard deviation
Table 2-5. Lengths of gradient test in C7B and Parogs by Detrended Correspondence Analysis (DCA)

<table>
<thead>
<tr>
<th>DCA</th>
<th>C7B_Pargos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lengths of gradient</td>
<td>2.579</td>
</tr>
</tbody>
</table>
Table 2-6. Permutation test of all constrained eigenvalues for microbial communities in phylum level (relative abundance %) by Redundancy analysis (RDA)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Variance explained</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>49%</td>
<td>0.001</td>
</tr>
<tr>
<td>Salinity</td>
<td>36%</td>
<td>0.001</td>
</tr>
<tr>
<td>ORP</td>
<td>66%</td>
<td>0.695</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>68%</td>
<td>0.8</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>59%</td>
<td>0.014</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>74%</td>
<td>0.099</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>74%</td>
<td>0.929</td>
</tr>
<tr>
<td></td>
<td>Network density</td>
<td>Proportion of positive connections</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>C7B_AH</td>
<td>0.17</td>
<td>0.74</td>
</tr>
<tr>
<td>C7B_BH</td>
<td>0.12</td>
<td>0.68</td>
</tr>
<tr>
<td>Pargos_HS</td>
<td>0.15</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Table 2-8. Hydrologically mixing model based on the endmembers in RDA analysis in figure 7A by using nonreactive Chloride trace (µM), and the values of α diversity in situ and form modeling prediction based on hydrological model.

<table>
<thead>
<tr>
<th></th>
<th>Fresh endmember</th>
<th>Salt endmember</th>
<th>Mixing zone (in situ)</th>
<th>Mixing zone (model)</th>
<th>Deviation (model/in situ)</th>
<th>Mixing model (Cl- based)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-</td>
<td>6.40</td>
<td>602.62</td>
<td>433.85</td>
<td>433.85</td>
<td>1</td>
<td>M_{Cl}=28.31%*C_{f_{Cl}}+71.69%*C_{s_{Cl}}</td>
</tr>
<tr>
<td>Chao1</td>
<td>1041.10</td>
<td>752.01</td>
<td>5212.59</td>
<td>833.85</td>
<td>0.16</td>
<td>M_{Chao1}=28.31%*C_{f_{Chao1}}+71.69%*C_{s_{Chao1}}</td>
</tr>
<tr>
<td>Shannon</td>
<td>5.98</td>
<td>5.31</td>
<td>8.38</td>
<td>5.50</td>
<td>0.66</td>
<td>M_{Shannon}=28.31%*C_{f_{Shannon}}+71.69%*C_{s_{Shannon}}</td>
</tr>
<tr>
<td>PD whole tree</td>
<td>51.43</td>
<td>39.88</td>
<td>211.47</td>
<td>43.15</td>
<td>0.20</td>
<td>M_{PD whole tree}=28.31%*C_{f_{PD whole tree}}+71.69%*C_{s_{PD whole tree}}</td>
</tr>
</tbody>
</table>
Figure 2-1. Location and Sampling sites. A) C7B. B) Spring-Pargos. Red circles are sampling depths from each site.
Figure 2-2. Precipitation pattern in Yucatan. Data was collected from 2000-2012 and provided by Meteorological Assimilation Data Ingest System (MADIS) at National Oceanic and Atmospheric Administration (NOAA) in Sampling sites. Asterisks (**) indicated significant difference between April and September at P<0.01 level.
Figure 2-3. Microbial community composition in C7B. A) Relative abundance of dominant (top 20) phylum groups. B) Proteobacteria. C) Dominant (top 10) shared genus.
Figure 2-4. Diversity analysis of microbial communities in C7B. A) β Diversity (PcoA) based on Unifrac distance of microbial communities. B) Venn diagram showing the shared and unshared of microbial communities at genus level. C) α diversity (Chao1, Shannon, and PD_whole_tree).
Figure 2-5. Correlation analysis between Environmental parameters and α diversity of microbial communities base on Pearson method. The correlation coefficient a significance level (P ≤ 0.05) was shown in the graph without cross.
Figure 2-6. Microbial community composition in Pargos. A) Relative abundance of dominant (top 20) phylum groups. B) Proteobacteria. C) Dominant (top 10) shared genus.
Figure 2-7. Diversity analysis of microbial communities in Pargos. A) β Diveristy (PcoA) base on Unifrac distance of microbial communities. B) Venn diagram showing shared and unshared of microbial communities at genus level. C) α diversity (Chao1, Shannon, and PD_whole_tree).
Figure 2-8. Correlation analysis between Environmental parameters and α diversity of microbial communities based on Pearson method. The correlation coefficient at a significance level (P ≤ 0.05) was shown in the graph without cross.
Figure 2-9. Microbial community clustering for C7B and Pargos. A) Redundancy discriminant analysis (RDA) based on the relative abundance of top 20 phyla and environmental factors. B) Network analysis for C7B and Pargos based on OTUs by using phylogenetic distance with maximum value of 0.99.
Figure 2.10. Co-occurrence network between microbial communities along a salinity gradient from C7B to Pargos. A) Network with Red and gray color represent negative and positive correlation, respectively. B) The proportion of connections for each phylum in the network.
Figure 2-11. Mixing model analysis. A) Endmembers: fresh endmember (i, C7B), salt endmember (ii, Pargos) and mixing zone (iii) based on RDA analysis. B) Comparison prediction value with insitu value. C) Residual value of top 20 genus
CHAPTER 3
MICROORGANISMS RELATED TO SULFUR CYCLING ACROSS A SALINITY
GRADIENT FROM CENOTE TO SUBMARINE GROUNDWATER DISCHARGE IN
YUCATAN

Background

The sulfur cycle, as one of the most important cycles linked with C and N cycles, provides an important energy source for microbial activities in sulfuric aquifer systems (Elshahed et al., 2003; Muyzer and Stams, 2008; Baker et al., 2015), such as in deep stratified groundwater systems (e.g. cenotes, caves) and related submarine groundwater discharge (SGD) springs. Studies on the sulfur cycle in deep groundwater systems can be traced back to the early 1990s (Stoessell et al., 1993; Socki et al., 2002; Engel, 2007; Pedersen, 2007; Pedersen et al., 2014), and similar studies for springs and SGD date back to the 1880s (Winogradsky, 1887; Trudinger and Swaine, 1979; Elshahed et al., 2003; Fishbain et al., 2003; Dillon et al., 2007; Chaudhary et al., 2009; Häusler et al., 2014; McAllister et al., 2015; Frolov et al., 2016). However, those previous studies discuss these two potentially connected systems separately and few, if any, reports try to place the groundwater and its related SGD together as a salinity gradient. In our study, we studied groundwater and spring systems to investigate the differences along the salinity gradient with respect to sulfur cycling and its related microbial communities.

In sulfuric habitats, the microbial communities related to sulfur cycling are clearly influenced by pH, salinity, concentrations of sulfide and sulfate, redox potential (ORP), light availability, and organic carbon content (Elshahed et al., 2003). Wawer (1997) and Nalven (2011) reported that salinity, organic carbon, H₂ and O₂, as well as some pollutants control the diversity and activities of sulfate reducing bacteria (SRB), and
Macalady et al. (2008) and Grünke et al. (2011) found that the concentration of $O_2$ and sulfide, as well as the morphological traits are important factors shaping the ecological niches for sulfur oxidizing bacteria (SOB). However, these studies did not synchronously compare the balance between SRB and SOB across salinity gradients. In our study, we studied the difference in ecological niches for SRB and SOB from groundwater to SGD along a steep environmental gradient, that includes gradients in pH, ORP, salinity, DOC, $SO_4^{2-}$, and light availability.

Deep stratified groundwater systems (e.g. cenotes, caves) and their related submarine groundwater discharge (SGD) springs provide very good natural ecosystems that can be used for studying the interactions within the bio-hydro-sphere, where the sulfur related microbial communities represent the biosphere, the sulfur related compounds and environmental conditions in water bodies represent the hydrosphere. They also provide good systems for interpreting the linkage between those microbial communities and their relevant ecological processes (Muyzer and Stams, 2008; Llorens-Marès et al., 2015). In our study, we will focus on the variation of sulfur cycles and their related microbial communities in a groundwater system in different seasons and in a spring system during different tides, together with the interactions between the two compartments to investigate the distribution of these organisms along the salinity gradient.

We hypothesize that sulfate reducing bacteria will be higher in C7B (high DOC and Low ORP) than that in Pargos (low DOC and high ORP), and Sulfide oxidizing bacteria representation will be highest at mouth of spring during low tide. The primary objectives of this study were to study the distribution pattern and niche partitioning of
potential microbial communities related to sulfate reduction and oxidation processes, as well as to evaluate the relative numbers of copies of SRB along the environmental gradient from groundwater (cenote) to an offshore spring.

**Materials and Methods**

**Site Description**

The sampling site is same to the Chapter 2

**Sample Collection**

The sample collection is same to the Chapter 2

**Samples Processing**

DNA extraction, quantitative PCR (qPCR) and sequencing:

1. DNA was extracted from filters using Powerwater® Sterivex DNA Isolation Kit (Mobio, Carlsbad, CA, USA) (Hollibaugh et al., 2014), and then diluted to $10^{-1}$ and stored at -20 °C.

2. Bacteria abundance was estimated using published primer 16S (Haems et al., 2003) by the quantitative polymerase chain reaction (qPCR) approach.

3. Sequencing: V3 and V4 regions with approximately ~460 bp of 16S rRNA was amplified by PCR and sequenced by Illumina MiSeq System (Klindworth et al., 2013) base on the protocol named “16S Metagenomic Sequencing Library Preparation-Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System”.

**Chemical analysis:** The YSI ProPlus sonde (calibrated daily) recorded salinity, pH, oxidation-reduction potential (ORP). Anion concentration of $\text{SO}_4^{2-}$, was analyzed with an automated Dionex DX500 Ion Chromatograph, $\text{H}_2\text{S}$ concentrations were analyzed in the field according to the method of Cline (1969). DOC concentrations were analyzed on a Shimadzu TOC-VCSN total organic carbon analyzer, and the coefficient of variance was less than 2%. All analyses were completed in the Department of Geological Sciences, UF.
Data Analysis

1. Sequences analysis: It was conducted by using pipeline for 16S rRNA analysis in QIIME (Caporaso et al., 2010), and after that, the potential sulfate reduction bacteria (SRB) and sulfide oxidation bacteria (SOB) was selected and presented in table 2 for further analysis based on publish papers (Voordouw et al., 1996; Friedrich et al., 2001; Cytryn et al., 2005; Rabus et al., 2006; Hubert and Voordouw, 2007; Frigaard and Dahl, 2009; Häusler et al., 2014; Eyice et al., 2015; Pokorna and Zabranska, 2015; Santana et al., 2016).

2. Co-occurrence network analysis: The relationship between microbial communities were analyzed by package Bioconductor and igraph network analysis in R using “Spearman correlation” analysis (McMurdie and Holmes, 2013).

Nucleotide Sequence Accession Numbers

Paired end Illumina 16S rRNA sequence data for all the water samples in this study were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRR5136460.

Results

Physical and Chemical Characteristics in C7B

In situ measurements of physiochemical characteristics in C7B and Pargos revealed clear differences with depth and tides (Figure 3-1). In C7B, a mixing zone of brackish water separates the top fresh water lens from underlying saltwater-haloconline (Stoessell et al., 1993), where a sharply increasing salinity was observed below the haloconline (depth of approximately 29m) down to the deeper areas during the two seasons. Compared with the top of C7B, the bottom of C7B was higher in DOC (Dissolved Organic Carbon), SO$_4^{2-}$, and HS$^-$ concentrations, yet lower in ORP and pH during both two seasons (Figure 3-1(A)). In Pargos, diurnal tides controlled the water movement through the spring area, during the low tide, brackish water discharged through spring channels with lower salinity, ORP, pH and SO$_4^{2-}$, but with higher DOC when compared with surface water. During high tides, saltwater intrudes into the spring
channels, with expected values of salinity, DOC and SO$_4^{2-}$ along the water profile all the way from the surface down to left channel. The concentrations of HS$^-$ fluctuated along the water profile during the two tides (Figure 3-1(B)). In summary, C7B exhibited a lower salinity, ORP, pH and SO$_4^{2-}$ concentrations, but higher DOC, HS$^-$, and SO$_4^{2-}$ concentrations than the submarine spring system (Pargos).

**Abundance, Composition and Variation of Sulfur Related Microbial Communities in C7B and Pargos**

The qPCR data (normalized $dsrB$: $dsrB$/16S rRNA gene copy numbers, indicantion of copy numbers sulfate reducing bacteria and total bacteria) for SRB and 16S rRNA gene sequences that matched known SRB and SOB are presented in Table 3-1 and Figures 3-2 and 3-3. Unfortunately, sequences at the surface and mouth during high tide of Pargos in the wet season, and 20m of C7B in the dry season, were not recovered, likely due to poor DNA isolation efficiencies. DNA sequences were obtained from those depths at high tide in dry season and are presented in Figures 3-2 and 3-3.

In C7B, the values of $dsrB$/16S rRNA gene copy numbers increased from 20m to 29m (the halocline), and then decreased from 29m to 32 m, which was partially consistent with the trends in relative abundances of SRB during the dry season, while the values of $dsrB$/16S rRNA gene copies increased from 20 m to 30 m and then decreased from 30 m to 32 m, which was consistent with the trends in relative abundance of SRB during the wet season. The relative abundance of SRB increased from 20 m to 30 m, and decreased from 30m to 32 m with *Deltaproteobacteria* group dominant during both seasons, and those SRB groups were more diverse below the halocline (29m) due to the presence of *Desulfitobacter* (phylum *Firmicutes*) and
Thermodesulfovibrionaceae (phylum Nitrospirae) groups when compared with samples taken above halocline. In general, the values of dsrB/16S rRNA gene sequence copies and relative abundance of SRB were enriched within the halocline (from 28 to 30m) compared with the areas above and below the halocline, and also higher in the wet season than in dry season at all depths (Figure 3-2). In Pargos, the values of dsrB/16S rRNA gene copies fluctuated along the depths with highest values at the mouth, higher values at left channel than that at the right channel during both low and high tides. During low tide, brackish water discharged through spring channels brought more SRB to the channels compared with ocean surface water, and a highest relative abundance of SRB was observed at the mouth with more diverse groups including Desulfitobacter, and Thermodesulfovibrionaceae, compared with other depths (Figure 3-2). During high tide, saltwater intruded into the spring channels with lower relative abundance of SRB compared with low tide, and also resulted in the absence of Desulfitobacter group in the right channel and at the mouth, and the highest relative abundance of SRB was observed at the mouth when compared with other depths. Generally, the values of dsrB/16S rRNA gene sequences and relative abundances of SRB were higher at low tide than that at high tide at all depths, with the Deltaproteobacteria group dominant in the whole spring system and exhibiting the highest relative abundance at mouth during both two tides.

In C7B, the relative abundances of SOB decreased during the dry season, and increased somewhat during the wet season around the halocline (from 28 m to 30 m depths), and the dominant SOB populations shifted from Betaproteobacteria SOB group above the halocline to Epsilonproteobacteria SOB group below the halocline. More
Firmicutes SOB were observed at 29m (halocline) in the wet season in comparison with the dry season, and a higher relative abundance of total SOB was observed in the wet season than in dry season (Figure 3-3). In Pargos, the relative abundance of SOB fluctuated along the depths during the two tides. During low tide, brackish water discharge through spring channels also brought more SOB to the right channel when compared with ocean surface water (Figure 3-3); during the high tide, saltwater intruded into the spring channels with lower relative abundance of SOB, which also resulted in a large decrease in Firmicutes SOB group at right channel when compared with low tide. Generally, the highest relative abundance of SOB and highest relative abundance of Epsilonproteobacteria SOB was observed at the mouth during both tides, and purple sulfur bacteria (PSB) were the dominant phototrophic sulfur bacteria observed in spring water in Pargos. Generally, SOB were more abundant than SRB at all sampling depths in both two sites (Figure 3-2 and Figure 3-3).

The Potential Sulfur-Disproportionation and Dimethylsulphide (DMS) Degradation Bacteria along the Salinity Gradient from C7B to Pargos.

Some genera observed in our study may be involved in processes related to sulfur-disproportionation and DMS degradation (Table 3-1) (Engel, 2007; Finster, 2008; González et al., 2010; Reisch et al., 2011; Eyice et al., 2015). The distribution pattern is presented in Figure 3-4. Four genera of SRB groups that may include members capable of sulfur-disproportionation were observed with a maximum relative abundance of less than 0.3%, and five genera in SOB groups potentially related to DMS degradation were observed with a maximum relative abundance of less than 0.06%. Higher relative abundances of genera related to sulfur-disproportionation was presented in marine system-spint Pargos in comparison with groundwater system-C7B with Desulfococcus
as the dominant group, except for the left channel during low tide in Pargos (Figure 3-4(A)), while the relative abundance of genera related to DMS degradation were similar between C7B and Pargos with a large variation in composition (Figure 3-4(B)). In C7B, *Methylotenera* (*Betaproteobacteria*) were more abundant above the halocline (20m-29m), and *Hyphomicrobium* (*Alphaproteobaceteria*) were dominant below the halocline during both dry and wet seasons.

A large tidal variation was noted in Pargos during different tides, at low tides, brackish water discharge through spring channels also brought more *Alphaprop_Hyphomicrobium* originated from the bottom of Cenotes to right channel and mouth when compared with ocean surface water, where *Methylotenera* (*Betaproteobacteria*) were dominant. At high tide, saltwater intruded into the spring mouth with higher relative abundance of *Thiobacillus* (*Betaproteobacteria*) in dry season, and *Gammaproteobacteria* groups related to DMS degradation dominated the channels in wet season (e.g. the gammaproteobacterial genera *Methylophaga* and *Thiomicrospira*) (Figure 3-4(B)).

**The Interactions between Microbial Communities along the Salinity Gradient from C7B to Pargos.**

To determine the changes in the interactions between bacteria related to sulfur cycling along the salinity and SO$_4^{2-}$ gradient form C7B to Pargos, three co-occurrence networks were constructed based on Spearman correlations conducted at the genus level for all the water samples collected from above halocline (samples from 20 m to 29 m), below the halocline (samples from 29 m to 32 m) in C7B, and high tide (samples from surface, mouth, and channels at high tide) in Pargos, and the correlation coefficients with value of $r>0.8$ at significant level (P<0.05) were chosen for constructing
the whole network (Figure 3-5). The subgroups with the strongest correlations were observed in the highest salinity zone in Pargos (connection density of 0.25) and highest values in total and average connections (Table 3-1, and Figure 3-5(A)). More than 94% of the connections were positive, and the proportion of positive connection decreased a little bit without significant changes, and the proportion of negative correlations increased somewhat along the salinity gradient from above the halocline in C7B to Pargos (Table 3-1, and Figure 3-5(B)). The proportion of positive connections with SRB is higher than that with SOB, while negative connections displayed the opposite trends in all salinity regions (Figure 3-5(B)). The proportion of positive connections with Deltaproteobacteria SRB and Epsilonproteobacteria SOB groups were dominant in all environmental conditions and generally decreased along the salinity gradient, while the proportion of positive connections with Alphaproteobacteria SOB, Betaproteobacteria SOB and Nitrospirae SRB groups generally increased along the salinity gradient. The positive connections with Firmicutes SOB and Firmicutes SRB groups were only apparent in C7B, but only negative connections with Firmicutes SOB and no connections with Firmicutes SRB were apparent in Pargos. In comparison with the phototropic sulfur bacteria PSB group, the proportion of positive connections with green sulfur bacteria (GSB) group was only higher below the halocline of C7B (Figure 3-5(B)), the genera in the GSB group involve positive connections in C7B that differ from Pargos (Figure 3-5(A)).

For the connections with potential groups related to sulfur-disproportionation and DMS degradation, there was no connection with sulfur-disproportionation related to bacteria above the halocline in C7B, and the connection with DMS degradation related
to *Hyphomicrobium (A_SOB2)* was along the salinity gradient, with highest number correlating with SRB in highest salinity zone in Pargos (Figure 3-5(A)). The product of DMS degradation was not completely oxidized by *Hyphomicrobium*, based on the connections between *Hyphomicrobium*, and sulfur-disproportionation related groups *Desulfobulbus (D_SRB8)* in Pargos and *Desulfovibrio (D_SRB13)* below the halocline in C7B (Figure 3-5(A)). A stronger connection was observed between *Desulfocapsa (D_SRB9)*, *Thiobacillus (B_SOB2)* and other sulfur related bacteria in Pargos when compared with C7B, and the connections with *Methylophaga (G_SOB2)*, *Thiomicrospira (G_SOB3)* were only observed in Pargos, while the connections with *Methylotenera (B_SOB4)* were only observed in C7B.

**Discussion**

**Conceptual Model of Sulfur Cycling in C7B and Pargos**

Based on the microbial communities related to sulfur cycling and the geochemical data, we built a conceptual model to interpret the difference in sulfur cycles between groundwater and offshore spring system.

C7B is a stratified cenote with anoxic water from the 20 m to 32 m, according to the ORP values. The halocline is at a depth of 29 m, where a sharp change in salinity occurs, and relatively higher sulfate (two times higher) at the bottom due to saline water intrusion. Sulfide oxidation with O$_2$ will be dominant the electron acceptor at the top of C7B as indicated by ORP, with depletion of O$_2$ and a likelihood of sulfide oxidation with NO$_3^-$ as the electron acceptor in the anoxic zone (Engel, 2007; Figure 3-6). However, below the halocline, no NO$_3^-$ was detectable, and organic material input from the mouth of the cenote would sink down below the halocline without complete oxidation due to low ORP, where fine organic material would settle at this layer due to the water density
difference and others would continue sink down to the bottom and accumulated (Socki et al., 2002; Pedersen, 2007). The combination of high concentrations of organic material, low ORP together with the high sulfate concentration below the halocline established a thermodynamically favorable condition for sulfate reduction (Socki et al., 2002; Pedersen, 2007), which result in a higher concentration of H$_2$S at the bottom in C7B (Figure 3-1(A); Figure 3-6). However, the halocline may also be viewed as a physical boundary to stop the H$_2$S exchange above and below the halocline until it was oxidized to H$_2$SO$_4$ by SOB at the halocline (Stoessell et al., 1993), which likely resulted in a low concentration of H$_2$S at the halocline in the wet season (Figure 3-1(A)). Furthermore, the halocline was proposed as a zone with possible sulfur reduction/oxidation and/or sulfur disproportionation by other studies (Thamdrup et al., 1993; Socki et al., 2002; Finster, 2008). It is not known at this time if sulfur disproportionation is a significant process in this site. Sulfur disproportionation may not be significant at this zone due to the absence of potential communities related to sulfur disproportionation (Figure 3-4(A)) and/or low iron and manganese contents in the water column (Pedersen, 2007; Finster, 2008), it may occur below the halocline due to the presence of potential communities related to sulfur disproportionation (Figures 3-4(A) and 3-6). The lower concentration of H$_2$S at the halocline may be attributed to lower sulfate reduction rates and higher sulfide oxidation rates due to the lower concentration of DOC (Figure 3-1(A)) (Al-Raei et al., 2009; Glombitza et al., 2013; Häusler et al., 2014).

A relatively higher abundance of SRB and SOB, and higher values of dsrB/16S rRNA gene copies were estimated at the mouth of Pargos (Figures 3-2 and 3-3). The
relatively high numbers of SOB correspond that with white snow-like material emanating from the mouth of the spring at low tide that is characteristic of sulfur oxidizers such as Arcobacter. Häusler et al. (2014) also reported that SRB and SOB in microbial mats of underwater springs were higher than that in the surrounding seawater. Tidal cycling is very important for sulfur cycling; during low tide, groundwater discharge to the mouth via the right channel exhibited low ORP and high DOC, which provided favorable conditions for sulfate reduction and increased the concentrations of $\text{H}_2\text{S}$ (Figures 3-1(B) and 3-6). During high tide, the water flow reversed with saltwater intrusion to the right channel, with an input of high ORP and low DOC, which is favorable for sulfide oxidation, resulting in lower concentrations of $\text{H}_2\text{S}$ (Figures 3-1(B) and Figure 3-6). A different situation for the mouth and left channel were observed, possibly due to the interference of a higher proportion of sulfur disproportionation communities in comparison with the right channel (Figure 3-4(A)).

The Effects of Salinity on Sulfur Related Microbial Communities along the Groundwater to Submarine Groundwater Discharge

Higher values of dsrB/16S rRNA ratios were observed in the halocline of C7B and at the Pargos mouth during low tides, in comparison with the high salinity zone at the bottom in C7B and at high tide in Pargos (Figure 3-2 and Figure 3-3), which was similar to other studies (Pedersen et al., 2014; Pachiadaki et al., 2014). Pedersen et al. (2014) found that SRB groups were dominant in the mixing layer in the deeper groundwater system, and Pachiadaki et al. (2014) found a decrease of $\text{dsrB}$ copies in the hypersaline zone below the halocline of a deep-sea hypersaline anoxic basin. van den Brand et al. (2015a and 2015b) also found that increased salinity decreased sulfate reduction rates and the inhibitory effect of salinity on sulfate reduction would be
alleviated after a long adaptation period. Meanwhile, among all the SRB groups, *Deltaproteobacteria* SRB were not only the dominant SRB group, but also the dominant SRB in networks (Figure 3-5), which may be attributed to their energy requirement from the cooperative metabolic interactions. Low energy production from their metabolism (Oren et al., 2011; Häusler et al., 2014) indicated that close cooperative loops within bacteria can result in indirect benefit to all species involved (Freilich et al. 2011).

In our study, SOB assemblages change along the salinity gradient with Betaproteobacteria SOB dominant in the low salinity zone and Epsilonproteobacteria SOB dominant in the high salinity areas (Figure 3-3), a finding that is consistent with other studies about the effects of salinity on controlling assemblage structures of SOB (Yang et al., 2013; Häusler et al., 2014). At the same time, salinity would change the sulfur oxidation rate, Keller (1969) found that sulfur oxidation activity was delayed and the oxidation rate was slightly affected by high salinity, and Suzuki et al. (1999) speculated that a lag period in sulfur oxidation at high salt concentrations may be caused by high osmotic pressures.

Sorokin et al. (2011) indicated that in order to cope with costly life at extreme conditions, both oxidative and reductive microorganisms should be capable to build a metabolism with a high- efficiency of dissimilatory conversions of inorganic sulfur compounds, and close cooperative loops between bacteria can result in indirect benefit to all species involved (Hibbing et al., 2010; Freilich et al., 2011). This may explain why a tighter connections between sulfur related microbial assemblages (Average connections in Table 3-1) were observed in higher salinity zones in Pargos (Table 3-1 and Figure 3-5(A)). However, a smaller change in relative abundance was observed in
SOB (around 2 fold change) than that in the SRB group (around 10 times change) during the tidal cycle (Figures 3-2 and 3-3), which may due to the different energy demands of these two groups. Häusler et al. (2014) found that the overall maintenance energy (e.g. cellular adjustments, osmotic equilibrium) for SRB population would increase under salinity fluctuation conditions in the spring system, which resulted in energy shortage for SRB cell division, and then limited the population size, while SOB may obtain sufficient energy to build up high biomass. In short, higher salinity and higher marine input will reduce the relative abundance of SRB and SOB, as well as sulfur oxidation rates and sulfate reduction rates, and DOC is very important for dissimilatory sulfate reduction.

Environmental Niches of SRB and SOB Groups

SRB were dominant by the *Deltaproteobacteria* groups independent of environmental conditions and showed a smaller niche differentiation in our study, even a good niche differentiation was found in other studies (Wawer, 1997; Nalven, 2011), while it is different for SOB. Firstly, pH controlled the total abundance of SOB, and higher relative abundance of SOB was found above the halocline of C7B and at low tide in Pargos due to neutral pH (around 7) and higher dissolved O$_2$ concentrations, because most sulfur oxidizers require neutral pH conditions to buffer metabolic acidity (Brimblecombe, 2005; Engel, 2007). Low pH related to high HS$^-$ by sulfate reducing process below the halocline in C7B during the wet season (Figure 3-1(A)), which was consistent with other studies of (Stoessell et al., 1993; Socki et al., 2002). Spring waters are rich in CaCO$_3$ where carbonate deposition and microbially mediated sulfide oxidation occur during the low tide in Pargos, and these processes would result in tufa deposits and whitish crusts (Douglas and Douglas, 2000; Chaudhary et al., 2009), then
discharged to the ocean that were observed during sampling. Different SOB groups exhibit different niche requirements for salinity and SO$_4^{2-}$; Macalady et al. (2008) and Grünke et al. (2011) reported some critical niche factors (e.g. oxygen, sulfide) among major groups of sulfur-oxidizing bacteria present in the cave waters. In our study, we found *Epsilonproteobacteria* SOB group preferred sulfidic areas with high salinity, such as below the halocline in C7B and in Pargos when compared with *Betaproteobacteria* SOB, which are adapted to low salinity and SO$_4^{2-}$ concentrations (Figure 3-1 and Figure 3-3). Campbell et al. (2006) also found that *Epsilonproteobacteria* were not only in sulfidic caves, but also in numerous other sulfur-rich habitats, including marine waters from terrestrial and marine sulfidic springs.

The intensity of light and the concentrations of H$_2$S controlled the interactions between other sulfur related bacteria and phototrophic sulfur bacteria (PSB, GSB), with a higher proportion of connections with GSB observed below the halocline of C7B in comparison with PSB (Figure 3-4(B)), which indicated a suitable electron donor (H$_2$S) and/or low intensity (red or green light) were suitable for GSB growth in deep groundwater systems (Parkin and Brock, 1980), because GSB can function under much lower light intensity conditions, which contributed to higher numbers of positive connections between other sulfur related bacteria with GSB than with PSG in our study (Tang et al., 2009).

**Firmicutes** SOB only show a higher proportion of positive connections with other sulfur related bacteria in groundwater system-C7B, yet only higher negative connections in Pargos (Figure 3-4(A) and 3-4(B)). The most likely explanation for this observation is
that *Firmicutes SOB* played a major role in dissimilative organic-sulfur oxidation in terrestrial environments rather than in marine systems (Santana et al., 2016).

The source and the concentration of DMS controls the interaction density between DMS degrading bacteria and other sulfur related bacteria, and the process related to sulfur-disproportionation and DMS degradation in marine system may be much more due to the higher connection density with *Desulfocapsa (D_SRB9)* and *Thiobacillus (B_SOB2)* groups due to the greater concentrations of DMS in marine systems (Figure 3-4(A) and Figure 3-4(B)) (González et al., 2010; Reisch et al., 2011; Eyice et al., 2015). The statement by Eyice et al. (2015) that “*Gamaproteobacterial* populations as more important DMS degraders in marine environment, *Betaproteobacterial Methylophilaceae* have a key role in DMS cycling in terrestrial environments” was also partially tested by the connections with *Methylophaga (G_SOB2)* and *Thiomicrospira (G_SOB3)* were only observed the marine system in Pargos, and the connections with *Methylotenera (B_SOB4)* were only observed in terrestrial groundwater system in C7B (Figure 3-4(A) and Figure 3-4(B)), but *Gamaproteobacterial* DMS degraders might not be the dominant DMS degraders in our study area due to lower connections in comparison with *Thiobacillus (B_SOB2)*. So, we speculated that SOB would be more sensitive to sea level change than SRB did in subterranean estuary base on the comparison of environmental niche partitioning between SRB and SOB.

**Summary**

This is the first study have been conducted in microbial sulfur cycling in STEs by systematically combining the terrestrial stratified groundwater with its related SGD. We find that halocline is very important for sulfur cycling in groundwater system, and the
bottom of groundwater possess a favorable thermodynamic conditions for supporting sulfate reduction, and SGD bring more SRB and SOB to the ocean water. It is inferred that niche partitioning plays more important role in SOB than SRB, and energy demands and DOC availability are possibly the key to open the door to understanding SRB activity and their interactions with other microorganisms. Seasonal and tidal variations in SRB and SOB, together with the interaction between these two groups along the salinity gradient possibly avail to speculate the effects of climate change together with sea level change on the spatial distribution of those sulfur related microbial communities. The methods (physiochemical analyses, qPCR, and high throughput 16S rRNA gene sequencing) used in this study provide insights into the potential linkages between the sulfur cycle and the microbial communities, and the variation in interactions between those sulfur related microbial communities along the environmental gradient, as well as the effects of SGD on coastal area in terms of sulfur process. The caveats for our study are that qPCR for DNA and 16S rRNA sequences cannot represent the activity of sulfur metabolism and the exact sulfur related microorganisms, and so further research with RT-qPCR and metatranscriptomic analysis would facilitate studies of gene activities and their regulation for sulfur dynamics in these water systems. Nevertheless, this study gives a holistic view on sulfur cycling for deep groundwater system and its related SGD, and will assist in a further understanding in the importance of environmental niches and microbial interactions in this system.
Table 3-1. Network analysis results for sulfur cycling

<table>
<thead>
<tr>
<th></th>
<th>Salinity (PSU)</th>
<th>SO$_4^{2-}$ (µmol/L)</th>
<th>Network density</th>
<th>Proportion of positive connections</th>
<th>Proportion of negative connections</th>
<th>Total connections</th>
<th>Average connections</th>
</tr>
</thead>
<tbody>
<tr>
<td>C7B_AH</td>
<td>0.74±0.10</td>
<td>0.37±0.02</td>
<td>0.14</td>
<td>94.7%</td>
<td>5.3%</td>
<td>38</td>
<td>1.6</td>
</tr>
<tr>
<td>C7B_BH</td>
<td>4.05±4.29</td>
<td>0.50±0.13</td>
<td>0.10</td>
<td>94.4%</td>
<td>5.6%</td>
<td>36</td>
<td>1.3</td>
</tr>
<tr>
<td>Pargos HS</td>
<td>32.04±1.92</td>
<td>28.13±2.18</td>
<td>0.25</td>
<td>94.6%</td>
<td>5.4%</td>
<td>168</td>
<td>4.5</td>
</tr>
<tr>
<td>Types</td>
<td>Abbreviation: Full name</td>
<td>Genus level</td>
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<tr>
<td>SRB</td>
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<tr>
<td>D_SRB:</td>
<td><em>Deltaproteobacteria</em> <em>SRB</em></td>
<td>Genus belongs to the order <em>Desulfuromonadales</em>, <em>Desulfobacteriales</em>, <em>Desulfarcuales</em> of <em>Deltaproteobacteria</em> (Voordouw et al., 1996; Rabus et al., 2006)</td>
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<tr>
<td>F_SRB:</td>
<td><em>Firmicutes</em> <em>SRB</em></td>
<td><em>Desulfitobacter</em> (Voordouw et al., 1996; Rabus et al., 2006)</td>
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<tr>
<td>N_SRB:</td>
<td><em>Nitrospirae</em> <em>SRB</em></td>
<td>Genus belongs to the family <em>Thermodesulfovibrionaceae</em> (Voordouw et al., 1996; Rabus et al., 2006)</td>
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<tr>
<td>A_SOB:</td>
<td><em>Alphaproteobacteria</em> <em>SOB</em></td>
<td><em>Rhodobacter</em>, and <em>Hyphomicrobium</em>, (Friedrich et al., 2001; Pokorna and Zabranska, 2015)</td>
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<tr>
<td>B_SOB:</td>
<td><em>Betaproteobacteria</em> <em>SOB</em></td>
<td><em>Hydrogenophaga</em>, <em>Thiobacillus</em>, and genus belong to <em>Methylophilaceae</em> (Friedrich et al., 2001; Cytryn et al., 2005; Eyice et al., 2015; Pokorna and Zabranska, 2015)</td>
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<tr>
<td>E_SOB:</td>
<td><em>Epsilonproteobacteria</em> <em>SOB</em></td>
<td><em>Arcobacter</em>, <em>Campylobacter</em>, <em>Sulfuricurvume</em>, <em>Sulfurimonas</em>, <em>Sulfurosipirillum</em> (Friedrich et al., 2001; Hubert and Voordouw, 2007; Häusler et al., 2014; Pokorna and Zabranska, 2015)</td>
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<tr>
<td>G_SOB:</td>
<td><em>Gammaroteobacteria</em> <em>SOB</em></td>
<td><em>Oceanospirillum</em>, <em>Methylophaga</em>, <em>Thiomicrrosipa</em>, <em>Beggiiota</em>, <em>Thiorthix</em> (Friedrich et al., 2001; Macalady et al., 2008; Grünke et al., 2011; Häusler et al., 2014; Pokorna and Zabranska, 2015)</td>
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</tr>
<tr>
<td>F_SOB:</td>
<td><em>Firmicutes</em> <em>SOB</em></td>
<td>Genus belong to family <em>Bacillaceae</em> (Friedrich et al., 2001; Santana et al., 2016)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GSB:</td>
<td>Green sulfur bacteria</td>
<td>Genus belong to family <em>Chlorobiaceae</em> (Frigaard and Dahl, 2009; Pokorna and Zabranska, 2015)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSB:</td>
<td>Purple sulfur bacteria</td>
<td>Genus belong to order <em>Chromatiaceae</em> (Frigaard and Dahl, 2009; Pokorna and Zabranska, 2015)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfur-disproportionation</td>
<td></td>
<td><em>Desulfococcus</em> (<em>D_SRB4</em>), <em>Desulfolublus</em> (<em>D_SRB8</em>), <em>Desulfoacapsa</em> (<em>D_SRB9</em>), <em>Desulfovibrio</em> (<em>D_SRB13</em>), (Engel, 2007; Finster, 2008)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Special group</td>
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</tr>
<tr>
<td>Dimethylsulphide degradation</td>
<td></td>
<td><em>Hyphomicrobium</em> (<em>A_SOB2</em>), <em>Thiobacillus</em> (<em>B_SOB2</em>), <em>Methylotenera</em> (<em>B_SOB4</em>), <em>Methylophaga</em> (<em>G_SOB2</em>), <em>Thiomicrrosipa</em> (<em>G_SOB3</em>), (González et al., 2010; Reisch et al., 2011; Eyice et al., 2015)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 3-1. Values of Salinity, ORP, pH, DOC (Dissolve Organic Carbon), as well as SO$_4^{2-}$, and HS$^-$; for water samples collected from study sites. A) C7B. B) Pargos.
Figure 3-2. Values of normalized dsrB gene (dsrB/16S), and relative abundance of microbial communities relate to potential sulfate reduction at genus level for water samples collected from C7B and Pargos.
Figure 3-3. Relative abundance of microbial communities relate to potential sulfur oxidation process at genus level for water samples collected from C7B and Pargos.
Figure 3-4. Relative abundance of microbial communities related to specific S cycling.
A) Potential sulfur-disproportionation in SRB group. B) Dimethylsulphide (DMS) degradation in SOB group.
Figure 3-5. Network analysis between SRB and SOB communities along salinity gradient from C7B to Pargos by spearman correlation. A) Network with red and gray lines represent negative and positive correlation, respectively. B) The proportion of positive (B1) and negative (B2) connections for SRB and SOB communities along salinity gradient from C7B to Pargos.
Figure 3-6. S cycling model in C7B and spring Pargos was built based on microbial and chemical data analysis.
CHAPTER 4
THE NITROGEN CYCLE IN A CENOTE AND SUBMARINE GROUNDWATER DISCHARGE IN YUCATAN PENINSULA

Background

Submarine groundwater discharge (SGD) refers to the flow of groundwater from continental margins to nearshore regions via diffuse seepage or spring discharge (Burnett et al., 2003; Moore et al., 2010). The region in the subsurface where fresh and saltwater mix is referred to as a subterranean estuary (STE). In coastal karst aquifers, such as those found in the Yucatan Peninsula, Mexico, most SGD occurs as nearshore or offshore springs discharge from networks of caves and conduits (Parra et al., 2015 and 2016). SGD may be more important than river discharge as a source of nutrients to near coastal waters on a global scale (Moore et al., 2010; Rogers and Casciotti 2010; Sawyer et al., 2016). One of the critical nutrient in coastal zones is nitrogen (N) (Null et al., 2014; Zehr and Kudela 2011; Brandes et al., 2007), which plays a pivotal role in ecosystem function (Jones et al., 2014; Tsiknia et al., 2015), such as nitrate (NO$_3^-$). It transported from groundwater to coastal water via SGD increased from 1.0 to 1.4 Tg/yr between 1950 and 2000 on a global scale largely from anthropogenic activities (Beusen et al., 2013), such that SGD plays a major role in coastal nutrient cycling (Santos et al., 2008).

SGD plays the most pronounced role in coastal nutrient cycling in karst environments lacking rivers, such as in the Yucatan Peninsula, where the waters in sinkholes are the one type of groundwaters and the sources of offshore SGD. The microbial groups that control the N cycle in groundwater may vary seasonally, therefore variably regulating the composition of dissolved inorganic nitrogen (DIN) delivery to coastal area via SGD. The composition, diversity, and cell numbers of microbial
communities suspended in groundwater have been reported to vary seasonally with shifts in flow, although similar community shifts did not occur in the sediments (Zhou et al., 2012). These seasonal impacts diminish with depth in unconfined aquifers (Lin et al., 2012), reflecting a more open system in the shallow zone compared with deeper zones in aquifers. Seasonal shifts in microbial communities may also occur due to seasonal inputs of nutrients and carbon from anthropogenic sources, and from differences in recharge between wet and dry seasons (Ben Maamar et al., 2015). Previous studies focused on microbial community structures in groundwater (Lin et al., 2012; Zhou et al., 2012; Ben Maamar et al., 2015), little work has been reported on spatial and temporal variation of microbial genes that control the N cycle in groundwater and SGD (Rogers 2010; Erler et al., 2014; Stoliker et al., 2016).

Meanwhile, hydrologic characteristics, including hydrologic mixing and linkages play very important roles in the delivery of the DIN and their related microbial community from groundwater to the coastal area via SGD (Gonneea and Charette 2014; Heiss and Michael 2014; Stoliker et al., 2016). The hydrologic mixing and linkages between groundwater and coastal water in subterranean estuaries may be enhanced by tidal pumping through conduits in karst systems in Yucatan (Martin et al., 2012; Heiss and Michael 2014; Parra et al., 2015 and 2016). During high tides, seawater can intrude into karst conduits (Parra et al., 2015 and 2016), shifting the groundwater-seawater interface inland, and cause mixing with the water bodies within flow pathway. Conversely, during discharge periods at low tides, the mixed groundwater and saltwater within flow pathway subsequently discharge to the ocean. Both mixed groundwater and saltwater discharge to coastal waters and saltwater intrusion into
aquifers may impact microbial N cycling as water chemistry and salinity changes
(Santoro et al. 2008; Santoro 2010).

During discharge periods at low tide, up to half of terrestrially derived N
(combined mass from runoff, riverine and SGD) may be released to the ocean via SGD
in some areas (Moore et al., 2006; Niencheski et al., 2007; Santos et al., 2008; Rogers
and Casciotti, 2010). Low-salinity, high-nutrient SGD plumes have been associated with
increased phytoplankton biomass in surface waters during discharge periods in coastal
Hawaii (Walker, 2012). Salinity variations play an important role in controlling the ratios,
abundances, and community compositions of nitrifiers and denitrifiers in sediments
(Rogers and Casciotti 2010; Bernhard et al., 2007 and 2010); However, the impacts
driven by mixing processes on microbial N cycling are understudied (Kroeger and
Charette, 2008), and the effects of salinity changes on microbial N cycling appear to be
inconsistent between studies in STEs. As an example, some studies report that copies
of AOA is higher in higher salinity zones (Abell et al., 2010; Jin et al., 2011), while
others found either no changes along salinity gradients or lower numbers of AOA in
higher salinity zones (Santoro et al., 2008; Mosier and Francis 2008). These studies
reflect how little is known of the microbial groups that control the response of the N-
cycle to hydrologic mixing, tidal variations, and changes in salinity in coastal aquifers.

To address some of these gaps in knowledge, we hypothesize N cycling differs
between groundwater and marine spring, and is not solely controlled by simple
hydrological mixing. The relative distribution of N cycling genes will be related to ORP
and NH4+ concentrations. And we studied the concentrations of microbial genes related
to the N cycle, and DIN concentrations at two sites: a stratified sinkhole approximately
25 km from the coast (Cenote Siete Bocas; C7B); and a submarine spring discharging (Pargos) approximately 0.5 km offshore. The two sites are not known to be directly connected; however, we assume chemical and microbial compositions in C7B and Pargos represent groundwater and SGD, respectively. Although not connection, the study area includes three distinct salinity zones.

Materials and Methods

Site Description

The sampling site is same to the Chapter 2

Sample Collection

The sample collection is same to the Chapter 2

Samples Processing

DNA Isolation and PCR: DNA was extracted from filters using Powerwater® Sterivex DNA Isolation Kit (Mobio, Carlsbad, CA, USA) (Hollibaugh et al., 2014). All primers and quantitative polymerase chain reaction (qPCR) primers and cycling parameters of N related functional genes are presented in Table 4-6 (nitrogen fixation, \textit{nifH}); nitrifiers (ammonia monooxygenase genes from ammonia oxidizing archaea, AOA; and bacteria AOB); denitrifiers (nitrite reductase, \textit{nirK} and \textit{nirS}; nitrous oxide reductase, \textit{nosZ}); and Dissimilatory Nitrate Reduction to Ammonium (DNRA), \textit{nrfA}). For the qPCR assays, all sample DNAs and standard DNAs were analyzed in duplicate by using the SYBR green qPCR protocols in a StepOne Plus real-time PCR system (Applied Biosystems, Thermo Fisher, Inc.) (Dang et al., 2013; Bae et al., 2014). Abundances of all genes were calculated as copies of genes per liter of water sample. Standard curves were generated by serial dilution ($10^{-3}$ to $10^{-6}$) of reference plasmids containing target genes constructed by cloning the appropriate amplification products into \textit{E. coli} hosts
using TOPO TA cloning kits (Life Technologies, Thermo Fisher Scientific Inc, USA). Concentrations and purity of plasmid DNAs were measured using Eppendorf biophotometer plus (VWR International LLC, USA) for qPCR analysis with A260/A280 ratios equal to between 1.7 to 2.0. Amplification efficiencies were evaluated as described by Bae et al. (2014), and were between 90.9% and 100%. Fidelity of amplification products was confirmed by melt curve analysis following each qPCR run.

Water samples were measured for ammonium and nitrate concentrations using a Seal AA3 Autoanalyzer (SEAL Analytical, Inc., WI, USA) continuous flow system following the methods of Strickland and Parsons (1978). The detection limits of NO$_3^-$ and NH$_4^+$ were 0.1 µmol L$^{-1}$ and 0.5 µmol L$^{-1}$, respectively. DOC concentrations were analyzed on a Shimadzu TOC-VCSN total organic carbon analyzer, and the coefficient of variance was less than 2%. All analyses were completed in the Department of Geological Sciences, University of Florida.

**Data analysis:** The spatial, seasonal and tidal ordination pattern were analyzed using ordination analysis in CANOCO 4.5 (ter Braak and Šmilauer, 2002; Legendrev et al., 2011), and the significant difference in copies of N cycling genes between different seasons and tides was tested by T-test in SPSS, and $P$ values smaller than 0.05 were considered to be significant. The relationships between the qPCR data and environmental factors were analyzed using Spearman correlation analysis in R (Gardener, 2012). The permutation test in CANOCO 4.5 was used to detect the important environmental factors contributing to the distribution of the N cycling genes (Knijnenburg et al., 2009; Phipson and Smyth, 2010).
Results

Differences in Water Compositions between C7B and Pargos

The average precipitation during the months we sampled (April and September) are around 66.9 and 171.1 mm (Figure 2-2), respectively, such that typically April represents the dry season and September represents the wet season. Salinity, ORP, and DOC, NH$_4^+$ and NO$_3^-$ concentrations at C7B changed little between dry and wet seasons (Figure 4-1(A)). Salinity, DOC, and NH$_4^+$ concentrations were constant from 20 m to 29 m below cenote openings, and increased from 29 m to 32 m. In contrast, NO$_3^-$ concentrations decreased slightly with depth from 20 m to 28 m, increased from 28 to 29 m, and decreased below detection limits ($\leq0.01\mu$mol) from 30 m to 32 m. ORP also decreased with depth, with an inflection at the halocline. In Pargos, samples collected at mouth and channels were higher in salinity and ORP, and exhibited lower concentrations of NH$_4^+$ and DOC during high tide than low tide ($P<0.01$; Table 4-1) (Figure 4-1 (B)). Concentrations of NO$_3^-$ were low (0.01µmol) across the tidal cycle. These results indicated that inland groundwater significantly differs in physiochemical composition from the coastal water.

Key N Cycling Gene Abundances in C7B during Different Seasons and in Pargos during Different Tides

With the exceptions of $nifH$ and $nosZ$, the copy numbers of N cycling genes in C7B were greater in the wet than dry season (Figure 4-2(A)). AOA, $nirK$ and $nirS$ numbers were somewhat greater above the halocline, while $nifH$, AOB, $nrfA$ and $nosZ$ were somewhat greater below the halocline during both seasons. Compared with non-significant changes in other genes, the abundances of $nrfA$ were 40 to 50 times greater in the wet than dry season, both above and below the halocline (Figure 4-2(A)).
In Pargos, all N cycling genes were greater at all depths during high tide when salt water intruded than low tide when groundwater discharged, with the exceptions of AOA at the right channel and mouth. Compared with small changes in other genes, the abundances of \textit{nifH} were 100 to 1000 times higher at the mouth and right channel during high tide, when salt water intrudes into the spring, than at low tide, when groundwater discharges. Copy numbers of N cycling genes differed more between high and low tides in the left channel than at other locations in the system (Figure 4-2(B)).

**The Ordination Pattern of C7B and Pargos Based on Key N Cycling Genes and Environmental Factors**

The ordination patterns of environmental parameters and genes in C7B and Pargos (Figure 4-3, all sites; Figure 4-4, C7B; Figure 4-5, Pargos) and Spearman Correlation analysis (Table 4-2, C7B; Table 4-3, Pargos) analyzed the relationships between key N cycling genes and environmental factors, yielding insight into the similarities and differences with respect to different seasons and different tides.

The spatial ordination patterns separating C7B and Pargos in wet season: The first and second axes of RDA (Figure 4-3) explained 95.2% and 2.6% of cumulative variance of the relationship, respectively. The first ordination (axis 1) was mostly contributed by NH$_4^+$, DOC ($P = 0.011$) and pH ($P = 0.004$) (Table 4-4), and the second ordination (axis) was mostly contributed by temperature, salinity, NO$_3^-$ and ORP (Table 4-4; Figure 4-3). Three distinct clusters were identified based on key N cycling genes and environmental gradients (Figure 4-3): A: the freshwater cluster was composed of samples from the 20 m, 28 m, 29 m, 30 m depths of C7B; B: the brackish water cluster was composed of samples from 32 m depth of C7B, and the right and left channels and
mouth of Pargos during low tide; C: the saltwater cluster was composed of samples from the right and left channels, and the mouth and surface in Pargos during high tide.

A strong relationship was observed between NO$_3^-$, NH$_4^+$, DOC, salinity and ORP moving from C7B to Pargos based on RDA analysis in Figure 4-3. The freshwater lens (C7B) harbored the highest concentrations of NO$_3^-$, the brackish water zone (32 m of C7B; Pargos mouth, and channels at low tide) harbored the highest concentrations of NH$_4^+$ and DOC, and the saline water zones (Pargos surface, mouth, and channels at high tide) exhibited the highest values of salinity and ORP. A clear boundary separating samples taken from above (20 m bws, 28 m bws of C7B) and below (30m bws of C7B) the halocline was observed; greater numbers of AOA and nirS were observed above the halocline. The composition of the more brackish water area (32 m bws of C7B) more closely reflected the conditions in the right channel of Pargos than either the mouth or left channel at low tide. The surface of Pargos was more similar to the mouth than to either of the channels during high tide, with greater numbers of AOB and nosZ observed in this area.

The seasonal ordination pattern in C7B: The first and second axes of the RDA (Figure 4-4) explain 84.6% and 13.4% of cumulative variance of the relationship, respectively, between the N cycling gene copies and physicochemical parameters at C7B between the wet and dry seasons. Salinity was a statistically significant contribution to the ordination pattern based on the abundance of key N cycling genes and environmental factors ($P=0.019$ for salinity; 1000 Monte Carlo permutations; Table 4-4). A greater difference was observed between samples from the dry season and wet season in C7B along Axis 1 (84.6% of the variation explained) than between samples
taken from above the halocline and below halocline along Axis 2 (13.4% % variation
explained). Samples taken at 32 m (3 m below the halocline) differed more than from
the other depths during the dry season, whereas samples taken at 30 m (1 m below the
halocline) differed more than other depths during the wet season (Figure 4-4). *nifH* and
*nosZ* concentrations were greater, and especially greater at 32 m bws in the dry season
than in the wet season, while other N cycling genes were greater (*AOA, nirK and nrfA*)
in the wet than dry season (Figure 4-4). Salinity was inversely related to most of the N
functional genes in C7B, with the exceptions of *nifH* and *nosZ* (Figure 4-4 and Table 4-
2). *AOA* concentrations were significantly correlated with salinity (-), ORP (+), NH$_4^+$ (-)
and NO$_3^-$ (+) ($P<0.05$). *nosZ* was significantly correlated with salinity (+) and ORP (-)
($P<0.01$), while other N functional genes (*AOB, nirK, nirS* and *nrfA*) were not
significantly correlated with any of those environmental factors (Table 4-2).

The tidal ordination pattern in Pargos: The first and second axes of RDA
explained 98 % and 1.1% of cumulative variance, respectively, in the N cycling gene
copies and physicochemical parameters in water from Pargos during high and low tides
(Figure 4-5). Among all the environmental factors measured, pH and NH$_4^+$ showed a
statistically significant contribution to the ordination pattern ($P=0.001$ for pH, $P=0.002$ for
NH$_4^+$; 1000 Monte Carlo permutations, Table 4-4). A larger difference was observed
between samples taken at low tide and at high tide in Pargos along Axis 1 coordinate
(98% variation explained) than between the different depths within tides presented
along the Axis 2 coordinate (1.1% variation explained). The key N cycling genes in
Pargos appeared to correlate with the movement of water; the right channel and mouth
of Pargos were much more similar to each other during low tide (during discharge),
while the surface was much more similar to the mouth, and the left channel was more similar to the right channel during high tide (Figure 4-5), strongly suggesting that the seawater is intruding into the channels at high tide. Excluding AOA, other key N cycling genes were significantly higher (nifH, nirK, nirS, nrfA and nosZ) during high tide than low tide (Figure 4-5). Salinity and ORP were positive, while NH₄⁺ concentrations were negatively correlated to most of the N functional genes, with the exception AOA. nifH concentrations were significantly positively correlated with ORP ($P<0.05$) and significantly negatively correlated with NH₄⁺($P<0.01$); AOB and nirK, nirS were significantly correlated with salinity (+) and NH₄⁺ concentrations (-) ($P<0.05$); nrfA was significantly correlated to NH₄⁺ (-) ($P<0.01$); nosZ was significantly positively correlated with salinity ($P<0.05$) (Table 4-3).

Discussion

The Effects of Hydrologic Conditions on the N-Nutrient and Key N Cycling Genes

Hydrologic conditions play an important role in shaping the composition of microbial communities (Moore et al., 2010; Lee et al., 2016). In our study area, RDA analysis in Figure 4-3 suggested that the hydrologic conditions separate the area into three different members along the flow pathway based on N-nutrients and N cycling genes: freshwater endmembers (A), mixed (B), and saline endmembers (C).

For the freshwater endmember, a clear seasonal variation in N cycling genes was observed with higher abundances of most N cycling genes in wet season (Figure 4-4). For the saline endmember, the abundance of N cycling genes in Pargos during different tides correlated with the movement of water (Figure 4-6), which developed in areas where the time scale for the movement of the water is smaller than the turnover times of the microbial communities (Crump et al. 2004; Santoro et al. 2010).
For the mixing zone, we employed a mixing model (Equation 2-1; Table 4-5) to estimate how the physical mixing process related to the distribution of N functional genes and the changes in DIN (Table 4-5). The sample at 32 m from C7B clustered together with the samples collected at low tide from Pargos (Figure 4-3), which suggested that the bottom of groundwater had greater impacts on N-nutrient and N cycling genes in offshore spring during discharge periods.

The values of all measured N cycling genes were lower than could be explained strictly by the mixing model in mixing zone (Table 4-5), indicating that the distribution of N cycling genes was controlled by environmental factors other than the physical mixing of fresh with salt water. The numbers of denitrifiers (nirK and nirS) showed a greater deviation from pure mixing than nitrifiers (AOA and AOB); however, the numbers of AOA and nosZ were much closer than the other N cycling genes to that predicted by pure mixing. Previous studies also found that shifts in ammonia-oxidizing assemblages may result from coastal hydrologic characteristics (Dang et al. 2008; Santoro et al. 2008). The concentrations of DIN were also not accurately described by simple mixing, indicating that DIN concentrations were likely affected by microbial processes along the flow path (Table 4-5). The concentrations of NH₄⁺ in the mixing zone were 9 times higher than predicted by mixing. It is likely that the accumulation of NH₄⁺ was mainly due to remineralization of organic matter along the flow path (Santos et al. 2008; Erler et al. 2014). The concentrations of NO₃⁻ were 57 times lower in the mixing zone than predicted by mixing alone. At this time, it is not known if NO₃⁻ was removed by denitrification or annamox (Kroeger and Charette 2008), although Erler et al. (2014) found 88% of the NO₃⁻ was removed by denitrification in deep groundwater zone, while
Lam et al. (2009) reported that anammox rather than denitrification was the likely prevalent pathway for consuming NO$_3^-$ in the oxygen deficient zone in the water column of a marine system.

**Conceptual Model for N Cycling in C7B and Pargos**

In order to explain the difference in N cycling in C7B and Pargos, we propose a conceptual model in Figure 4-6 based on the distribution of N cycling genes and DIN concentrations.

In C7B, AOA was significantly negatively correlated with NH$_4^+$, and positively correlated with NO$_3^-$ (Table 4-2), suggesting that nitrification was controlled by AOA in C7B. The elevated NO$_3^-$ concentrations and lower NH$_4^+$ concentrations above the halocline (Figure 4-3) in both seasons suggested that nitrification may be more favorable than denitrification above the C7B halocline (Figure 4-6), although it is quite likely that a significant amount of the NO$_3^-$ observed was due to nitrification of NH$_4^+$ within the vadose zone during recharge from terrestrial runoff (Erler et al., 2014). In an analogous situation, Ben Maamar et al. (2015) showed that low organic carbon and Fe concentrations, coupled with high ORP, led to low rates of denitrification in surficial groundwater, and other models also indicated that nitrate removal by denitrification is limited in an oxidized, nitrate rich freshwater SGD plume in groundwater discharge area (Spiteri et al., 2008). The opposite trend was observed below the halocline, where NO$_3^-$ were was not detected, with moderate to extremely high concentrations of NH$_4^+$, which suggested a relatively higher NO$_3^-$ consumption (e.g. denitrification, DNRA, and/or anammox) than NO$_3^-$ production (e.g. nitrification) by microbial processes and diffusion of NO$_3^-$ from above to below the halocline. Relatively high concentrations of NH$_4^+$ observed below the halocline may be mainly due to remineralization of organic matter.
(Erler et al., 2014), which was consistent with the result from the mixing model in Table 4-5 and the RDA analysis in Figure 4-3.

In Pargos, AOB numbers were significantly negatively correlated with NH$_4^+$ concentrations (Table 4-3), indicating that nitrification was likely controlled by AOB, which was consistent with other studies (Santoro et al., 2008; Bagchi et al., 2014). AOB numbers were significantly positively correlated with both nirK and nirS, suggesting that the by-products from AOB might be denitrified by the hosts of both nirK and nirS (Table 4-3), and nirS hosts were identified as being a dominant denitrifier by a previous study in a higher salinity zone (Rogers, 2010). The significant positive correlation between nitrifiers and denitrifiers (Table 4-3), as well as the tight cluster (Figure 4-5), suggests nitrification and denitrification are coupled between the spring channels and mouth in Pargos at high tides, with little accumulation of either NH$_4^+$ or NO$_3^-$ observed (Figure 4-5 and Figure 4-6).

We speculate that the low redox potentials with depth in C7B rather than increased salinity was responsible for the spatial separation and decoupling of nitrification-denitrification in the freshwater lens. In the spring, tidal pumping causes water to flow in and out of the spring to generate reversible redox potential gradients, such that there may be a critical zone with appropriate ORP during the tidal cycle that can support coupled nitrification and denitrification process in Pargos (e.g., Rogers and Casciotti, 2010).

**The Effects of SGD on the N-Nutrient and Key N Cycling Genes**

Hydrologic conditions play an important role in shaping the composition of microbial communities (Moore et al., 2010; Lee et al., 2016), and communities develop in areas where the time scale for the movement of the water is greater than the turnover
times of the microbial communities (Crump et al., 2004; Santoro et al., 2010). In our study area, RDA analysis suggested that the abundance of N cycling genes in Pargos during different tides correlated with the movement of water (Figure 4-5). Samples from 32 m bws of C7B clustered more closely with samples in the right channel than with the left channel and mouth in Pargos during low tides (Figure 4-3). This similarity suggests that groundwater similar in composition to C7B contributed to the transport of microbial communities to the SGD at Pargos. Dang et al. (2008) also found that freshwater discharge impacted the distribution of AOA assemblages, and Santoro et al. (2008) speculated that a shift in ammonia-oxidizing microbial communities may have been due to a coastal hydrology linkage. Lower copy numbers of most N cycling genes during SGD at low tide may not promote N nutrient removal at the channels before discharging to the ocean at low tide in Pargos (Figure 4-5).

**Responses of Key N Cycling Genes to Steep Salinity Gradient from C7B to Pargos**

We found that most N cycling genes, with the exceptions of *nifH* and *nosZ*, were negatively related to salinity in C7B, but were positively related to salinity in Pargos (Table 4-2 and 4-3). The effects of salinity on N cycling genes vary from study to study. For example, Santoro (2008) found higher numbers of *AOB* in higher salinity regions and nearly constant *AOA* along the salinity gradient in a subterranean estuary. However, Wang and Gu (2014) reported that different *AOA* might have different requirements for salinity, and Francis et al. (2005) pointed out that differences in the distribution and diversity of *AOB* in estuarine systems between sites may not be salinity-driven, but that *AOA* were more likely associated with salinity. Bernhard et al. (2007) also found that *AOB* exhibited a broad range of salinity tolerances, and Abell et al.
(2010) and Jin et al. (2011) ascribed the positive relationship between salinity and AOA to the high concentration of NH$_4^+$ at high salinity sites. In our study, we found lower AOA and higher AOB in higher salinity regions (Figure 4-3), a finding that is consistent with the results of Mosier and Francis (2008). Some studies suggested that denitrifiers and DNRA differed in salinity tolerance in different environments (Abell et al., 2010; Song et al., 2014), such that negative correlations between salinity and nirK, nirS, nrfA copy numbers in C7B may be attributed to the possibility that denitrifiers and DNRA in freshwater are less tolerant to salinity than those in the marine system (Tables 4-2 and 4-3). Copy numbers of nosZ were positively correlated with salinity ($P<0.05$) in both C7B and Pargos (Tables 4-2 and 4-3); Yang et al. (2015) also found a positive relationship between nosZ and salinity.

There are three possible explanations for the contradictory effects of salinity on the abundance and distribution of N cycling genes: (1) Different microbial communities may respond differently to environmental factors (Ligi et al., 2014), such that the site was the dominant factor differentiating communities for some key N cycling microbes (Walker et al., 2008). Francis et al. (2003) also pointed out that salinity appeared to play a role, but no single parameter can completely explain the microbial diversity pattern; (2) The contribution of each environmental factor to microbial abundance and distribution is not clear due to the potential covariable effects, e.g., salinity and NO$_3^-$ negatively co-varied, while DOC and NH$_4^+$ positively co-varied, across the entire study area (Figs. 4-4 and 4-5), as well as counteractive effects, e.g., the relationships between salinity, DOC, NH$_4^+$ were opposite in different sites in our study area (Figs. 4-4 and 4-5) (Dang et al., 2008; Castro et al., 2010); (3) Community composition may be
selected for members that can adapt to specific parameters when environmental conditions change (Hemme et al., 2010; Mosher et al., 2012), which resulted in a different response of the same microbe to the same factor in different environments. Future studies regarding the effects of salinity on microbial processes should focus on site specificity and the mechanisms for adapting to changes; no single factor is likely to be the long-term driver for controlling microbial processes.

**Summary**

This study combined seasonal and tidal variations to study the effects of hydrologic source on the abundance of key N cycling genes in subterranean estuary water system. The results demonstrated that the abundance of N functional genes in the C7B and Pargos depended on seasonal and tidal changes, and showed a clear distribution pattern along the steep ORP, N-nutrient and salinity gradients in the study area. Differences in salinities affect the freshwater and marine system differently as seen with the different responses of N cycling genes to salinity changes in C7B and Pargos. The N cycling was controlled by weakly uncoupled AOA-\textit{nirK} model of nitrification-denitrification in C7B and strongly coupled \textit{AOB-(nirK+nirS)} model of nitrification-denitrification in Pargos. Conflicting results from different studies regarding the effects of salinity on the abundance and distribution of microbial N cycling genes may be due to the covariable effects between salinity and other factors, and the adaptive capacity of microbes to changing environmental conditions, such that the overall environmental conditions may play an important role in explaining those effects.
Table 4-1. Average values of physiochemical parameters for water samples collected from C7B in April (dry season) and September (wet season), and Pargos during low and high tides in September (wet season), 2014.

<table>
<thead>
<tr>
<th></th>
<th>C7B Dry season</th>
<th>C7B Wet season</th>
<th>P(T-test) (Dry-Wet)</th>
<th>Pargos Low tide</th>
<th>Pargos High tide</th>
<th>P(T-test) (High-Low)</th>
<th>P(T-test) (C7B-Pargos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp (°C)</td>
<td>26.8±1.4</td>
<td>25.6±0.7</td>
<td>0.11</td>
<td>30.0±0.2</td>
<td>29.9±0.17</td>
<td>0.50</td>
<td>&lt;0.01 (**)</td>
</tr>
<tr>
<td>pH</td>
<td>6.8±0.4</td>
<td>7.1±0.4</td>
<td>0.30</td>
<td>7.4±0.03</td>
<td>8.0±0.09</td>
<td>&lt;0.01 (**)</td>
<td>&lt;0.01 (**)</td>
</tr>
<tr>
<td>Salinity (PSU)</td>
<td>3.5±4.6</td>
<td>2.6±3.8</td>
<td>0.74</td>
<td>24.6±0.06</td>
<td>32.8±0.04</td>
<td>&lt;0.01 (**)</td>
<td>&lt;0.01 (**)</td>
</tr>
<tr>
<td>ORP (mV)</td>
<td>-255.0±67.1</td>
<td>-215.8±95.5</td>
<td>0.47</td>
<td>-16.6±10.0</td>
<td>77.1±14.5</td>
<td>&lt;0.01 (**)</td>
<td>&lt;0.01 (**)</td>
</tr>
<tr>
<td>NH₄⁺ (μmol/L)</td>
<td>168.8±326.2</td>
<td>226.1±453.2</td>
<td>0.82</td>
<td>45.6±9.2</td>
<td>5.0±2.4</td>
<td>&lt;0.01 (**)</td>
<td>0.17</td>
</tr>
<tr>
<td>NO₃⁻ (μmol/L)</td>
<td>23.7±27.6</td>
<td>34.6±34.8</td>
<td>0.63</td>
<td>≤0.01</td>
<td>≤0.01</td>
<td>0.68</td>
<td>0.01 (**)</td>
</tr>
<tr>
<td>DOC (μmol/L)</td>
<td>173.5±152.1</td>
<td>139.0±112.4</td>
<td>0.72</td>
<td>135.3±45.0</td>
<td>58.0±1.0</td>
<td>0.02 (*)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Temp: Temperature; ORP: Oxidation and Reduction potential; DOC: Dissolve organic carbon; Asterisks (*) indicated significant difference between April and September at P<0.05 level; Asterisks (**) indicated significant difference between April and September at P<0.01 level.
Table 4-2. Spearman correlation coefficients between N-cycling genes (Copies/L) and environmental factors in C7B by combining samples from dry season (April, 2014) and wet season (September, 2014)

<table>
<thead>
<tr>
<th></th>
<th>nifH</th>
<th>AOA</th>
<th>AOB</th>
<th>nirK</th>
<th>nirS</th>
<th>nrfA</th>
<th>nosZ</th>
<th>salinity</th>
<th>ORP</th>
<th>NH₄⁺</th>
<th>NO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifH</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOA</td>
<td>-0.417</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOB</td>
<td>0.267</td>
<td>0.217</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nirK</td>
<td>-0.35</td>
<td>0.700*</td>
<td>0.45</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nirS</td>
<td>0.117</td>
<td>0.233</td>
<td>-0.183</td>
<td>0.3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nrfA</td>
<td>-0.35</td>
<td>0.617</td>
<td>0.417</td>
<td>0.900**</td>
<td>0.183</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nosZ</td>
<td>0.933**</td>
<td>-0.6</td>
<td>0.3</td>
<td>-0.383</td>
<td>0.067</td>
<td>-0.35</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>salinity</td>
<td>0.5</td>
<td>-.917**</td>
<td>-0.033</td>
<td>-0.517</td>
<td>-0.3</td>
<td>-0.367</td>
<td>0.667*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORP</td>
<td>-0.5</td>
<td>0.883**</td>
<td>-0.167</td>
<td>0.483</td>
<td>0.35</td>
<td>0.3</td>
<td>-0.683*</td>
<td>-0.950**</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.25</td>
<td>-0.717*</td>
<td>0.167</td>
<td>-0.217</td>
<td>-0.3</td>
<td>0.4</td>
<td>0.833**</td>
<td>-0.900**</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>-0.452</td>
<td>0.703*</td>
<td>-0.042</td>
<td>0.142</td>
<td>0.209</td>
<td>0.017</td>
<td>-0.611</td>
<td>-0.854**</td>
<td>0.787*</td>
<td>0.728*</td>
<td>1</td>
</tr>
</tbody>
</table>

ORP: Oxidation and Reduction potential; Positive value means positive correlation; negative value means negative correlation; *: Correlation was significant at the 0.05 level (2-tailed); **: Correlation was significant at the 0.01 level (2-tailed).
Table 4-3. Spearman correlation coefficients between key N cycling genes (copies/L) and environmental factors in Pargos by combining samples from low and high tides in wet season (September, 2014)

<table>
<thead>
<tr>
<th></th>
<th>nifH</th>
<th>AOA</th>
<th>AOB</th>
<th>nirK</th>
<th>nirS</th>
<th>nrfA</th>
<th>nosZ</th>
<th>salinity</th>
<th>ORP</th>
<th>NH₄⁺</th>
<th>NO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifH</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AOA</td>
<td>0.036</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>AOB</td>
<td>0.964**</td>
<td>0.086</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nirK</td>
<td>0.964**</td>
<td>0.086</td>
<td>1.000**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nirS</td>
<td>0.964**</td>
<td>0.086</td>
<td>1.000**</td>
<td>1.000**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nrfA</td>
<td>0.964**</td>
<td>-0.036</td>
<td>0.893**</td>
<td>0.893**</td>
<td>0.893**</td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>nosZ</td>
<td>0.857*</td>
<td>-0.143</td>
<td>0.929**</td>
<td>0.929**</td>
<td>0.929**</td>
<td>0.821*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>salinity</td>
<td>0.714</td>
<td>-0.464</td>
<td>0.821*</td>
<td>0.821*</td>
<td>0.821*</td>
<td>0.643</td>
<td>0.821*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORP</td>
<td>0.786*</td>
<td>-0.25</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.714</td>
<td>0.607</td>
<td>0.786*</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>-.929**</td>
<td>-0.036</td>
<td>-0.821*</td>
<td>-0.821*</td>
<td>-0.821*</td>
<td>-0.964**</td>
<td>-0.714</td>
<td>-0.571</td>
<td>-0.786*</td>
<td>1</td>
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<tr>
<td>NO₃⁻</td>
<td>0.107</td>
<td>0.143</td>
<td>-0.071</td>
<td>-0.071</td>
<td>-0.071</td>
<td>0.214</td>
<td>0.036</td>
<td>-0.393</td>
<td>-0.107</td>
<td>-0.25</td>
<td>1</td>
</tr>
</tbody>
</table>

ORP: Oxidation and Reduction potential
Positive value means positive correlation; negative value means negative correlation;
* Correlation was significant at the 0.05 level (2-tailed);
** Correlation was significant at the 0.01 level (2-tailed).
Table 4-4. Permutation test of all constrained eigenvalues for nitrogen functional genes (copies/L) by Redundancy analysis (RDA)

<table>
<thead>
<tr>
<th></th>
<th>Variance explained for C7B</th>
<th>P value for C7B</th>
<th>Variance explained for Pargos</th>
<th>P value for Pargos</th>
<th>Variance explained for all</th>
<th>P value for all</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>96%</td>
<td>0.45</td>
<td>100%</td>
<td>1.000</td>
<td>97%</td>
<td>0.28</td>
</tr>
<tr>
<td>pH</td>
<td>33%</td>
<td>0.057</td>
<td>95%</td>
<td>0.001</td>
<td>93%</td>
<td>0.004</td>
</tr>
<tr>
<td>Salinity</td>
<td>94%</td>
<td>0.019</td>
<td>98%</td>
<td>0.45</td>
<td>96%</td>
<td>0.60</td>
</tr>
<tr>
<td>ORP</td>
<td>100%</td>
<td>1.000</td>
<td>NTV</td>
<td>NTV</td>
<td>99%</td>
<td>0.54</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>98%</td>
<td>0.39</td>
<td>98%</td>
<td>0.002</td>
<td>95%</td>
<td>0.12</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>71%</td>
<td>0.250</td>
<td>NTV</td>
<td>NTV</td>
<td>95%</td>
<td>0.34</td>
</tr>
<tr>
<td>DOC</td>
<td>78%</td>
<td>0.34</td>
<td>99%</td>
<td>0.086</td>
<td>39%</td>
<td>0.011</td>
</tr>
</tbody>
</table>

NTV: no test value
Table 4-5. Hydrological mixing model based on the endmembers in RDA analysis in figure 1 by using nonreactive Chloride trace (µM), and the values of N functional genes (copies/L) and concentrations of DIN (µM) in situ and form modeling prediction based on hydrological model.

<table>
<thead>
<tr>
<th></th>
<th>Fresh endmember</th>
<th>Salt endmember</th>
<th>Mixing zone (in situ)</th>
<th>Mixing zone (model)</th>
<th>Deviation (model/in situ)</th>
<th>Mixing model (Cl⁻ based)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>6.40</td>
<td>602.62</td>
<td>433.85</td>
<td>433.85</td>
<td>1</td>
<td>M_{cl}=28.31%*C_{f, cl}+71.69%*C_{s, cl}</td>
</tr>
<tr>
<td>nifH</td>
<td>9.90E+05</td>
<td>3.38E+07</td>
<td>9.53E+04</td>
<td>2.45E+07</td>
<td>257.28</td>
<td>M_{nifH}=28.31%*C_{f, nifH}+71.69%*C_{s, nifH}</td>
</tr>
<tr>
<td>nrfA</td>
<td>8.46E+06</td>
<td>1.72E+07</td>
<td>2.45E+06</td>
<td>1.47E+07</td>
<td>6.02</td>
<td>M_{nrfA}=28.31%*C_{f, nrfA}+71.69%*C_{s, nrfA}</td>
</tr>
<tr>
<td>AOA</td>
<td>1.91E+07</td>
<td>6.89E+05</td>
<td>1.69E+06</td>
<td>5.90E+06</td>
<td>3.50</td>
<td>M_{AOA}=28.31%*C_{f, AOA}+71.69%*C_{s, AOA}</td>
</tr>
<tr>
<td>AOB</td>
<td>5.86E+04</td>
<td>1.21E+05</td>
<td>4.69E+04</td>
<td>1.03E+05</td>
<td>2.19</td>
<td>M_{AOB}=28.31%*C_{f, AOB}+71.69%*C_{s, AOB}</td>
</tr>
<tr>
<td>nirK</td>
<td>9.89E+07</td>
<td>1.74E+08</td>
<td>1.41E+07</td>
<td>1.53E+08</td>
<td>10.83</td>
<td>M_{nirK}=28.31%*C_{f, nirK}+71.69%*C_{s, nirK}</td>
</tr>
<tr>
<td>nirS</td>
<td>2.73E+06</td>
<td>4.98E+05</td>
<td>9.73E+04</td>
<td>1.13E+06</td>
<td>11.63</td>
<td>M_{nirS}=28.31%*C_{f, nirS}+71.69%*C_{s, nirS}</td>
</tr>
<tr>
<td>nosZ</td>
<td>6.60E+04</td>
<td>1.85E+05</td>
<td>1.33E+05</td>
<td>1.51E+05</td>
<td>1.14</td>
<td>M_{nosZ}=28.31%*C_{f, nosZ}+71.69%*C_{s, nosZ}</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>10.09</td>
<td>2.08</td>
<td>39.42</td>
<td>4.35</td>
<td>0.11</td>
<td>M_{NH4}=28.31%*C_{f, NH4}+71.69%*C_{s, NH4}</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>57.10</td>
<td>0.12</td>
<td>0.28</td>
<td>16.25</td>
<td>57.48</td>
<td>M_{NO3}=28.31%*C_{f, NO3}+71.69%*C_{s, NO3}</td>
</tr>
</tbody>
</table>
Table 4-6. Primers and thermal cycling conditions for Nitrogen functional genes

<table>
<thead>
<tr>
<th>Sequence</th>
<th>bp</th>
<th>QPCR Cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifH → N₂ fixation</td>
<td>400</td>
<td>QPCR→95°C 3min→(95°C, 30s ; 59°C, -1°C↓/ each cycle, 30s; 72°C, 1min) ×6x→ (95°C, 30s; 55°C, 30s; 72°C, 1min; 83°C, 15s→'read') ×32x→final 95°C, 15s; 60°C, 1min; +0.5 °C→'read'; 95°C, 15s→'read'(Mehta et al., 2003)</td>
</tr>
<tr>
<td>AOAmi876F→5'-ATGGCTGGTGCAGCAGCAG-3'</td>
<td>632</td>
<td>QPCR→95°C for 3min→(95°C, 30 s; 60°C, 45 s; 72°C, 45 s; 80°C, 15s→'read') ×40→final 95°C, 15s; 60°C, 1min; +0.5 °C→'read'; 95°C, 15s→'read'(Jin et al., 2011; Tourna et al., 2008)</td>
</tr>
<tr>
<td>nirK→Nitrite reductase NO₂⁻→NO</td>
<td>165</td>
<td>QPCR→95°C 3min→(95°C, 30 s; 63°C, 30s - 1°C↓/ each cycle; 72°C, 30s) ×6x→(95°C, 30s; 60°C, 30s; 72°C, 30s; 81°C, 15s→'read') ×34x→final 95°C, 15s; 60°C, 1min; +0.5 °C→'read'; 95°C, 15s→'read'(Henry et al., 2004)</td>
</tr>
<tr>
<td>nirS→Nitrite reductase NO₂⁻→NO</td>
<td>400</td>
<td>QPCR→95°C 3min→(95°C, 30 s; 63°C, 30s - 1°C↓/ each cycle; 72°C, 30s) ×6x→(95°C, 30s; 60°C, 30s; 72°C, 30s; 81°C, 15s→'read') ×34x→final 95°C, 15s; 60°C, 1min; +0.5 °C→'read'; 95°C, 15s→'read'(Henry et al., 2004)</td>
</tr>
<tr>
<td>nosZ→Nitrous oxide reductase N₂O→N₂</td>
<td></td>
<td>QPCR→95°C 15min→(95°C, 15s; 67°C, 30s - 1°C↓/ each cycle; 72°C, 30s; 83°C, 15s→'read') ×6x→(95°C, 15s; 62°C, 15s; 72°C, 30s; 83°C, 15s) ×40x→final 95°C, 15s; 65°C, 15s +0.5 °C→'read'; 95°C, 15s→'read'(Henry et al., 2006)</td>
</tr>
<tr>
<td>16S→Bacteria</td>
<td>353</td>
<td>QPCR→√ 95°C 3min→(95°C, 30s ; 52°C, 45s; 72°C, 30s; 80°C, 15s→'read') ×40x→final 95°C, 15s; 60°C, 1min; +0.5 °C→'read'; 95°C, 15s→'read'(Harms et al., 2003)</td>
</tr>
</tbody>
</table>
Figure 4-1. Values of Salinity, ORP, concentrations of DOC (Dissolved Organic Carbon), NH$_4^+$ and NO$_3^-$ for water samples collected from study sites. A) C7B. B) Pargos. The ‘0’ values for concentration of NO$_3^-$ below detection limit.
Figure 4-2. Copy numbers of N-cycling genes measured in water samples collected from study sites. A) C7B. B) Spring Pargos. Dash arrows represent the wet season in C7B, high tide in Pargos; solid arrows represent the dry season in C7B, low tide in Pargos, respectively.
Figure 4-3. RDA triplot showing correlation between N cycling gene copies and physicochemical parameters measured in water samples collected from Cenote 7 Bocas (closed circles) and spring Pargos (open circles). A) Samples cluster from C7B. B) Samples cluster from high tide (HT) in Pargos. C) samples cluster from low tide (LT) in Pargos.
Figure 4-4. RDA triplot showing correlation between N cycling gene copies and physicochemical parameters measured in water samples collected from C7B. Asterisks (*) indicated significant difference between different seasons (T-test with $P < 0.05$). A) Samples cluster from dry season (D) in C7B; B) Samples cluster from wet season (W) in C7B.
Figure 4-5. RDA triplot showing correlation between N cycling gene copies and physicochemical parameters measured in water samples collected from spring Pargos. Asterisks (*) indicated significant difference between different tides (T-test with $P < 0.05$). A) Samples cluster from high tide (HT) in Pargos. B) Samples cluster from low tide (LT) in Pargos.
Figure 4-6. N cycling model in C7B and spring Pargos. Yellow line in C7B indicates the position of halocline.
CHAPTER 5
THE VARIATION OF NITROGEN CYCLING GENES ALONG THE SEEPAGE FACE AND RIVER DISCHARGE AREA IN IRL

Background

There are two main terrestrially derived fresh flows to coastal areas; one is submarine groundwater discharge (SGD), and another one is river discharge (RD) (Taniguchi et al., 2002; Destouni et al., 2008; Mulligan and Charette, 2009). SGD is the groundwater flow from continental margins presented as diffuse seepage or spring discharge through the seabed to the coastal area, which is driven by hydraulic forcing between the continental and oceanic aquifers (Burnett et al., 2003; Moore et al., 2010); River discharge is surface flow to the ocean, which includes large amount of sediment (mainly as suspended sediment) in addition to water (Phillips and Slattery, 2006; Paola, 2011).

Recently, SGD input has been considered to be more important than riverine input to coastal areas due to higher flux of nutrients (e.g. nitrogen-nutrients) and carbon, such that SGD plays a main role in coastal biogeochemical cycling (Moore et al., 2006 and 2010; Santos et al., 2008; Rogers and Casciotti, 2010). Santos et al. (2008) reported that the distribution of dissolved nitrogen species were consistent with a sequence of reactions in the surface layer where nitrate was initially depleted because of denitrification, ammonium accumulation, and some dissolved organic carbon (DOC) remained due to organic matter remineralization. Charette et al. (2013) found that dissolved inorganic nitrogen (DIN) from SGD flux was 3 times lower than from river flux. Coastal rivers serve as vital conduits in DOC transportation to coastal areas (Leech et al., 2016), and the DOC is around 10 times higher from river discharge when compared with submarine groundwater discharge (SGD) (Szymczycha, and Pempkowiak, 2016).
Dai et al. (2012) indicated that riverine dissolved organic carbon (DOC) fluxes were highest from South America. Beusen et al. (2013) reported that the direct comparison of RD to SGD is difficult due to the difference in coverage range of discharge, and few field studies have been conducted. Certainly, all those geochemical N cycling are driven by microbial based processes (Falkowski et al., 2008; Santoro, 2010; Algar and Vallin, 2014), which are particularly complicated to model due to numerous functional genes involved in and different groups functioning differently in same conditions (Bowen et al., 2014). It is necessary to study how the different loading of N-nutrients and DOC from SGD and RD affect the whole N cycling at the genetic level, and thereupon, how these microbially based processes may provide insight into the functioning of the entire N cycle, and the potential fate of N-nutrients input to the coastal area via SGD and RD in coastal areas.

The coupled processes of nitrification and denitrification play an essential role in the fate of N-nutrients input to coastal areas (Jäntti et al., 2011; Hines et al., 2012 and 2015; Zhang et al., 2015; Marchant et al., 2016). After Francis et al. (2005) proposed that ammonium oxidizing archaea (AOA) may be more widespread than AOB in marine sediments and played an important role in coupled nitrification-denitrification process, Bowen et al. (2014) confirmed that AOA were numerous than ammonium oxidizing bacteria (AOB) in total gene abundance and gene expression levels in marine sediments. They also found that the normalized gene expression (cDNA:DNA) of AOB was approximately 10 times higher than AOA, suggesting that AOB may play a disproportionately large role relative to their total abundance. Some studies argue that AOB are more important in ammonia oxidation process (Bernhard et al., 2010; Wang et
al., 2011; Wang et al., 2013a). Zhang et al. (2014) found a hint of coupled nitrification (all amoA)-denitrification (nirS) in a riverine estuary; However, Bowen et al. (2014) said that more attention should be placed on gene expression, rather than total gene abundance, because inactive genes could obscure the linkage between gene abundance and ecosystem function. Little information is available to prove the indication of coupled nitrification-denitrification process, or identify which type of amoA (AOA or AOB) is more important to the coupling process at the level of gene expression; therefore it is necessary to conduct in situ field studies to investigate the competition between nitrifiers and the genes involved in coupled nitrification-denitrification processes in coastal areas.

In addition to the coupling of nitrification and denitrification processes, an important process is the complete denitrification process to N₂, which is one of the crucial processes that controls N₂O emission (de Wilde and de Bie, 2000), by nitrite reductase (nirK and nirS) and nitrous oxide reductase (nosZ). There have been some genetic based studies in inland soil and lake systems (Wang et al., 2012; Harter et al., 2014; Saarenheimo et al., 2015) and some isotope based studies in coastal aquifer systems (Marchant et al., 2016; Young et al., 2016) to illustrate the N₂O emission via denitrification, yet little genetic based information is available for coastal areas.

To address these issues, we obtained surface sediments from SGD areas in the IRL (IRL) of Florida, and studied the microbial ecology of N cycling by quantifying the N cycling genes at both gene abundance and gene expression levels, and analyzed the correlations between them to investigate the coupled nitrification-denitrification process. We hypothesized that: (1) The different loading of N-nutrients and DOC from SGD and
RD in coastal areas will change the total number and active numbers of the different N cycling genes, but will not change the distribution pattern and the competition pattern of N cycling; (2) AOB rather than AOA dominates in marine sediments at both gene abundance and gene expression level, as was reported by Santoro and Francis (2008) and Bagchi et al. (2014), and plays a key role in coupling with denitrifiers in coastal areas. (3) Our genetic based analyses will support the isotopic based results from Young et al. (2016), that most NO$_3^-$ will be reduced to N$_2$ due to high DOC.

**Materials and Methods**

**Site Description**

IRL is a long transition estuary between terrestrial and marine system in the Atlantic coastal area of central Florida (Figure 5-1), which receives fresh water mainly from precipitation, urban storm water, and river discharge (Figure 5-1(B)) as well as terrestrial SGD (Figure 5-1(C)). Tidal variation is small in IRL and thus has little effect on the discharge magnitude and the freshwater-saltwater boundary of terrestrial SGD, or on river discharge (Martin et al., 2007; Roy et al., 2013). Eau Gallie North (EGN) is one type of SGD area where flow rates decrease from around several, sometimes tenths of centimeters per day at the coastline, to no flow at the freshwater-saltwater boundary (Martin et al., 2007; Roy et al., 2010), and the freshwater-saltwater boundary is between 22.5 m and 30 m from shoreline, which moves back and forth depending on storms, hurricanes, and variation of discharge (Roy et al., 2010, 2011 and 2013). Eau Gallie River is divided into several sections for human use (Hoffman et al., 1974), which contributes around 25% of the fresh water influx with a large amount of nutrients to the northern area of the Indian River. The lower portion of the river will become saline when the prevailing wind is easterly, meanwhile it is a large source of biomass for the Indian
River which is supported by the high concentrations of organic sediments on the river bottom (Hoffman et al., 1974).

Sample Collection

Triplicate surface sediment cores were collected at sites 0, 15, 17.5, 20, 22.5, 30 m (EGN0, EGN15, EGN17.5, EGN20, EGN22.5, EGN30) from shoreline within SGD area of Eau Gallie North (EGN), as well as at EGT and EGMD within river discharge area of Eau Gallie River in August, 2015 (Figure 5-1). All the sediments for DNA and RNA isolation were immediately frozen on dry ice and transported to the lab. At the same time, nutrient water samples were filtered and frozen until analysis, dissolved organic carbon (DOC) samples were filtered into pre-combusted amber vials and acidified in the field with HCl to a pH<2 and stored at 4°C.

Samples Processing and Data Analysis

DNA Isolation and PCR: DNA was extracted from sediments using Powersoil DNA Isolation Kit (Mobio, Carlsbad, CA, USA). All primers and quantitative polymerase chain reaction (qPCR) primers and cycling parameters of N related functional genes are presented in Table 4-6 (Nitrogen fixation, nifH; ammonia oxidizing archaea (AOA) and bacteria (AOB); denitrifiers, nirK and nirS; Dissimilatory Nitrate Reduction to Ammonium (DNRA), nrfA; nitrous oxide reduction, nosZ). For the qPCR and RT- qPCR assays, all sample DNAs and cDNAs together with standard DNAs were analyzed in duplicate by using the SYBR green protocols in a StepOne Plus real-time PCR system (Applied Biosystems) (Dang et al., 2013; Bea et al., 2014). Abundances of all genes were calculated as copies of genes per gram of sediment. Standard curves with $R^2>0.999$ were generated by serial dilution from $10^{-4}$ to $10^{-8}$ of reference plasmids containing target genes constructed by cloning the appropriate amplification products into E. coli.
hosts using TOPO TA cloning kits (Life Technologies, Thermo Fisher Scientific Inc, USA).

The YSI Professional Plus Meter (calibrated daily) recorded salinity, pH, and oxidation-reduction potential (ORP) in situ. Ammonium and nitrate concentrations were analyzed by Seal AA3 Autoanalyzer continuous flow system following the methods of Strickland and Parsons (1978). The detection limits of NO$_3^-$ and NH$_4^+$ were 0.1 µmol L$^{-1}$ and 0.5 µmol L$^{-1}$, respectively. DOC concentrations were analyzed on a Shimadzu TOC-VCSN total organic carbon analyzer, and the coefficient of variance was less than 2%. All analyses were completed in the Department of Geological Sciences, UF.

**Data analysis:** Principal component analysis (PCA) was employed using ordination analysis in CANOCO 4.5 program (ter Braak and Šmilauer, 2002), and the significant difference in copies of N cycling genes between seepage face in Eau Gallie North (EGN) and muck area in Eau Gallie River (Figure 5-1) was tested by T-test, the significant difference in copies of different N cycling genes within the same site was tested by Tukey-Kramer honestly significant difference (HSD) of one-way analysis of variance (ANOVA) in SPSS program, and $P$ value smaller than 0.05 was considered as significant.

**Results**

**Physicochemical Characteristics in IRL**

The mean concentration of NH$_4^+$ was around 65.1 µM and 146.6 µM, and the mean concentration of NO$_3^-$ was around 2.6 µM and 0 µM in seepage face and muck area, respectively (Figure 5-2), and Young (2013) studied the nitrate attenuation mechanisms in SGD area and found that more nitrate was denitrified during SGD to the mud cap sediments than during SGD through sand sediments. NH$_4^+$ was significantly
higher than NO$_3^-$ in both sites (T-test with $P < 0.05$), and NH$_4^+$ was significantly higher in the muck area than in the seepage face (T-test with $P < 0.05$). The mean concentration of DOC around 383.3 µM and 567.5 µM in seepage face and muck area, respectively, which was significant higher in muck area than that in seepage face (Figure 5-2, T-test with $P < 0.05$).

**Number of Total and Active Bacteria in IRL**

The total number and their relative activities were quantified by qPCR and RT-qPCR (Figure 5-3) using the 16S rRNA gene primers presented in Table 4-5. The total number of bacteria had significant difference along the seepage face and muck area, (HSD of ANOVA with $P > 0.05$), while the relative activites (mRNA copies) only showed a significant difference among some sites along the seepage face, with significantly higher numbers of transcripts at 20m from shoreline (HSD of ANOVA with $P < 0.05$).

The total number and the active number of bacteria were significantly higher in the muck area than in the seepage face (HSD of ANOVA with $P < 0.05$), while the ratio of active to total bacteria didn’t show significant differences between these two areas in general, excluding 30m from shoreline in seepage face.

**The Distribution Pattern of N Cycling Genes in IRL**

The N cycling genes were quantified by qPCR (DNA) and RT-qPCR (cDNA; from mRNA) using the N cycling genes listed in Table S1, and the results are presented in Figures 5-4 and 5-5. The general distribution pattern of the total and active N cycling genes in the seepage face and muck area were similar, with the nitrifiers AOA and AOB significantly lower than the NH$_4^+$ producers $niiH$ and $nrfA$, as well as the denitrification genes $nirK$, $nirS$, and $nosZ$ at both total and active levels (HSD of ANOVA with $P < 0.05$). For each N cycling gene, the total number was generally 1000 times higher than
the active number, excluding nosZ, which had a highest expression ratio. nitH was the major NH₄⁺ active producer and significantly higher than nrfA (HSD of ANOVA with P < 0.05); AOB was the only active nitrifier; nirK and nosZ were the main active denitrifiers and significant higher than nirS (HSD of ANOVA with P < 0.05) and the active nosZ was higher than the total active nir (nirK and nirS) even with lowest total number among all the denitrifiers; among all the nitrite reductases, the total and active number of nrfA was lower than nir (nirK and nirS) for all samples collected from seepage face, while the active nrfA was higher than nir (nirK and nirS) even with lower total numbers of nrfA in comparison with nirK/S for all samples collected from the muck area (Figure 5-5).

The PCA analysis of the seepage face and muck area in IRL indicated the difference between these two areas based on N cycling genes (Figure 5-6), and Person correlation analysis was conducted to describe the correlation between each N cycling genes (Tables 5-1 and 5-2). The numbers of the total and active N cycling genes in the seepage face were significantly lower than in the muck area (T-test with P < 0.05), and those two sites were clearly separated along axis 1 gradient with the variation explaining more than 94% (Figure 5-6). The muck area showed a higher variation than the seepage face along both axes, and the spatial variation was larger based on the active N cycling genes than based on total numbers of N cycling genes, which was especially obviously presented for samples collected from muck area at the mouth of the river, and Bowen et al. (2014) also found a bigger variation for the gene expression than that for gene abundance of N cycling genes in their incubation study. All the N cycling genes showed significantly positive correlations between each other both at total and active levels in our study area (Pearson correlation, P<0.05).
Discussion

The Effects of DOC on Microbial Activity and N Cycling Genes

Effects on Microbial activity: The concentration of DOC had big effects on both the growth (including growth rate and efficiency) and the composition of microbial communities (Eiler et al., 2003; Xu et al., 2014; Le et al., 2016). In our study, the total and relative activities of bacteria together with the total and relative activities of N cycling genes were significantly higher in the muck area than in seepage face area even with large variations among the replicates in muck area (Figures 5-3 and 5-5), which indicated that higher DOC input via riverine discharge exerted stronger effects on the abundance and activity of bacteria in the coastal estuary area than SGD area with sediments as a filter. Xu et al. (2014) also found that riverine DOC input significantly increased bacterial production.

Among all the N cycling genes, the total and relative activities of nitrifiers were significantly lower than other genes, especially the denitrifiers (Figure 5-4). This may be because nitrifiers are chemoautotrophic and obtain energy from ammonium oxidation rather than from organic carbon (Verhagen and Laanbroek, 1991), while most denitrifiers are heterotrophic and carbon is important for their activity. Bowen et al. (2014) said that carbon is likely to be the ultimate factor limiting complete denitrification, and the limitation was ultimately observed at the genetic level. So, the significantly higher relative activity of nitrifiers in the muck area was due to significantly higher concentrations of ammonium (Figure 5-6), and higher DOC in the ocean water supported higher activities of denitrification together with N immobilization by heterotrophic microbes, where less ammonium can support nitrifying bacteria activity (Strauss and Lamberti, 2002; Booth et al., 2005). This may explain the low relative
activities of nitrifiers in comparison with denitrifiers. Montano et al. (2007) also found that autotrophic nitrification is magnified by decreases in C availability for heterotrophic microbial activity due to the decreased N immobilization by heterotrophic microbes under lower C availability.

**Effects on nitrate reductase** (*nrfA, nir [nirK and nirS]*): DNRA bacteria (*nrfA*) and denitrifiers (*nirK* and *nirS*) are regulated by similar environmental conditions and competition for electron donor and nitrate in the environment (Sgouridisa et al., 2011). C/N ratios has a significant influence on the dominant N reduction pathway, such that DNRA will be the major pathway at higher ratio of C to N, whereas denitrification dominates in environments that are rich in nitrate while low in electron donors (Mohan et al., 2004; Algar and Vallin, 2014). Behrendt et al. (2013) found that nitrate reduction was clearly dominated by denitrification rather than by DNRA in marine sediments, irrespective of the electron donors (e.g. organic carbon, sulfide, or iron). Our results indicate that the N reduction process will shift from denitrification dominant to DNRA dominant when the environment switch form the seepage face (low DOC, high NO$_3^-$) to muck area (high DOC, extremely low or no NO$_3^-$) (Figure 5-5), and we speculated that low ratios of C:N due to high nitrate concentration and low labile C for DNRA process contributed active denitrifiers higher than active DNRA in seepage face, and more efficiently scavenge nitrate from the environment by DNRA, as the higher affinity for nitrate contributes to DNRA outnumbering denitrifiers under environment conditions similar to the muck area (Dong et al., 2011).
Competition between Nitrifiers (AOA and AOB) and Coupled Nitrification and Denitrification Process

Recently, numerous studies have indicated that AOA are more numerous than AOB under certain conditions, which countered the notion of AOB as the only nitrifier type responsible for ammonia oxidation (Hatzenpichler, 2012; Pett-Ridge et al., 2013; Wang and Gu. 2014). This may be attributed to the mixtrophic AOA with higher affinity for \( \text{NH}_4^+ \) and oxygen, such that they may be more competitive than the autotrophic AOB under environmental conditions with lower oxygen, \( \text{NH}_4^+ \), or pH (Francis et al., 2005; Leininger et al., 2006; Martens-Habbena et al., 2009; Rogers and Casciotti, 2010; Kim et al., 2012; Pett-Ridge et al., 2013; Wang et al., 2013a; Bowen et al. 2014; Wang and Gu., 2014). Wang and Gu. (2014) found in an incubation experiment that acidic conditions had a small effect on AOA in comparison with AOB, and high nitrification rates in acidic conditions may be associated with higher abundance of AOA rather than AOB. AOA showed a higher affinity for \( \text{NH}_4^+ \) at low pH, which decreased the availability of \( \text{NH}_4^+ \) and AOB usually showed a very low abundance or were even undetectable in acidic conditions. In our study, we found that AOB outnumbered AOA both at the gene abundance and gene expression level (active AOA was undetectable) under the environmental condition with near neutral pH, high redox potential and appropriate concentration of \( \text{NH}_4^+ \) in the lagoon system (Figure 5-4). This indicated that AOB rather than AOA was closely correlated with ammonia oxidation and was consistent with some other studies conducted over a wide range of study areas under different conditions (Bernhard et al., 2010; Wang et al., 2011; Wang et al., 2013a). Santoro and Francis (2008) and Bagchi et al. (2014) pointed out that AOA were numerically dominant over
AOB in freshwater system, while AOB outcompeted AOA in marine system by several orders of magnitude.

Variation in ORP in subtidal permeable sediments would favor coupled aerobic and anaerobic processes in coastal areas (Marchant et al., 2016), and coupled N processes were very important to remove (coupled nitrification to denitrification and Anammox, and coupled DNRA to Anammox) and nitrification coupled with DNRA (Rütting et al., 2008; Jäntti et al., 2011; Hines et al., 2012 and 2015; Zhang et al., 2015). We found that active nitrifier AOB was significantly correlated with all active denitrifiers (nirK, nirS, nosZ) and active nrfA in our study (Table 1 and 2), which indicated that nitrification-denitrification and nitrification-DNRA may be closely coupled. The angle between AOB and nirK was much smaller (overlapped) than that between AOB and nrfA in PCA analysis (Figure 5-5), which indicated that nitrification-denitrification coupled more closely than nitrification-DNRA in IRL. Marchant et al. (2016) found that a closer correlation between nitrification and denitrification leads to extensive N-nutrient loss rather than coupled nitrification-DNRA, which recirculates N-nutrients, in permeable sediments in coastal areas. Hines et al. (2012) investigated the coupled N process by modeling and further confirmed that nitrification plays a crucial role in N removal, and coupled nitrification-denitrification accounted for a higher proportion than other coupled processes in their study estuary, even though Zhang et al. (2015) found that coupled nitrification-DNRA played an very important role in N conservation in neutral and alkaline conditions in rice field due to the syntrophic requirement for providing more available N-nutrient for plants. Meanwhile, our results also suggested that AOB rather than AOA play an important role in coupled nitrification-denitrification in brackish
sedimentary system in our study area, and we tested the hypothesis summarized in Francis et al. (2005), confirming that AOB outcompete AOA as the major nitrifier consistent with Bagchi et al. (2014) at gene expression levels based on their in situ study in coastal area.

**Potential N₂O Emission in IRL**

N₂O is a byproduct or intermediate product during nitrification, DNRA and denitrification processes, which have been less reported in coastal systems (Wong et al., 2013a; Marchant et al., 2016; Young et al., 2016). In our study, we represented the N₂O reduction by using total and active gene nosZ in coastal estuary related to the SGD and river discharge. Wang et al. (2012) also studied N₂O reduction by using nosZ, and found that nosZ controlled N₂O reduction along the littoral gradient in a eutrophic freshwater lake. Active nosZ was higher than nir (nirK and nirS) in both seepage face and muck area (Figure 5-5), suggesting complete N₂O reduction and little N₂O emission in our study area. This may have been related to the very low concentration of NO₃⁻ in both sampling sites; while nosZ/nir(nirK and nirS) >1 may be due to nirK and nirS-type denitrifiers being currently underestimated in PCR-dependent surveys due to primer coverage limitations, which may attribute to the higher measurement of expression of nosZ than total nirK+nirS in our study (Wei et al., 2015; Helen et al., 2016). Harter et al., (2014) and Saarenheimo et al. (2015) used the ratios of nosZ/nir(nirK and nirS) to predict N₂O emission in agriculture area and lake system, and found that the environmental condition with higher NO₃⁻ and low carbon was accompanied by lower nosZ/nir ratios, Marchant et al. (2016) confirmed the relationship by using stable isotope techniques, and found a net N₂O consumption only until nitrate was limited in costal system, and Young et al. (2016) demonstrated that higher DOC favored the complete
denitrification process and increased N2 production and they predicted that sea level rise may offset N2O flux to the atmosphere in the SGD area as DOC inputs from seawater driving the complete denitrification process.

Saarenheimo et al. (2015) speculated that nitrification contributed to N2O production in oxic-anoxic interface, and Burgos et al. (2015) reported that environmental conditions in coastal sediments are favorable for nitrate reduction via denitrification due to high loading of organic matter and low oxygen concentrations. They reported that N2O production was positively correlated with oxygen utilization, which may be attributed to nitrification. de Wilde and de Bie (2000) also found that nitrification in the water column was the main source of N2O in an estuary, and then Santoro et al. (2010) calculated that nitrification can produce around 1.5 to 4 times of local N2O flux from deep water. Santoro et al. (2011) found a signature of N2O produced by marine ammonia-oxidizing archaea (AOA) by the isotope technique in coastal areas, and Marchant et al. (2016) confirmed that N2O production was partially from nitrification in the coastal subtidal permeable sediments in South-East North Sea. Considering the DOC/NO3− ratios between the seepage face and muck area, we predicted that N2O production would be higher in seepage face than in the muck area. Wong et al. (2013) also found that SGD had the highest N2O fluxes (around 20 fold higher than riverine input) observed in estuaries.

**Summary**

To our knowledge, this is the first genetic based study of the response of the N cycle to the different loading of N-nutrient and DOC from SGD and RD in coastal waters. Our data further confirm the previous geochemical/isotopic based researches (Santos et al., 2008; Young et al., 2016), and support the idea from Bagchi et al. (2014)
that $AOB$ dominates in marine sediments and also identify that $AOB$ rather than $AOA$ closely couples with all denitrifiers at the gene expression level in coastal areas. We also found that DOC significantly affects the total and active number of the N cycling genes, and governs the complete denitrification process that contributed to the N$_2$O emission. This is consistent with most other genetic and geochemical/isotopic based studies (Harter et al., 2014; Saarenheimo et al., 2015; Marchant et al., 2016; Young et al., 2016). All of those findings provide insight into the underlying mechanism of whole N cycling in sediments controlled by microorganisms in IRL, and help to determine the potential fate of N-nutrients input to the coastal as well as predict the most likely pathway for N$_2$O emission in this area.
Table 5-1. Pearson correlation coefficient between total N cycling genes (Copies/L) for all the samples collected from IRL in August, 2015

<table>
<thead>
<tr>
<th></th>
<th>nifH</th>
<th>nrfA</th>
<th>AOA</th>
<th>AOB</th>
<th>nirK</th>
<th>nirS</th>
<th>nosZ</th>
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<td></td>
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<td>.977**</td>
<td>.987**</td>
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<td>.963**</td>
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<tr>
<td>nosZ</td>
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<td>.946**</td>
<td>.879**</td>
<td>.857**</td>
<td>.840**</td>
<td>.903**</td>
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</tr>
</tbody>
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Positive value means positive correlation; negative value means negative correlation;
* Correlation was significant at the 0.05 level (2-tailed);
** Correlation was significant at the 0.01 level (2-tailed).
Table 5-2. Pearson correlation coefficient between active N cycling genes (Copies/L) for all the samples collected from IRL in August, 2015

<table>
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<th>AOB</th>
<th>nirK</th>
<th>nirS</th>
<th>nosZ</th>
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<tr>
<td>nrfA</td>
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<td></td>
<td></td>
</tr>
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<td>.600*</td>
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<td>nosZ</td>
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<td>.665*</td>
<td>1</td>
</tr>
</tbody>
</table>

Positive value means positive correlation; negative value means negative correlation;
*. Correlation was significant at the 0.05 level (2-tailed);
**. Correlation was significant at the 0.01 level (2-tailed).
Figure 5-1. Location map of the IRL system. A) The southern portion of Mosquito Lagoon, BRL, and the northern portion of IRL. B) Sampling muck site in Eau Gallie River. C) Sampling site in nearshore seepage face (adapted from Martin et al., 2007).
Figure 5-2. Concentration of NH₄⁺ and NO₃⁻ as well as DOC for sediments collected from seepage face and muck area in Eau Gallie River of IRL in August, 2015. Different letters represent significant difference (Tukey-Kramer (HSD) of ANOVA with $P < 0.05$).
Figure 5-3. Copy number of 16S rRNA genes for DNA and cDNA measured in sediments collected from seepage face and muck area in Eau Gallie River of IRL in August, 2015. The different letters indicate significant differences among each N cycling genes (Tukey-Kramer (HSD) of ANOVA with $P < 0.05$)
Figure 5-4. Copy number of N cycling genes for DNA and cDNA measured in sediments collected from seepage face and muck area in Eau Gallie River of IRL in August, 2015. The different letters indicate significant differences among each N cycling genes (Tukey-Kramer (HSD) of ANOVA with $P < 0.05$)
Figure 5-5. The ratios of \( \text{nosZ/nir(nirK+nirS)} \) and \( \text{nrfA/nir(nirK+nirS)} \) measured in sediments collected from seepage face and muck area in Eau Gallie River of IRL in August, 2015.
Figure 5-6. PCA biplot showing distribution pattern of sampling sites based on N cycling gene measured in sediments collected from Eau Gallie River of IRL (IRL) in August, 2015. Asterisks (*) indicates the significant difference of the values between seepage face and muck area (T-test with \( P < 0.05 \)).
CHAPTER 6
MICROBIAL NITROGEN CYCLING RESPONSE TO THE GROUNDWATER DISCHARGE AND SALTWATER INTRUSION ALONG THE SEEPAGE FACE IN BRL, FLORIDA

Background

Subterranean estuaries (STEs) are active N cycling zones, where permeable sediments along the freshwater-saltwater interface play a very important role in regulating nitrogen (N) transformation from groundwater to coastal waters (Gu et al., 2007; Mills et al., 2008; Santoro, 2010; Torres et al., 2012). As the sea level fluctuates due to tidal cycling, the microbes will experience different hydrologic processes including but not limited to submarine groundwater discharge (SGD) or saltwater intrusion, and consequently different physicochemical characteristics (e.g. salinity, pH, ORP, and N-nutrients). Preisner et al. (2016) showed an overall compositional and functional sensitivity of microbial communities to environmental condition changes in their study, and found the activity of N cycling genes changes significantly before and after environmental disturbance related to the salinity changes. Changes in these abiotic factors will change the microbial activity related to N cycling, consequently change the N nutrient status in this area in a short term, which including release of adsorbed ammonium, lower nitrate concentrations after salt intrusion (Weston et al., 2006; Canavan et al., 2007). In terms of the global changes in sea level and human activities related to the freshwater withdrawal, the long term effects of the shifting hydrological processes on the N cycling processes should be predicted and well understood in the future (Santoro, 2010).

The prediction and understanding of the responses of microbial community as well as activity to the changes in these processes are usually studied in laboratory
manipulation experiments via flow through reactors after recognizing the shortage of slurry incubations (Laverman et al., 2006; Wankel et al., 2011). Recently, there have been many studies using flow-through incubation to investigate changes in microbial community structures (Edmonds et al., 2009), and physicochemical processes related to the carbon, N, Sulfur, Phosphorus cycling (Weston et al., 2006; Laverman et al., 2007; Gu et al., 2007; Torres et al., 2012; Zhao et al., 2013), as well as microbial carbon and N transformation processes (Hunter et al., 2006; Laverman et al., 2006; Wankel, et al., 2011; Hatamoto et al., 2014). Most of those studies use artificial freshwater and saltwater with salinity range smaller than 10 psu for tidal freshwater sediments (Weston et al., 2006; Laverman et al., 2007; Edmonds et al., 2009), or they just conduct study with field seawater flows through incubation with salinity range larger than 29 psu for permeable or freshwater sediments in coastal area (Hunter et al., 2006; Canavan et al., 2007; Wankel, et al., 2011; Torres et al., 2012). Whereas, little information is available about how the whole microbial N cycling responds to the shifts between SGD and saltwater intrusion via flow-through experiments combining field freshwater and seawater together in permeable sediments within the saltwater-freshwater interface in the STEs.

Previous studies have demonstrated that the difference in microbial community structures and activities were most strongly controlled by physical gradients such as depth, sediment grain size, and sediment porosity in coastal areas (Hunter et al., 2006; Mills et al., 2008; Highton et al., 2016). One hypothesized model proposed by Santoro (2010) was inconclusive about the composition of microbial community with mixtures of of two end members (fresh and saltwater) or specific intermediate salinity members in
the saltwater-freshwater interface. We here hypothesize that the composition of microbial community related to N cycling in surficial sandy sediments should contain a mixture of the two end members and show stable and diverse genetic adaption to the multiple changes in environmental conditions, and we also hypothesize that microbial activity related to the N cycling in different depths with different grain size should respond differently to those changes in environmental conditions. Again, Edmonds et al. (2009) stated that microbial responses to environmental changes were mostly attributed to shifts in the metabolic pathways rather than changes in the community gene reservoir, but it is still unclear whether those metabolic changes in N cycling are significant or not in the different layers in STEs, so we will test the significance in the response by studying the activities of the N cycle in the different layers that are associated with different depths.

N cycling genes sharing similar same functions (e.g., nitrifiers, AOA and AOB; denitrifers, nirK and nirS) will also respond differently to environmental changes, but little is known about their relative response to changes in salinity (Zhang et al., 2013). Zhang et al. (2013) found in their soil incubation study that gene abundances of AOB was the most responsive of N cycling genes, followed by denitrifiers nosZ and nirS, while other N cycling genes were less sensitive to the environmental changes. Preisner et al. (2016) conducted fundamental research to test the response of different N cycling genes to salinity changes at mRNA level, however, there are few reports, if any, on how microbial N cycling genes respond and how the distribution pattern of N cycling genes responds to the hydrological shifting between freshwater and saltwater intrusion in STEs.
This study will provide knowledge on how microbial N cycling respond to the shifts between SGD and saltwater intrusion via flow-through experiments by combing field freshwater and seawater together for different types of sediments collected from the saltwater-freshwater interface in BRL, Florida, USA. We hypothesize that: (1) the N cycling genes are more sensitive to the changes in hydrological shifts between SGD and saltwater intrusion in the deeper silty sediments than in the shallower sandy sediments; (2) the relative responsiveness is different among the different N cycling genes in the same sediments; (3) the distribution pattern of N cycling genes will not change due to the hydrological shifts between SGD and saltwater intrusion, such that the N removal capacity will not change substantially.

**Materials and Methods**

**Site Description**

BRL is a long transition estuary between terrestrial and marine systems in the Atlantic coastal area of central Florida (Figure 6-1), which is one of three interconnected lagoons in the IRL Basin and receives fresh water mainly from precipitation, overland runoff, stream flows (small), and terrestrial SGD (Gao, 2009). Tidal variation is small in IRL Basin and thus has little effect on the discharge magnitude and the freshwater-saltwater boundary of terrestrial SGD (Martin et al., 2007; Roy et al., 2013).

**Sample Collection**

Triplicate sediment cores were collected at sites 1m, 11m, 21m, 45 m (BRL1, BRL11, BRL21, BRL45) from the shoreline within the seepage face of the BRL in July, 2016 (Figure 6-1). All cores were separated into two layers (sandy layer, from 10-20 cm; silty layer, from 20-50cm; see Table 6-1), and replicates from each layer were well mixed and divided into two parts: one part was immediately frozen on dry ice and
transported to the lab for DNA and RNA isolation; one part was kept fresh and transported to the lab for water-flow incubation experiments. Ambient pore water for use in water-flow incubation experiments was collected at depth of around 10-50 cm from each site (we failed to get ambient pore water from site 1), and lagoon water were collected above the seepage face from 1-45 m from the shore.

**Water-Flow Column Experiment**

For the stock of K\(^{15}\)NO\(_3\), 0.2g of K\(^{15}\)NO\(_3\) was weighed out and diluted into 2 liters of deionized water (NO\(_3^-\) =0.99*10\(^{-3}\)mol/L), and then 300 ml of stock solution was mixed into 6L of seawater, and 150 ml of stock solution was added into each of the 3L ambient pore water vessels. Finally, the actual concentration of \(^{15}\)N addition was 48.5 µM (49.5µM) and was at the same magnitude as the setup in study from Wankel et al. (2011).

Sediment slurry with site pore water was added to 50 mL polycarbonate columns with top and bottom ends connected with tubing, and then were put into the frames (Figure 6-2). The inlet tubing were connected with the pumps and stretched to the container with water (ambient pore water or lagoon water). The ambient pore water (group 1) and lagoon water (group 2) was continuously pumped through the tubing in an up-flow direction by a peristaltic pump set at a speed of 1.5ml/min for 48 hours at room temperature, with first 24 hours equilibration based on previous study from Wankel et al. (2011). The measured flux was around 0.02 ml/min in the field, which is much slower than our incubations. Even with the high flow rate relative to that in the field, we can still compare the difference in the effects of SGD and saltwater intrusion on microbial community structure and activity. Water samples were collected at different time periods (0, 2, 6, 10, 20, 24, 48 h) to determine the changes in NH\(_4^+\) and NO\(_3^-\) concentrations.
After 48 hours all the cores from the columns were frozen at -80°C until nucleic acid isolation.

**The Isolation Efficiency Test**

To test the effects of salinity on the isolation efficiency, we incubated the same sediments with one in the high salinity solution, and another one in fresh water for 48 hours at the room temperature. After incubation, we added extra standard plasmid with AOA (ammonia oxidation archaea) amplicon (10 µl, around 2.3*10^9 copies/µl) into these two incubated sediments before starting DNA isolation to test the effects of salinity on the isolation efficiency by calculation the copies of bacterial 16S rRNA genes and AOA via qPCR.

**Samples Processing and Data Analysis**

DNA and RNA were extracted from sediments using Powersoil DNA and RNA Isolation Kits (Mobio, Carlsbad, CA, USA). All primers and quantitative polymerase chain reaction (qPCR) primers and cycling parameters of N related functional genes are presented in Table 4-6: ammonia oxidizing archaea (AOA) and bacteria (AOB); denitrifiers, nirK and nirS; nitrous oxide reduction, nosZ; Dissimilatory Nitrate Reduction to Ammonium (DNRA), nrfA). For the qPCR and RT- qPCR assays, all sample DNAs and cDNAs (transfer RNA to cDNA using SuperScript III first-strand synthesis supermix from Invitrogen, Carlsbad, CA; Bae et al., 2014), together with standard DNAs were analyzed in duplicate by using the SYBR green protocols in a StepOne Plus real-time PCR system (Applied Biosystems) (Dang et al., 2013; Bea et al., 2014). Abundances of all genes were calculated as copies of genes per gram of sediment. Standard curves were generated by serial dilution (10^-3 to 10^-8) of reference plasmids containing target genes constructed by cloning the appropriate amplification products into *E. coli* hosts.
using TOPO TA cloning kits (Life Technologies, Thermo Fisher Scientific Inc, USA), and concentrations and purity of plasmid DNAs were measured using Eppendorf biophotometer plus (VWR International LLC, USA) for qPCR analysis with an A260/A280 ratio equal to between 1.7 to 2.0. Amplification efficiencies were evaluated as described by Bae et al. (2014), and were between 89% and 94%. Fidelity of amplification products was confirmed by melt curve analysis following each qPCR run.

The YSI Professional Plus Meter (calibrated daily) recorded salinity, pH, and oxidation-reduction potential (ORP) in situ. Ammonium and nitrate concentrations were analyzed by Seal AA3 Autoanalyzer continuous flow system following the methods of Strickland and Parsons (1978). The detection limits of NO$_3^-$ and NH$_4^+$ were 0.1 µmol L$^{-1}$ and 0.5 µmol L$^{-1}$, respectively. TN and TOC concentrations were analyzed on total kjeldahl nitrogen and a Shimadzu TOC-VCSN total organic carbon analyzer, respectively, and the coefficient of variance was less than 2%. All analyses were completed in the Department of Geological Sciences, UF.

The Principal component analysis (PCA) was employed using ordination analysis in CANOCO 4.5 program (ter Braak and Šmilauer, 2002), and the significant difference in copies of N cycling genes between ambient pore water and lagoon water flows was tested by T-test and $P$ value smaller than 0.05 was considered as significant.

Results

**Physicochemical and Microbial Characteristics of Water Collected from Field in BRL**

The lagoon water had 2 to 10 times higher salinity than ambient pore water, and salinity gradient of ambient pore water was from 1.78 to 12.82 psu at sandy layer, and from 1.61 to 9.81 PSU in the silt layer at the seepage face from 1 to 45m (Fig. 6-1 and
Meanwhile, lagoon water had higher pH (weak alkaline) than pore water (neutral), as well as higher ORP, while it had a significantly lower concentration of NH$_4^+$ compared with ambient pore water. The field water used for the water-flow column experiments had undetectable concentrations of NO$_3^-$, and ratios of TOC to TN were higher than 10 for most of the field water, with the highest ratio equal to 22.36 in lagoon water (Table 6-1).

The DNA copies of 16S rRNA were similar along the seepage face at 1m to 45m from shoreline at both sandy and silty layers (Figure 6-4 (A)), while the cDNA copies of 16S were 100 times lower for silty layers at 11 and 45 m from shoreline (Figure 6-4(B)). The pattern of N functional genes (DNA) showed higher copy numbers of NH$_4^+$ producers ($nifH$ and $nrfA$), significantly lower copies of nitrifiers (AOA and AOB), and higher copies of denitrifiers ($nirK$, $nirS$ and $nosZ$) (Figure 6-4(C)), and most cDNA of those N functional genes were detected in the field except for AOA and $nirS$ (Figure 6-4(D)).

**Physicochemical and Microbial Characteristics of Samples Collected from Water-Flow Column Experiments**

The lower concentrations of NH$_4^+$ and NO$_3^-$ were presented in all the water-flow column experiments (Figure 6-5). No big effect on the decreased trends of NO$_3^-$ with different types of water flow through all the columns (Figure 6-5(B, D)), while the decreased trends in NH$_4^+$ concentrations were alleviated in lagoon water flow through all the columns (Figure 6-5(A, C)).

After testing the effects of salinity on the isolation efficiency, we didn’t find significant differences in the DNA copies of bacterial (16S rRNA genes) between the salt and fresh water samples collected from the same sediment after 48 hours
incubation (Figure 6-3 (A)), which suggested that the salinity will not affect nucleic acid isolation efficiency during the samples processing. The DNA and cDNA copies of 16S rRNA were higher in all water-flow experiments compared to field samples, especially in the samples from silty group (Figures 6-6(A, B) and 6-4). The DNA copies of bacteria showed a significant difference between samples collected from columns with ambient pore water and lagoon water flows for both two sediments (Figure 6-6(A); T-test with \( P<0.05 \)); While the cDNA copies of bacteria just showed significant higher values in silty sediment samples collected from columns with ambient pore water flows than that with lagoon water flows, no significant difference was found for sandy sediments (Figure 6-6(B); T-test with \( P>0.05 \)).

After testing the effects of salinity on the isolation efficiency for AOA (ammonia oxidation archaea), we didn’t find significant differences between the salt and fresh water samples collected from the same sediment after 48 hours incubation (Figure 6-3(B)), and the concentration of AOA was around \( 10^9 \) copies/per units (\( \mu l \) or g) and didn’t showed significant changes from the beginning when AOA was added to the samples to the end of the isolation, which suggested that the salinity and the duration of the isolation will not affect the nucleic acid isolation efficiency, and differences in quantification functional genes would attribute to the difference treatments in experiments. We detected activity for most N cycling genes listed in Table 4-5 in our water-flow column experiments, except for AOA (Figure 6-7). The pattern of N functional genes (DNA and cDNA) was similar to that in the field; however, higher copies of \( \text{NH}_4^+ \) producers (\( \text{nifH} \) and \( \text{nrfA} \)), significantly lower copies of nitrifiers (AOA and AOB), and higher copies of denitrifiers (\( \text{nirK} \), \( \text{nirS} \) and \( \text{nosZ} \)) were observed (Figure 6-7).
DNA copies of AOB were higher than AOA in most samples (Figure 6-7(A, C)). No significant differences were found for DNA and/or cDNA copy numbers of ammonia oxidizers between the samples collected from the ambient pore water and lagoon water flow experiments for same type of sediment (Figure 6-7), while significantly higher numbers of DNA copies of ammonia oxidizers were observed in sandy sediments than that in silty sediments (Figure 6-8(A); Tukey-Kramer test of ANOVA with \( P<0.05 \)).

The DNA copies of all of the target genes involved in denitrification (\( nirK \), \( nirS \), and \( nosZ \)) showed significant decreases after lagoon water in comparison with ambient pore water flow for silty sediments (Figure 6-7(C); T-test with \( P<0.05 \)), while only the DNA copies of \( nirS \) were significantly decreased after lagoon water flow through the sandy sediments (Figure 6-7(A); T-test with \( P>0.05 \)).

The cDNA copies of \( nirS \) and \( nosZ \) were significantly lower in lagoon water flow experiments in comparison with ambient pore water flow experiments for silty groups (Figure 6-7(D); T-test with \( P<0.05 \)); however, no cDNA copies of denitrifiers showed significant difference between different water flow through experiments for sandy groups (Figure 6-7(B); T-test with \( P>0.05 \)). \( nirS \) was generally higher than \( nirK \) for most of samples collected from water flow column experiments (Figure 6-7(A, C)), which was consistent with other studies in estuary sediments (Jones and Hallin, 2010; Lee and Francis, 2017). Higher DNA copy numbers of \( nirK \), \( nirS \), and \( nosZ \) were observed in ambient pore water in comparison with lagoon water flow through columns (Figure 6-9(A)), and the DNA copies of \( nosZ \) were lower than total DNA copies of \( nirK \) and \( nirS \) for all types of samples (Figure 6-9(A)), while the cDNA copies of \( nosZ \) were higher than total copies of \( nirK \) and \( nirS \) for most of these samples (Figure 6-9(C)). The DNA and
cDNA copies of nirS and nosZ were consistently responsive to the hydrological changes in our study (Figure 6-8), which may be due to higher frequency of co-occurrence with each other (Jones et al., 2008; Graf et al., 2014).

The DNA copies of nrfA gene didn’t show significant differences between samples collected from ambient pore water flow and lagoon water flow through experiments for all types of sediments (Figure 6-7(A, C)), and the cDNA copies of nrfA were not detected in all the samples and were significantly lower (Figure 6-7(D); T-test with P<0.05) in samples collected from lagoon water flow experiments in comparison from ambient pore water flow experiments only for silty group (Figure 6-7(D); T-test with P<0.05). The DNA copies of nrfA were higher than nir(K+S) in all samples collected from lagoon water flow experiments, while they were lower than nir(K+S) in most of samples collected from ambient pore water flow experiments (Figure 6-9(C)). However, cDNA copies of nrfA were lower than nir(K+S) in most samples in water flow through column experiments (Figure 6-9(D)), and significantly higher nrfA cDNA copies were present in ambient water flow through the silty sediments compared with lagoon water flow through the sandy sediments (Figure 6-8).

The difference between these treatments based on all N cycling genes as a whole was showed in Figure 6-10 by PCA analysis. Smaller variation was observed for the effects of lagoon water compared with the effects of ambient pore water on the suite of target N cycling genes at both the DNA and cDNA levels, and the effects of lagoon water on the N cycling genes were similar for all type of sediments, while the effects of ambient pore water varied from samples to samples with larger effects on silty group than that on the sandy group both at DNA and cDNA levels. As a whole, the N cycling
genes increased under the water-flow experiments with ambient water when comparing with that with lagoon water at both DNA and cDNA levels, and this effect was much more obvious for the silty sediments.

**Discussion**

**The Response of Bacteria to the SGD and Saltwater Intrusion**

In order to examine the microbial response to SGD and saltwater intrusion in the different depths due to tidal fluctuation, we accordingly quantified the total number (DNA) and active number (cDNA) of bacteria in the different sediment layers (sandy sediments and silty sediments). We found that the responses of the total bacteria activity (16S rRNA cDNA) to the SGD and saltwater intrusion were different in different layers. Compared with non-significant responses to the water-flow column experiments shifting from ambient water to lagoon water flows in sandy sediments, a significant and stronger response with higher values of 16S rRNA cDNA copy numbers in ambient water (water from SGD) in silty group (Figure 6-6(B)). This may have been due to the higher salt stress for bacteria in silty sediments, where they needed more energy for osmotic adaptation during saltwater intrusion and thereupon resulted in energy shortage for cell division and function (Oren, 2011). The greater positive responses of microbial community to SGD were also founded in other studies, which may due to nutrient enrichment and low salinity in SGD in comparison with saltwater (Garcés et al., 2011; Oren, 2011; Carlson and Wiegner, 2016).

A significantly higher number of 16S rRNA DNA copy number was observed in samples collected from ambient water than those collected from lagoon water experiments (Figure 6-6(A)) for both types of sediments in our lab study, which was again supported by in situ groundwater addition experiments from Garcés et al. (2011).
Garcia et al. (2011) found that groundwater additions increased the biomass of all major autotrophic groups in a coastal area, which was also supported by a field study from Lee et al. (2016), who found that seawater exhibited a lower richness and diversity of microbial communities than discharging groundwater in situ.

Furthermore, all the column sediments generally exhibited higher DNA and cDNA copies of bacteria than field sediments in our study (Figures 6-4 and 6-7). This finding is consistent with previous research reported by Hunter et al. (2006), who analyzed the microbial diversity in flow-through column experiments and found higher diversities in all column-derived sediments than in the field samples. They attributed this finding to the frequently changing environmental conditions that restricted bacterial growth in the field. Additionally, Laverman et al. (2006) reported that microbial reaction rates would usually be overestimated due to the enhancement of the accessibility to organic matter and electrons in water-flow experiments with sediment slurries in comparison with field study. They also speculated that the relatively higher flow rates in the flow experiments in comparison with in the field will also supply more sufficient nutrients and carbon source per time scale, which enhanced the growth of bacteria.

**The Response of N Cycling Genes to SGD and Saltwater Intrusion**

Highton et al. (2016) indicated that the changes in nitrogen functional genes are strongly related to depth, sediment grain size, and sediment porosity. In order to examine the response of the nitrogen functional genes to tidal fluctuations, we measured the total number and relative activities of key nitrogen functional genes in the different sediment layers (sandy sediments and silty sediments).
The response of nitrifiers (aoa and aob) to SGD and saltwater intrusion:

mRNA from AOA was below the detection limit even with the evidence of DNA of AOA in both field samples and samples from water flow column experiments (Figures 6-4 and 6-7), and very low numbers of copies of AOB were detected in a small number of samples. The inhibition of nitrifiers in water flow through column experiments was also documented by Weston et al. (2006). The absence or low amount of nitrification activity in soils rich in organic matter has often been reported, and the critical C/N ratios of 11.6 and 9.6 greatly contribute to the competition between the heterotrophic bacteria and chemolithotrophic ammonium oxidizing species (Verhagen and Laanbroek, 1991). In our study, most of the C/N ratios were higher than 10 (Table 1); it is likely that the heterotrophic bacteria will consume all mineral nitrogen present in the water flow column for assimilation into the cell material. Hutchins et al. (2009) studied the microbial metabolic responses to groundwater discharge and also found that the addition of groundwater stimulated the heterotrophic over autotrophic metabolism of microbes. The range of DNA copies of AOA and AOB were similar to the previous incubation study from Wankel et al. (2011), and fewer DNA copies of AOA and AOB were observed in silty sediments when compared with sandy sediments. This may be due to the lower ORP and concentration of NH$_4^+$ (Wang et al., 2013a). The observation that DNA copies of AOB were higher than AOA may be due to AOB preference for the marine system, which is consistent with reports from Wankel et al. (2011) and Bagchi et al. (2014).

The response of denitrifiers (nirK, nirS, and nosZ) to SGD and saltwater intrusion

The responses of denitrifiers to the SGD and saltwater intrusion were similar to the total bacteria, with no significant effects observed for sandy sediments, while the DNA and cDNA copies of nirS, and nosZ were significantly suppressed by saltwater
intrusion in silty sediments (Figure 6-7(B, D)), except for the cDNA copy numbers of nirK, which was not sensitive to the changes in the water flow conditions at all. These findings are consistent with results of Zhang et al. (2013), who found nirK to be the least sensitive N cycling gene to the changes in environmental conditions. Zhao et al. (2013) said that osmotic stress rather than ionic toxicity and oxidative stress primarily controlled the microbial activity when salt stress occurred, so we supposed that the different response of denitrifiers in different sediments to the SGD and saltwater intrusion may be due to the different adaptive capacity to changes in osmotic stress in different sediments. Other studies have also reported the suppression of denitrification under saltwater flow or incubation conditions for fresh sediments where it was assumed that microbes possessing the adaptive capacity to the changes in osmotic stress were present (Weston et al., 2006; Seo et al., 2008). Laverman et al. (2007) found that denitrification was not significantly affected, although it slightly decreased by an additional 10 psu of salt in their study via fresh sediment flow-through reactor.

Greater cDNA copies of nosZ than nir(nirK and nirS) in both field samples and samples from water flow column experiments (Figure 6-7) indicated high N$_2$O reduction and low N$_2$O emission in the study area independent on the water flow conditions, which was possibly related to the very low concentration of NO$_3^-$ in sampling sites (Harter et al., 2014; Saarenheimo et al., 2015; Marchant et al., 2016; Young et al., 2016). Burgos et al. (2015) reported that coastal sediments were good for nitrate reduction during denitrification process due to high loading of organic matter and low oxygen concentration, and Kandeler et al. (2006) and Zhang et al. (2015) also found higher copies of nosZ compared with nir(nirK+nirS). Graf et al. (2014) reported that
organisms containing nosZ without nir genes were found in Bacteroidetes and Firmicutes, and that they would be the primary N₂O consumers. Higher cDNA copy numbers of nosZ than nir may be also because nirK and nirS were underestimated by RT-qPCR due to potential primer coverage limitations (Wei et al., 2015; Helen et al., 2016).

**The response of DNRA (nrfA) to SGD and saltwater intrusion**

The responses of DNRA to the SGD and saltwater intrusion were similar to the total bacteria and denitrifiers, no big effects for sandy sediments, while the cDNA copies of nrfA were significantly suppressed by saltwater intrusion in silty sediments. DNA copies of nrfA were higher than nir (nirK+nirS) for all samples under lagoon water flow experiments (Figure 6-9), which indicate the growth of DNRA over denitrifiers was favored in lagoon water, likely due to higher C/N ratios (Table 1) than in ambient pore water (Algar and Vallino, 2014; Van et al., 2015). The cDNA copies of nrfA were lower than nir (nirK+nirS) for most samples even with higher DNA copies of nrfA than nir (nirK+nirS) under lagoon water flow experiments. Laverman et al. (2007) found that around 50% of nitrate reduction accounted for complete denitrification in comparison with another pathway (e.g. incomplete denitrification, Anammox, DNRA) under both fresh and saltwater flow conditions, and Canavan et al. (2007) also found that the seawater flow experiments favor denitrification over DNRA process. The controlling factors of the competition between denitrification and DNRA activities need a better understanding in future studies. Also, it is worthy to mention that it is not appropriate to use the ratios of NH₄⁺ production rates to NO₃⁻ reduction rates to quantify the competition between denitrification and DNRA (dissimilatory NO₃⁻ reduction to NH₄⁺) processes (e.g. Laverman et al., 2006; Yang et al., 2012), because NH₄⁺ production is
not only controlled by DNRA, but also controlled by nitrogen fixation and decomposition of organic matter, and released from sediment due to high ionic strength. NO$_3^-$ reduction is controlled by denitrification, DNRA, Anammox processes, such that more precise and correct methods should be used in future studies, such as the microbial N cycling gene makers and $^{15}$N isotope.

**The Pathways for NH$_4^+$ and NO$_3^-$ Removal in the Sediments in BRL**

Based on the response of N cycling genes to the SGD and saltwater intrusion in our water flow through study, we speculated that the decrease in concentrations of NH$_4^+$ was mainly attributal to assimilation for biomass growth (Laverman et al., 2006) rather than nitrification (Figure 6-7). When compared with samples from ambient pore water flow through experiments, the decreased nitrification rate in lagoon water flow through experiments (Figure 6-5(A, C)) would be caused both by low DNA copies of 16S rRNA genes (Figure 6-6(A)) and the release of adsorbed ammonium under higher ionic strength conditions (Weston et al., 2006; Canavan et al., 2007).

We also supposed that the decrease in concentrations of NO$_3^-$ may be mainly attributed to denitrification and partially to DNRA in our study, and the decreased coefficients were similar in the same layer, even close in different layers with first-order reaction (Figure 6-5(B, D)), which indicated that there were not significantly different in the NO$_3^-$ removal capacity at spatial and hydrological scales, and Yang et al., (2012) also found a similar NO$_3^-$ removal capacity described by first-order reactions in different layer of sediment in river sediment with sufficient NO$_3^-$ and organic matter. This confirmed changes in water chemistry from seawater intrusion may not substantially change the N removal capacity of sedimentary microbial processes as hypothesized by Hines et al. (2015). The NO$_3^-$ removal rate would be lower in the water flow through
column experiments in the laboratory due to the higher flow rate in comparison with field (Gu et al., 2007; Spiteri et al., 2008).

Summary

To our knowledge, this is the first paper to discover the effects of ambient pore water (from SGD in the field) and lagoon water (from seawater in the field) on the gene copy number of total bacteria and N cycling genes in different layers of sediment in STEs by water-flow column experiments both at DNA and cDNA levels, and reflect the different response of the microbial activity to the changes in hydrological process in different layers even within half meters, suggesting the zone was very thin (smaller than 20cm based on our study) in the STEs to buffer the changes in the environmental conditions due to hydrological processes shifts caused by global changes in sea level and human activities. Meanwhile, coupling of Nitrification-Denitrification and DNRA-Anammox are two important processes to remove surplus NH$_4^+$ in STEs (Hines et al., 2015), while the lower copy numbers of AOA and AOB may weaken coupling Nitrification with Denitrification, and enhance coupling DNRA with Anammox for N removal in our study area. The enhancement of DNRA-Anammox may more likely happen in silty sediments under lagoon water flow conditions, which is consistent with the modeling results from Hines et al. (2015), who found that seawater intrusion may support coupling of DNRA-Anammox and decoupling of nitrification to denitrification in sediments. The entire N removal capacity would not change significantly, which was confirmed by the decreased coefficients for NO$_3^-$ in Fig 2-B, D.

Santoro (2010) proposed two hypotheses to explain the response of microbial communities in the freshwater-saltwater interface to the SGD and saltwater intrusion, and we hypothesized that the microbial community in sandy sediments in our study area
would contain a mixture of fresh and salt adapted microbes, which contributed no significant changes in the activity of total bacteria and N cycling genes. The mixture may be due to a mutually complementary function where one component was suppressed by shifting from ambient pore water to lagoon water flows. We do not know if organisms specifically adapted to intermediate salinity were present in our study, Edmonds et al. (2009) indicated that the microbial community in sediments didn’t change even over the course of one month incubation with continuous saltwater flow, and the taxa didn’t become more “marine-like” in the seawater-amended sediment either. The course of a tidal cycle in BRL is likely to be shorter than the turnover of the microbial community.
Table 6-1. Physicochemical characteristics of the ambient water (AW) and lagoon water (LW) using for the water-flow column experiment collected from BRL

<table>
<thead>
<tr>
<th>Site</th>
<th>Layer/Depth (cm)</th>
<th>Salinity (PSU)</th>
<th>pH</th>
<th>ORP</th>
<th>NH₄⁺ (µM)</th>
<th>NO₃⁻ (µM)</th>
<th>TOC/TN</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRL 11Sa</td>
<td>Sand /10-20</td>
<td>1.78</td>
<td>6.88</td>
<td>-79.70</td>
<td>102.37</td>
<td>-0.57</td>
<td>13.14</td>
</tr>
<tr>
<td>BRL 21Sa</td>
<td>Sand/ 10-20</td>
<td>4.90</td>
<td>6.86</td>
<td>-193.10</td>
<td>156.05</td>
<td>-0.48</td>
<td>8.12</td>
</tr>
<tr>
<td>BRL 45Sa</td>
<td>Sand /10-20</td>
<td>12.82</td>
<td>7.18</td>
<td>-218.00</td>
<td>120.26</td>
<td>-0.42</td>
<td>8.84</td>
</tr>
<tr>
<td>BRL 1Si</td>
<td>Slit/ 5-20</td>
<td>5.08</td>
<td>6.14</td>
<td>-247.00</td>
<td>173.61</td>
<td>-0.30</td>
<td>11.74</td>
</tr>
<tr>
<td>BRL 11Si</td>
<td>Slit /20-35</td>
<td>1.61</td>
<td>6.90</td>
<td>-87.90</td>
<td>99.80</td>
<td>0.63</td>
<td>13.69</td>
</tr>
<tr>
<td>BRL 45Si</td>
<td>Slit/ 30-50</td>
<td>9.81</td>
<td>7.01</td>
<td>-230.30</td>
<td>99.12</td>
<td>-0.43</td>
<td>10.42</td>
</tr>
<tr>
<td>LW</td>
<td>surface</td>
<td>20.66</td>
<td>7.86</td>
<td>-40</td>
<td>4.44</td>
<td>-0.34</td>
<td>22.36</td>
</tr>
</tbody>
</table>

Sa: sandy sediment; Si: silty sediment; OPR: oxidation and reduction potential; TOC: total organic carbon; TN: total nitrogen
Figure 6-1. Location map of the BRL, and the Sampling site in BRL (adapted from Martin et al., 2007).
Figure 6-2. The setup of the water-flow experiments. A) The ambient water (group 1) and lagoon water (group 2) were pumped through the tubing at a rate of 1.5 ml/min; B) triplicates for each sample; C) water samples were collected during different periods.
Figure 6-3. The isolation efficiency test for different treatments (AW: ambient water; LW: Lagoon water). A) Isolation efficiency test for 16S rRNA gene. B) Isolation efficiency test for AOA. Same letter indicates the no significant differences between AW and LW treatments (T-test, two sides).
Figure 6-4. Copy number of functional genes measured in sediment samples collected from BRL. A) DNA of 16S. B) cDNA of 16S rRNA gene. C) DNA of N cycling genes. D) cDNA of N cycling genes (sa: sandy sediments; si: silty sediments; 11-45m: the distance from shoreline; all the replicates are mixed in the field, and subsample for incubation study in the lab).
Figure 6-5. The changes in concentrations of NH₄⁺ and NO₃⁻ measured in water samples collected from water-flow column experiments. A) Concentrations of NH₄⁺ in sandy sediments. B) Concentrations of NO₃⁻ in sandy sediments. C) Concentrations of NH₄⁺ in silty sediments, D) Concentrations of NO₃⁻ in silty sediments.
Figure 6-6. Copy number of 16S rRNA gene measured in sediment samples collected from water-flow column experiments. A) DNA copies of 16S rRNA gene. B) cDNA copies of 16S rRNA gene. (sa: sandy sediments; si: silty sediments; 11-45m: the distance from shoreline). Different letters indicate significant differences between AW and LW treatments base on 16S copy number at $P < 0.05$ (T-test; two sides).
Figure 6-7. Copy number of N cycling genes measured in sediment samples collected from water-flow column experiments. A) DNA copies of N cycling genes in sandy sediments. B) cDNA copies of N cycling genes in sandy sediments. C) DNA copies of N cycling genes in silty sediments. D) cDNA copies of N cycling genes in silty sediments. Different letters indicate significant differences between AW and LW treatments based on copy number of N cycling genes at $P < 0.05$ (T-test; two sides).
Figure 6-8. Boxplot (n=8 for each treatment) of copy number of N cycling genes. A) DNA. B) cDNA. (sa: sandy sediments; si: silty sediments). Different letters indicate significant differences among all samples at $P < 0.05$ (Tukey-Kramer (HSD) test of ANOVA).
Figure 6-9. The correlation between N functional genes. A) DNA ratio of nir(K+S) to nosZ. B) cDNA ratio of nir(K+S) to nosZ. C) DNA ratio of nir(K+S) to nrfA. D) cDNA ratio of nir(K+S) to nrfA. (n= 8 for each treatment; sa: sandy sediments; si: silty sediments).
Figure 6-10. PCA plot showing the distribution pattern of samples based on N cycling gene (DNA and cDNA) measured in sediments collected from water-flow column. (sa: sandy sediments; si: silty sediments).
CHAPTER 7
CONCLUSIONS

The primary focuses of this research were to quantify the composition and structure of microbial communities and their related N cycling genes in two different SGD areas, including one channelized spring discharge in Yucatan and one permeable seepage face in IRL. This work sought to understand:

1. Chapters 2, 3, and 4: How the composition and structure of microbial community, as well as N cycling genes, vary from terrestrial groundwater to marine spring, and the impact of a large scale of environmental gradient (e.g. salinity and nutrient gradients) play in governing microbial community structure and composition, including the whole N cycling process in a karst aquifer in the Yucatan, Mexico.

2. Chapters 5 and 6: The distribution pattern of microbial N cycling genes, and how microbial N cycling responds to the shifts between SGD and saltwater intrusion at the saltwater-freshwater interface in a diffuse seepage area in IRL (IRL) Basin, FL, USA.

The Study in The Yucatan, Mexico (Chapter 2, 3 and 4)

In Yucatan, ground water is hydrologically connected to offshore springs via underground conduits, and the flow path may include cenotes that may be several kms inland. For those cenotes, we usually find a higher ORP and low DOC at the top of groundwater, while it is opposite at the bottom of the cenote. For those offshore springs in marine water, the hydrological processes are much controlled by diurnal tidal cycling; during low tide, terrestrial groundwater is discharged to the ocean via submarine groundwater as an offshore spring, which usually contains high nitrogen nutrients, DOC, low ORP and salinity. During the high tidal reversal, sea water intrudes to the conduits, which usually has low nitrogen nutrients, DOC and high ORP and salinity. Under those environmental conditions, we hypothesize that the composition, structure and interaction of microbial communities, as well as N cycling differ from sinkhole to offshore springs and are driven by environmental gradient and ecological niche partitioning (e.g.
salinity, nitrogen nutrients, ORP and DOC). Based on the hypotheses, we characterized microbial communities by 16S rRNA amplicon sequencing and microbial N functional genes and sulfur reducing bacteria (dsrB) by qPCR in water samples collected from a stratified water-filled sinkhole (Cenote Siete Bocas, C7B) and an offshore submarine spring (Pargos) in the Yucatan Peninsula, and proposed a conceptual model to decipher the N cycling process in the two systems.

**Microbial Community (Chapter 2 and 3)**

We found that samples from the sinkhole and offshore spring cluster together in a PCoA, and a sample from the right channel at low tide clusters between the sinkhole and spring, suggesting a potential connection between some sinkholes similar to C7B and Pargos. In C7B, we found that spatial variation was greater than temporal variation, while in Pargos tidal variation was greater in comparison with seasonal variation.

The composition of the microbial communities was dominated by *Proterobacteria*, and differs from sinkhole to offshore spring, where the representative group is *Firmicutes* in sinkhole rather than *Cyanobacteria* in offshore spring. More diverse metabolic processes are noted in the sinkhole, which includes S oxidation, two types of methane oxidation, photosynthesis, and complex C metabolism, while two major metabolism processes are noted in offshore spring, including S oxidation and photosynthesis. A higher proportion of complex C metabolism was observed in offshore spring in low tide samples, which was mostly attributed to groundwater discharge. *Alphaproteobacteria* is the dominant class in the low salinity zone, *Gammaproteobacteria* is the dominant connection in the high salinity zone. SRB are dominant by the *Deltaproteobacteria* groups independent of environmental conditions, while the composition of SOB is sensitive to the environmental conditions, which
indicates that SOB would be more sensitive to climate change and sea level change than SRB would be. A greater number of taxa related to potential sulfur-disproportionation and DMS degradation were roughly identified in the marine system in comparison with the groundwater system.

The interaction networks within microbial communities are based on correlation analysis along the environmental gradient. *Actinobacteria, Betaproteobacteria, Firmicutes,* and *Planctomycetes* show higher proportions of connections in C7B than that in high salinity zones of Pargos. A higher proportion of connections with *Cyanobacteria* is found above the halocline in C7B and in Pargos in our study, and the connections with *Nitrospirae* are only present above the halocline in C7B. Meanwhile, a more highly centralized cluster and connection density of some individual groups, together with higher positive connections are noted in the low salinity zones. An increase in negative correlation is found with increasing salinity concentrations. These findings suggest that the fundamental assembly of microbial communities varies along the environmental gradient, and suggest that the ecological niche separation with depth and salinity drives the different interactions within microbial communities. A tighter connection between sulfur related microbial groups are observed in higher salinity zone, which may due to a high-energy efficiency requirement.

**N Cycling (Chapter 4)**

We detected a clear seasonal variation in sinkhole and tidal variation in offshore springs with respect to N cycling genes, with AOA as the dominant nitrifier in cenote, and AOB dominates in offshore spring.

The model cenote (C7B) is an open-mouth and a very stratified groundwater system, with a clear halocline at depth of 29m. The model offshore spring (Pargos) is
affected by diurnal tides and a homogenous system. We detected a loose coupling of nitrification with denitrification in the cenote, where nitrification dominates above the halocline with NO$_3^-$ accumulation, and denitrification dominates below the halocline with NH$_4^+$ accumulation; while a close coupling of nitrification with denitrification was observed in Pargos, with no accumulation of N-nutrient, suggesting a faster turn-over of N-nutrients in spring compared to groundwater.

The Study in Indian River Basin (IRB), FL, USA (Chapter 5 and 6)
Indian River Basin is a silica sandy sedimentary seepage ecosystem and different from the calcium carbonate channelized ecosystem in Yucatan. Fresh water has higher nutrients, low DOC, ORP and salinity from terrestrial SGD via sediments. Tidal variation is smaller in IRB compared with Yucatan, and thus has little effect on the discharge magnitude and the freshwater-saltwater boundary in this area. Based on environmental conditions, we characterized N functional genes by qPCR and RT-qPCR in sediment samples collected from IRB in field and water-flow through column experiments in laboratory. We hypothesized that: (1) The pattern of N functional genes (DNA and cDNA) is similar along the seepage face; and (2) the microbial activities related to N cycling differently respond to the changes in hydrological process in different sediment layers.

N Cycling in the Field (Chapter 5):
In this chapter, we sampled the surface sediments in the seepage face along the salinity gradient in Eau Gallie North (EGN) and riverine sediments in Eau Gallie River (EGR) from IRL. We found that the pattern of N functional genes (DNA and cDNA) is similar along the seepage faces and riverine sediments, and shows higher numbers of copies of NH$_4^+$ producers (*nifH* and *nrfA*), significantly lower numbers of nitrifiers (AOA
and AOB), and higher numbers of denitrifiers (nirK, nirS and nosZ), which accounts for the accumulation of NH$_4^+$ in the sampling area, active nitrifiers AOB (active AOA were undetectable). Relative activities of denitrifiers (nirK, nirS and nosZ) were significantly positively correlated with each other, and nosZ is higher than total nirK and nirS, which suggests complete denitrification coupled with nitrification process transferring NH$_4^+$ to N$_2$ in this area.

**N Cycling in Water Flow Through Column Experiments (Chapter 6):**

In this chapter, we sampled the surface sediments together with the filed pore water and lagoon water in the seepage face along the salinity gradient in BRL and set up water flow through column experiments. We found that the microbial activities related to N cycling respond differently to the changes in hydrological processes in different layers with no significant impacts observed for the first layer (0-20 cm), and significant impacts were observed for the second layer, especially for the denitrification process.

Seawater intrusion may support coupling of DNRA with Anammox and some decoupling of nitrification to denitrification in sediments, such that NO$_3^-$ removal may be mainly attributed to complete denitrification (NO$_3^-$ to N$_2$) and partially to DNRA. The different responses of N cycling genes to SGD and saltwater intrusion in the seepage face suggests that the zone may be very thin to buffer the changes in environmental conditions due to hydrological processes shifts; however, changes in hydrological process would not change the relative abundance of N cycling genes at either the DNA and cDNA level, as well as the removal capacity of sedimentary microbial processes.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Laibin Huang was born and raised in Huangshi village, Nancheng county, Fuzhou City, Jiangxi Province, China. In 2009, He received his bachelor’s degree in ecology from the University of Science and Technology, Beijing. In 2012, he obtained his master’s degree in environmental ecology from Beijing Normal University. In 2013, he came to University of Florida to pursue his PhD degree in environmental microbiology.