June 2020

Microbial Community Structures in Three Bahamian Blue Holes

Meghan J. Gordon
University of South Florida

Microbial Community Structures in Three Bahamian Blue Holes

by

Meghan J. Gordon

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
with a concentration in Cell & Molecular Biology
Department of Cell Biology, Microbiology and Molecular Biology
College of Arts and Sciences
University of South Florida

Major Professor: James Garey, Ph.D.
Kathleen Scott, Ph.D.
Diane TeStrake, Ph.D.

Date of Approval:
June 26, 2020

Keywords: 16S rDNA, microbial ecology, photosynthesis, San Salvador

ACKNOWLEDGEMENTS

Special thanks to my graduate thesis advisor Dr. James Garey for all of his time and guidance on this project. I would also like to thank my committee members Dr. Kathleen Scott and Dr. Diane TeStrake, my lab members Madison Davis, Robert Scharping, Chelsea Dinon and Arian Farid, and my father and go-to "IT guy," Ted Gordon.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

This study used 16S rDNA metagenomics and water chemistry to conduct an examination of microbial community dynamics and biogeochemistry in three physically adjacent, sunlit blue holes with variable hydrologic regimes on San Salvador Island, Bahamas. Church and Watling's Blue Holes are holomictic with relatively clear waters, while Inkwell Blue Hole hosts density stratification and waters stained brown with tannins. Based on water color and clarity and physicochemical profiles, I hypothesized Church and Watling's Blue Holes would be dominated by oxigenic photoautotrophs, and that the bottom layer of Inkwell would be characterized by euxinic (anoxic and sulfidic) conditions and host primarily sulfur-reducing bacteria. Microbial community profiles were dominated by 16S sequences associated with photoheterotrophic organisms in Watling's and with anoxygenic purple sulfur bacteria in Church. Communities in the surface and halocline of Inkwell were dominated by sequences affiliated with cyanobacteria and photoheterotrophic organisms, while the bottom waters were dominated by *Arcobacter*, with fewer numbers of the sulfate-reducting bacteria and the green sulfur bacteria *Chlorobium*. Sequences provisionally identified as anoxygenic purple and green sulfur bacterial clades dominated the photosynthetic communities in Inkwell's bottom layer, reminiscent of bacterial populations found at greater depths in other stratified sinkholes. Results of this study suggest Inkwell presents an underrepresented ecosystem in current literature that might serve as a natural laboratory for research regarding competition between anoxygenic and oxygenic photosynthesic microorganisms and sulfur cycling dynamics.

INTRODUCTION

Blue holes are water-filled vertical depressions formed by dissolution/collapse processes and are prominent features in karst worldwide. The term "blue hole," coined in reference to their blue appearance when seen from above, is used to refer to geological features with highly variable hydrological regimes and water chemistries[1]. While blue holes share a characteristic rounded morphology, depth and diameter can vary greatly according to the timing and means of their formation[2]. On an island as small as San Salvador, the blue holes are relatively shallow and formed during glacioeustatic sea-level lowstands[3-6]. Their mixed and sometimes complex water chemistries depend on the means by which water enters and leaves the hole, as well as depth, evapotranspiration potential, solar insolation, water mixing and surface features affecting water quality (such as vegetation and pumping)[7]. Meteoric fresh water can enter a hole during or after rainfall, either directly or through a shallow freshwater spring draining meteoric water from a nearby source. Where present, marine water can also enter more directly through solutionally-enlarged openings, or conduits. If a blue hole is sufficiently deep and in the absence of water mixing, stratification may occur, in which fresh, meteorically sourced water sits atop denser brackish or marine water, separated by a mixing zone or halocline.

Numerous studies using Mg/Ca and Mg/Cl ratios, stable isotopes, dye tracing and salinity have been carried out on San Salvador in order to describe the geology and hydrology of the island[3, 4, 6, 8-13]. San Salvador has a subtropical climate and an annual rainfall of approximately 1125mm,

with the majority of precipitation occurring during the wet season from May to January. With an evaporation potential of 1300mm/year, the island has a negative annual water budget. During the rainy season, when there is a temporary groundwater surplus, meteoric water forms a freshwater lens on top of denser brackish groundwater. However, higher-permeability karstified regions of bedrock and the presence of conduits has led to a thinning of the island's freshwater lens. In addition, the arid climate causes evaporative upconing of deep saline water below topographic troughs that partition the lens.

Several studies have now been conducted in an effort to characterize the hydrochemistry and water sourcing in blue holes on San Salvador. Vermette et al (2001) noted that the salinities of 37-42 ppt and tidal range of 0.6-0.9 meters in Watling's Blue Hole, taken together with high Mg^{++}/Ca^{++} ratios, water clarity, and apparent water column mixing, are consistent with conduit-sourced marine water inputs[14]. The clarity of Watling's water is also consistent with results from Davis and Johnson (1989), who found that San Salvadoran lakes with connecting conduits were clear and algae-free[8]. Results from this study can confirm the presence of an active conduit in the bottom of Watling's Blue Hole. However, a preliminary characterization of the physicochemical profiles and water chemistries within Watling's, Church and Inkwell Blue Holes from 2013 measured Watling's salinity in the 20-25 ppt range, results which were duplicated in this study. A recent study using $\delta^{18}O$ and $\delta^{2}H$ compositions of Inkwell and Church waters found that, although both holes are physically influenced by tidal forcing, the waters in each hole are of meteoric origin, and not the result of mixing of fresh meteoric water with deep marine waters[15]. Whether Watling's brackish salinity is similarly due to the salinization of

meteoric water through contact with surrounding karst-associated mineral deposits or to marine water inputs remains unclear.

Blue holes serve as natural laboratories in which to study fundamental relationships between microbiota and the environment. Studies suggest that, even when geographically adjacent or hosting only relatively minor differences in water chemistry, blue hole ecosystems can support significantly different microbial communities and biogeochemistry[7]. In Florida, our lab has found that the microbial communities present in the water column, sediment and biofilms of three blue holes relatively close to one another were completely different, although there were parallels in community structure when analyzing metabolic potential (unpublished data). Several studies have been conducted on microbial communities in Bahamian blue holes, but they have focused on deeper, permanently stratified systems on larger islands. Gonzalez et al and Macalady et al found microbial communities dominated by anoxygenic phototrophs of the *Chlorobi* clade, with smaller numbers of *Deltaproteobacteria*, in three blue holes on Andros and Abaco Islands[7, 16]. Anoxygenic phototrophic members of the *Gammaproteobacteria* were also found to be responsible for the dark color of the so-called "black holes" of South Andros Island, Bahamas[17]. Results of these studies are discussed in more detail in the Discussion section below. Several studies have examined anoxygenic photosynthesis and sulfur cycling dynamics in Bahamian blue holes, again in relatively deep systems hosting permanent stratification[18, 19].

In this study, I used 16S rDNA metagenomics techniques to analyze the prokaryotic microbial community composition present in three adjacent, sunlit blue holes during March 2019. Each site has a unique hydrologic regime and physical profile. Taxonomic identifications were used to

predict metabolic potentials and estimate nutrient cycling dynamics in each blue hole. Vertical physicochemical profiles of each blue hole, along with nutrient levels and water inflow data, were used to correlate environmental conditions and microbial community profiles. These data provide the foundation for future biogeochemisty and photosynthesis research in these systems.

METHODS

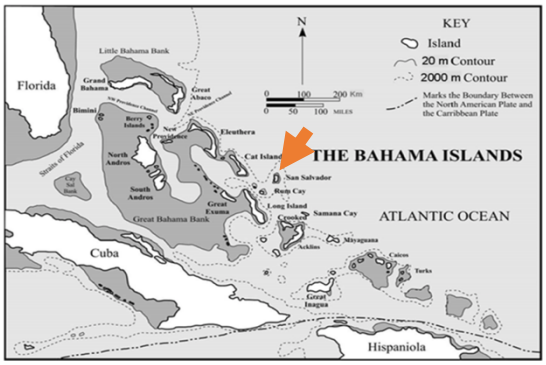
Site descriptions

San Salvador is a carbonate platform island in the eastern Bahamas ranging 14 km North-South and 8 km East-West (**Figure 1**). Church, Inkwell and Watling's Blue Holes are all located on the southern end of the island. Watling's Blue Hole is the furthest south, and Church Blue Hole and Inkwell Blue Hole are approximately 300m northeast and 670m north of Watling's, respectively.

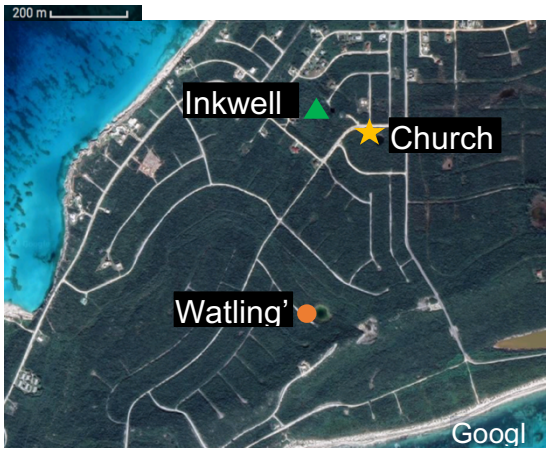
Inkwell is approximately 550m from the coast and has a maximum depth of ~9m **(Figure 2)**. It has the smallest diameter of the three sites studied, approximately 16m, and the entire perimeter is lined with dense vegetation. There is a large conduit located at ~5m depth on the southwestern side. From the surface its waters appear dark brown. Water visibility ranges from under one meter at the surface to ~3m at depth. It is the only blue hole in this study that hosts a density stratified system. A 1.5m freshwater layer sits atop a 1.5-2m mixing zone/halocline, with saltier microoxic water underneath.

Church Blue Hole is the site furthest inland, approximately 750m. It is ~32m in diameter and bowl-shaped, with a maximum depth of ~4m. Vegetation is present, especially on the southern perimeter, but not as dense as at Inkwell. There is a conduit at the center of the pond that is filled with sediment and not actively transporting water.

Watling's Blue Hole has the largest diameter, approximately 63m, and the least surrounding vegetation of the sites in this study. Its maximum depth is roughly the same as Inkwell's, ~ 9m, making it the largest hole we studied as well. Surface visibility was highest at this site. There is a large conduit located near the center of the hole at a depth of ~7.5m.



Larson, Erik B. and Mylroie, John E.



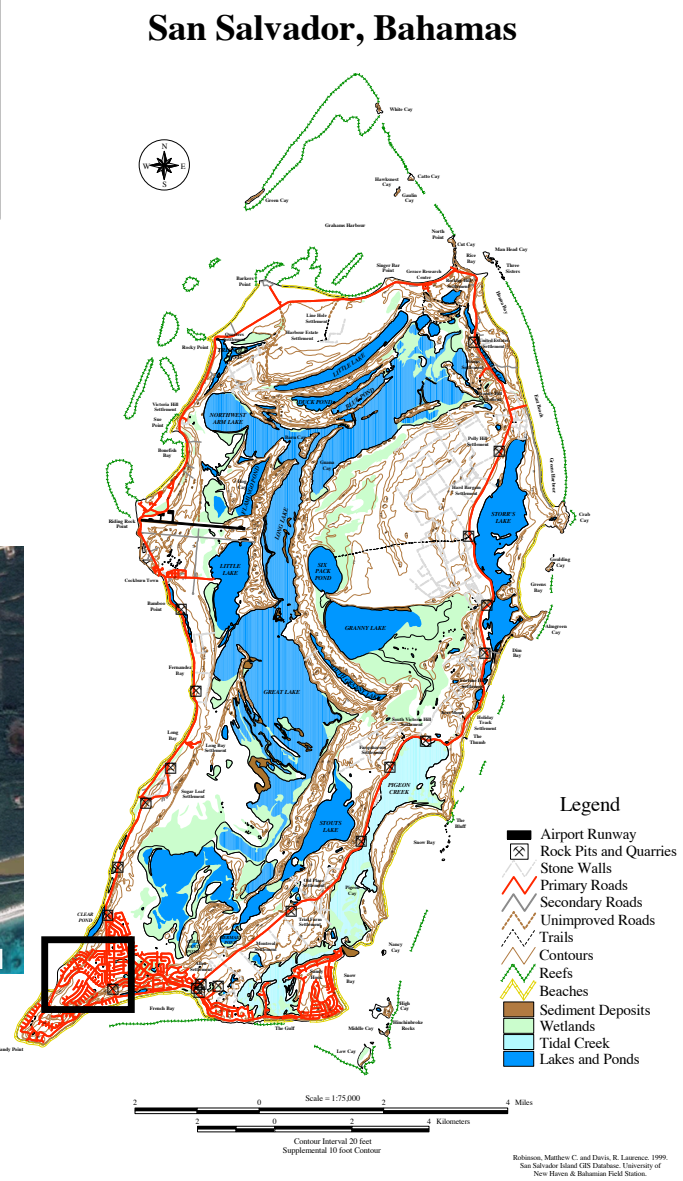


Figure 1: San Salvador, Bahamas, and the location of the three blue holes in this study, Inkwell, Church and Watling's Blue Holes.

Sampling and physicochemistry measurements

Sample collection was completed by scientific divers on SCUBA in March 2019. DNA samples were collected on 0.2mm filters (Thermo Scientific Nalgene) using sterile 60 ml BD syringes with Luer-Lok tips that were opened at depth. Triplicate samples were taken at a depth of six meters in both Watling's and Church blue holes. Due to the stratification at Inkwell, triplicate samples were obtained from surface, two-meter and nine-meter depths. Sample filters were frozen until DNA extraction could be performed. Vertical profiles of water temperature, pH, dissolved oxygen and salinity were obtained by divers prior to sample collection using a Hydrolab DS5X datasonde, calibrated before use according to manufacturer protocols.

Hydrochemistry measurements

Water chemistry samples were collected concurrently with biological samples, at the same depths and in triplicate. Sulfate, sulfide, nitrate, nitrite, and ammonium concentrations were quantified using HACH kits and a HACH DR2000 spectrophotometer according to manufacturer protocols. Samples were kept in a cooler on ice until analyses were performed within hours of collection.

Time-series conduit flow measurements

Divers deployed an acoustic doppler velocimeter (ADV) and Hydrolab at approximately 10:30 AM on March 14 at the conduit opening at the bottom of Watling's Blue hole. They were retrieved March 16 at approximately 4:30 PM. During their deployment, these instruments measured depth, salinity, temperature, dissolved oxygen and pH. The ADV continuously measured water velocity along three spatial axes, and the resulting unit vectors were combined into a single velocity vector. Uncertainty for raw velocity measurements was ± 0.1%. Velocity measurements were then multiplied by conduit cross-sectional area to calculate discharge volumes and directions. Discharge measurements were averaged

over twenty-minute intervals to reduce noise. Timeseries profiles for Watling's conduit were then compared to profiles gathered at Inkwell and Church in March 2017 using similar methods.

DNA extraction and sequencing

DNA sample extraction from filters was accomplished using QIAamp PowerFecal DNA Kit according to manufacturer protocols. 16S rRNA metagenomic profiles were obtained using an Illumina MiSeq platform and 2x300 base pair paired-end protocol and the primers recommended by Earth Microbiome (515F: 5'-GTGYCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACNVGGGTWTCTAAT-3').

Metagenome assembly and binning

Amplicon forward and reverse sequences were assembled into contigs, aligned and filtered for quality using Mothur 1.40.5[20]. Chimeras were removed during several steps of processing by screening sequence lengths. Quality sequences were clustered into OTUs based on 97% identity for bacteria and 60% identity for archaea. Sequences were then rarified across samples to adjust for discrepancies in sampling efficiency using the "sub.sample" command in mothur with the options 'persample=T' and 'size=[sequence count of the replicate that experienced the lowest sequencing efficiency]'.

Bioinformatic and statistical analyses

Bacterial OTUs with less than twenty sequences in a sample were removed from further analysis (less than two sequences for the archaea). Quality-filtered sequences were aligned to the Silva 132 prokaryotic database and assigned a taxonomic identity using Mothur 1.40.5 according to published protocols[20]. A representative sequence from each bacterial OTU was also run against the NCBI database using BLASTn to obtain a secondary identification. Taxonomic identifications for each OTU were assigned according to the database match with the highest percent identity at the genus level. OTUs corresponding to identical genera were combined in the final taxonomic tables. Both the Silva and NCBI taxonomic identities of

each OTU were considered during analysis of bacterial metabolic potentials, provided they had a percent identity exceeding 90%. PCO plots of square-root transformed sequence data and the BioEnv analysis are based Bray Curtis similarities and were created using Primer v7 software (Primer-E Ltd., Albany, New Zealand). Rarefaction curves were created using Mothur in order to assess species richness.

RESULTS

Water Chemistry and Hydrology

Physicochemical profiles confirm the presence of density stratification in Inkwell, as evidenced by changes in dissolved oxygen, temperature and salinity, with a halocline extending from approximately 2-4 meters depth at the time of sampling (**Figure 2**). Stratification is absent in Watling's and Church. Temperatures were 25C in Church and Watling's and 23-24C in the top and bottom layers of Inkwell, with a maximum temperature of 27C in the halocline. Watling's salinity is slightly higher than that of Church and Inkwell bottom, although the waters in all three sites fall within the brackish range (0.5 to 35 ppt). Inkwell salinity shows a gradual but distinct stratification pattern, with fresher surface water (9 ppt) on top of more saline bottom waters (~18ppt). Dissolved oxygen levels were

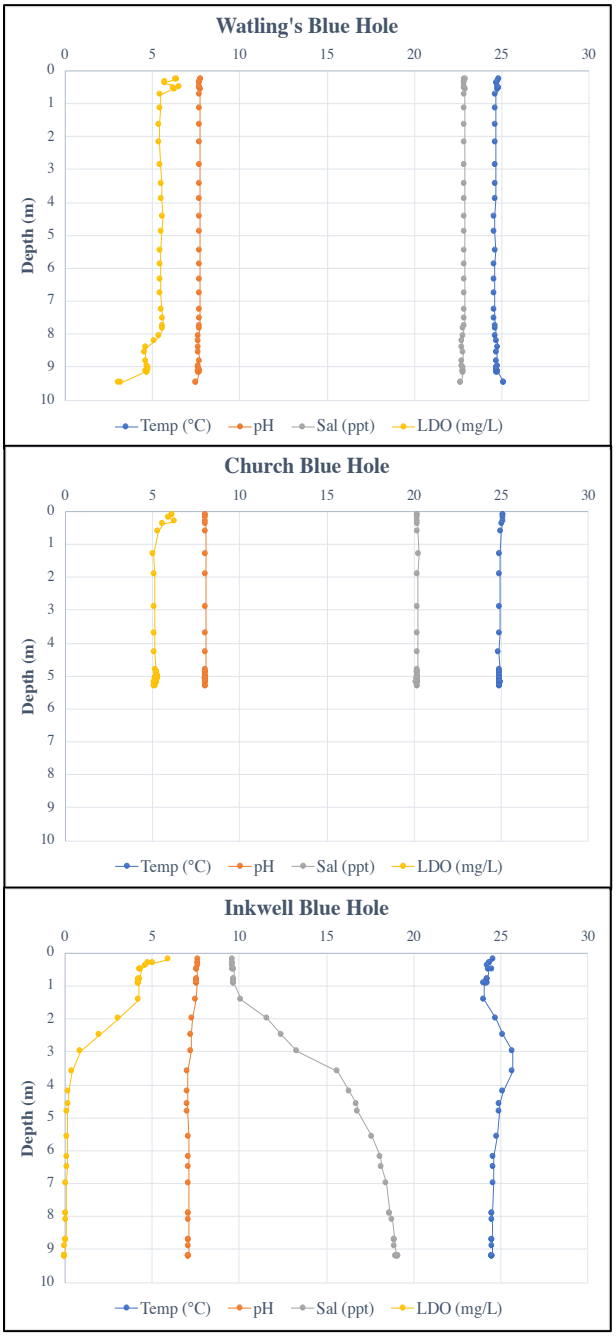


Figure 2: Vertical profiles of temperature, pH, salinity and dissolved oxygen (LDO) in Watling's, Church and Inkwell Blue Holes, March 2019.

approximately 6 mg/L in Watling's, 5 mg/L in Church and 4 mg/L in Inkwell surface waters, falling to near zero just below the halocline in Inkwell. The drop in DO seen in Watling's profile below eight meters depth represents water in the conduit opening.

Time-series conduit flow data from the ADV shows a distinct tidal pattern in Watling's conduit (**Figure 3**), similar to what was seen in Inkwell's conduit in 2017 using the same methods[21]. Salinity in Watling's conduit waters, while displaying a shallow tidal pattern that lagged slightly behind the discharge pattern, remained between 21.56 and 23.97 ppt. Temperature remained between 24.47 and 26.25C. Nutrient levels are provided in **Table 1**. Field work restrictions limited our collected measurements to sulfate, sulfide, nitrate, nitrite and ammonium.

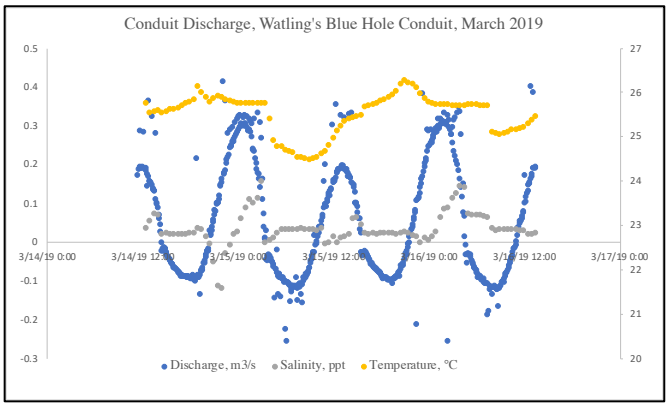


Figure 3: Conduit discharge in Watling's Blue Hole over 48-hour period. Discharge (m^3/s) is shown in blue, temperature (°C) in yellow, and salinity (ppt) in grey.

Table 1: Sulfate, Nitrate, Nitrite and Ammonium concentrations in Watling's, Church, and Inkwell blue holes at the time of biological sampling, March 2019. *Sulfide levels were below detectable limits in all samples.* Plus/minus values represent standard deviations. Sample size *n*=3

	WATLING'S	CHURCH	INKWELL SURFACE	INKWELL HALOCLINE	INKWELL BOTTOM
Sulfate (mg/l)	1160 ± 68	956 ± 15	734 ± 0	855 ± 51	1250 ± 38
Sulfide (mg/l)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Nitrate-N (mg/l)	0.61 ± 0.33	0.68 ± 0.02	0.53 ± 0.12	0.81 ± 0.09	0.22 ± 0.04
Nitrite-N (mg/l)	0.12 ± 0.03	0.07 ± 0.07	0.03 ± 0.00	0.00 ± 0.00	0.14 ± 0.04
Ammonium-N (mg/l)	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.10	0.07 ± 0.03	0.48 ± 0.17

Biology

Principle coordinate analysis accounting for 41.8% and 26% of variation along the x- and y-axes, respectively, show the highest degree of similarity between bacterial OTUS present in Watling's and Church Blue Holes (**Figure 4A**). Inkwell surface and middle (halocline) layers retain at least 20% similarity in this analysis, while Inkwell's bottom layer is the most different from any other sample (under 5% similarity). Coordinate analysis of archaeal OTUs, accounting for 18.9% and 10.9% of variation along the x- and y-axes, respectively, shows a greater degree of variation among the archaea than the bacteria (**Figure 4B**). However, as with the bacteria, archaeal OTUs are the most similar between Watling's and Church and different between Inkwell bottom and all other samples.

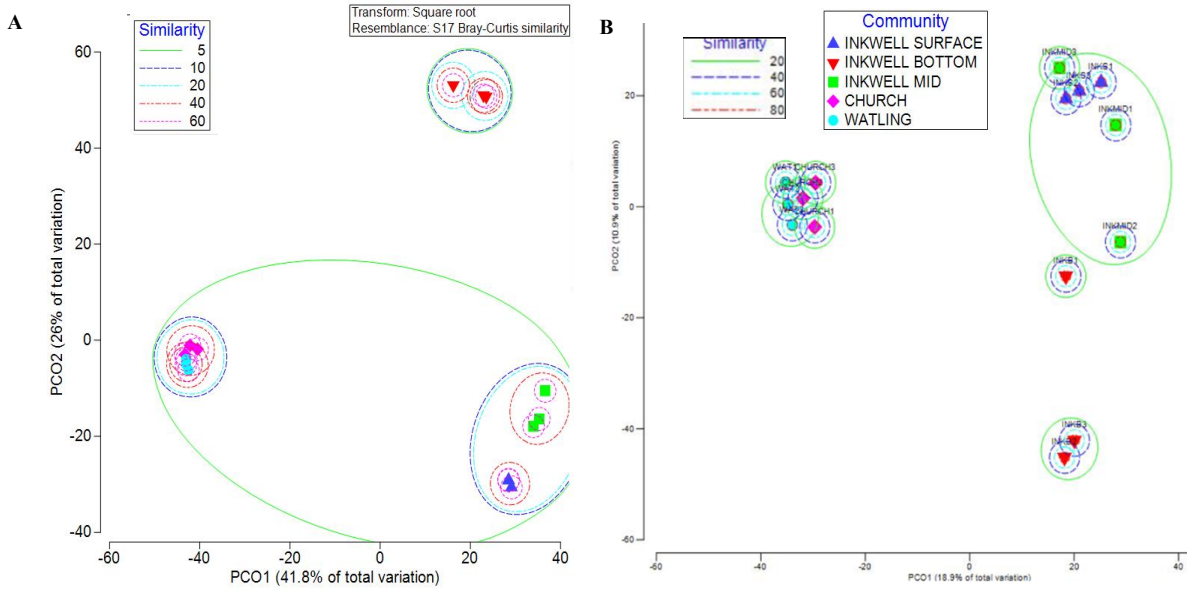


Figure 4: PCO plots of A) bacteria, and B) archaea based on Bray-Curtis similarity between OTUs across all samples. Each sample is represented by a different color, with the three replicates for each sample shown.

Figure 5 shows the PCO plot of bacterial OTUs overlain with environmental data, while **Table 2** shows the results of BEST analysis. In figure 7, vectors indicate direction of increase of the labeled abiotic environmental factor. Variation between the Watling's-Church cluster (Fig. 5) and Inkwell bottom correlates clearly with dissolved oxygen (LDO) and ammonium. Dissolved oxygen was below detectable limits in the bottom of Inkwell and ammonia was below detectable limits in both Watling's and Church, while at a maximum (0.48 ± 0.17) in Inkwell bottom (Table 1). The BEST analysis also identified a correlation between pH and biological variation. This variation appears primarily between the Watling's-Church cluster and the Inkwell samples. Average pH values used for these analyses were as follows: Church 8.06, Watling's 7.69, Inkwell bottom 7.62, Inkwell surface 7.56, Inkwell halocline 7.18. Variations between Inkwell surface and halocline are correlated with differences in nitrate, LDO and ammonia. Variation among Inkwell's layers correlated with nitrate and sulfate levels.

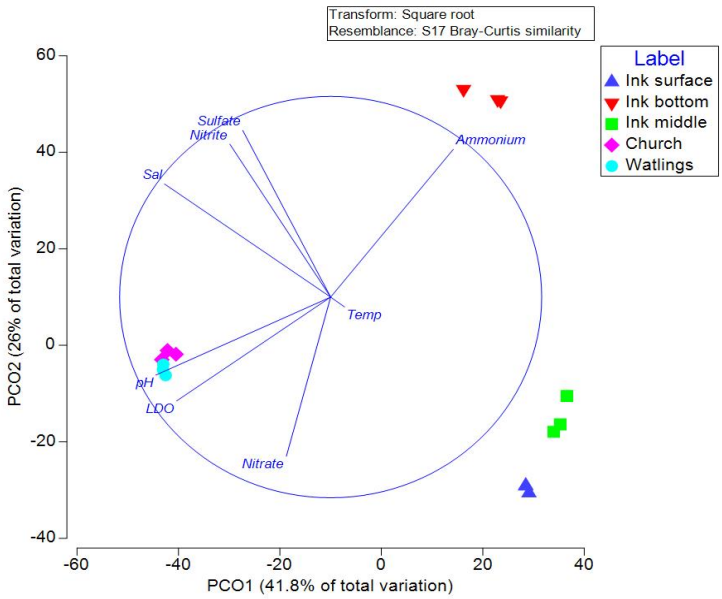


Figure 5: PCO plot of bacterial OTUs with vector overlays of abiotic environmental factors. Vectors indicate direction of increase of the associated abiotic factor.

Table 2: BEST analysis correlating OTUs and abiotic data.

BEST Biota and/or Environment matching		
Parameters	**Best results**	
Correlation method: Spearman rank	*No.Vars*	*Corr. Selections*
Method: BIOENV	1	0.860 LDO
Maximum number of variables: 1	1	0.812 pH
Analyse between: Samples	1	0.675 Ammonium
Resemblance measure: D1 Euclidean distance	1	0.578 Nitrate
	1	0.371 Nitrite
	1	0.323 Sulfate
	1	0.300 Sal
	1	0.043 Temp

Table 3: Number of OTUs and sequence abundance data before and after rarefication. Sample sequence totals represent the average number of sequences across the three replicates of each sample.

			Sample sequence totals				
		OTUs Total	***Watling's***	***Church***	***Inkwell surface***	***Inkwell halocline***	***Inkwell bottom***
Bacteria	Before rarifying	39, 865	101,469	88, 265	104,005	97,636	66,593
	Rarified	19,219	21,198	21,232	21,149	21,197	20,883
	Analyzed		**17,560 67 OTUs**	**18,538 41 OTUs**	**18,110 77 OTUs**	**18,438 115 OTUs**	**13,430 83 OTUs**
Archaea	Before rarifying	5,835	2,094	1,515	1,010	1,400	5,140
	Rarified	2,704	485	485	485	485	485
	Analyzed		**238 37 OTUs**	**171 44 OTUs**	**189 52 OTUs**	**176 44 OTUs**	**180 36 OTUs**

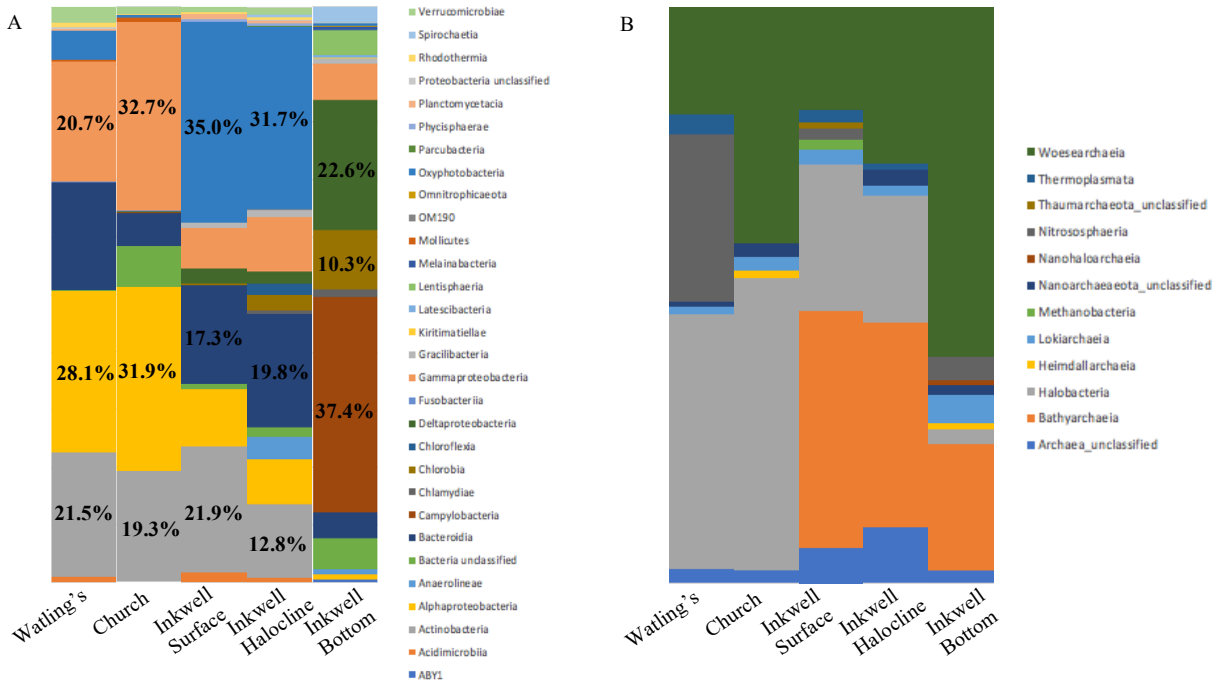


Figure 6: Relative abundance of A) bacterial, and B) archaeal classes across samples identified by 16S rDNA amplicons. Percent labels represent relative abundance values.

The number of sequences and OTUs before and after rarifying, as well as the numbers that were used for the remaining analyses, are summarized in **Table 3**.

Microbial community taxonomic identifications are summarized in **Figure 6** by Class and in **Table 4** by Genera. For full lists of genera present in each sample see **Supplementary Tables 1A-5A**. Watling's and Church Blue Holes were both dominated by *Alphaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria*, although not in the same order of abundance. Inkwell's surface and halocline were dominated by *Oxyphotobacteria*, followed by *Actinobacteria* and *Bacteroidia*. Inkwell's bottom layer was predominantly *Camplyobacteria*, followed by *Deltaproteobacteria* and *Chlorobia*.

In terms of archaea, Watling's and Church were both dominated by *Halobacteria*. These were followed by *Nitrososphaeria* in Watling's and by the *Woesearchaeia* in Church. *Bathyarchaiea* were only detected in Inkwell, where they were predominant in the surface and halocline. They were second in abundance in Inkwell's bottom, following the *Woesearchaeia*.

DISCUSSION

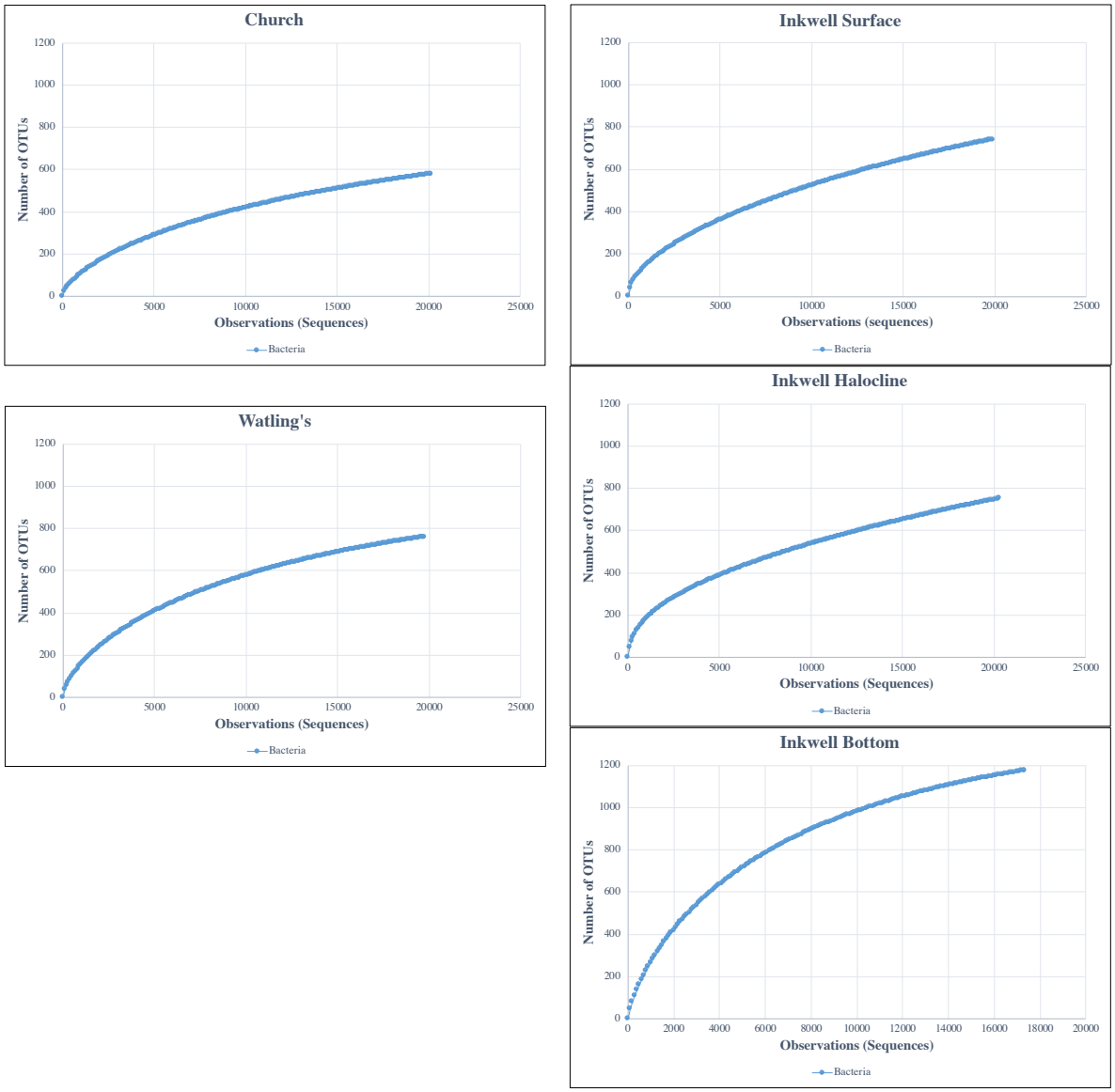
Rarefaction curves for biological samples are shown in **Figure 7**. The bottom of Inkwell appeared to host the greatest species richness. However, a clear asymptote cannot be seen in any of the rarefaction curves, likely due to the sequence rarification method used, high numbers of rare and/or unidentified taxa, or a combination of the above.

Watling's Blue Hole is significantly larger (both in surface area and depth) than Church and hosts an active conduit, whereas water flows in and out of Church primarily through the surrounding limestone matrix[15]. Water in Watling's in noticeably clearer than in the other two sites, with a slightly higher salinity. Both Watling's and Church host groups provisionally identified as *SAR11 Clade III*, *Aquiluna* and *Rhodoluna* among their top five OTUs. The *SAR11* belong to the *Pelagibacteria* clade (Clade III being characteristic of brackish waters) and feed on dissolved organic carbon and nitrogen[22]. The latter two genera are photoheterotrophs able to assimilate a variety of substrate. While Watling's and Church communities cluster closely together in a principle coordinate analysis, differences in their community dynamics become more apparent when analyzing their photosynthetic organisms (**Table 5**). In Church, photosynthesis is most likely predominantly carried out by purple sulfur bacteria in the family *Ectothiorhodospiraceae*, represented as the second most abundant OTU (19.6%) in Church and completely absent from Watling's. Cyanobacteria in Church include *Gleocapsa* (0.3%) and *Leptolyngbya* (0.2%). While there were small numbers of *Synechococcus* (4.6%) and *Gleocapsa*

Table 4: Top ten provisionally identified genera in each sample. 'SEQ': total number of sequences bearing identification, 'REL. ABD.': relative abundance, 'OTUs': OTUs corresponding to genus. Full lists of genera provided in Supplemental Tables 1A-5A.

	GENUS	% IDENTITY	SEQ	REL. ABD. (%)	OTUs
Watling's	Candidatus Aquiluna	97	1858	10.6	1
	SAR11 Clade III	100	1540	8.8	2
	Candidatus Rhodoluna	98	1518	8.6	7
	Wenxinia	93	1270	7.2	9
	Cellulophaga	95	1113	6.3	11
	AEGEAN-169 marine group	100	1096	6.2	16
	Congregibacter	96	909	5.2	18
	Synechococcus	99, 99, 100	801	4.6	3, 166, 227
	HIMB11	98	725	4.1	20
	Deep-sea mussel NZ3 thioautotrophic gill symbiont	95	635	3.6	22
Church	SAR11 Clade III	100	4749	25.6	2
	Ectothiorhodospiraceae_uncultured	93	3642	19.6	4
	Candidatus Aquiluna	97	2238	12.1	1
	Firmicutes_unclassified	91	1308	7.1	14
	Candidatus Rhodoluna	98	1245	6.7	7
	MWH-UniP1 aquatic group	100	1112	6.0	12
	Wenxinia	93	1068	5.8	9
	Litoricola	100, 100	622	3.4	19, 70
	WHC2-2	98	437	2.4	17
	Cellulophaga	95	330	1.8	11
Inkwell surface	Synechococcus	98, 98, 99, 97	4632	25.6	143, 5, 3, 165
	Candidatus Aquiluna	97	2156	11.9	1
	Cyanobium	98, 99	1703	9.4	6, 40
	PeM15	100, 100, 93, 94	1290	7.1	10, 60, 92, 131
	Ectothiorhodospiraceae_uncultured	93	664	3.7	4
	Flavobacteriales_unclassified	85, 99, 91	583	3.2	23, 49, 89
	Candidatus Rhodoluna	98	528	2.9	7
	Fluviicola	100, 100	488	2.7	28, 126
	Phaeocystidibacter	92, 91, 92	396	2.2	56, 69, 91
	Paracoccus	97	383	2.1	31
Inkwell halocline	Cyanobium	95, 97, 99, 99	3489	18.9	6, 5, 40, 146
	Synechococcus	97, 98	2356	12.8	165, 3
	Candidatus Aquiluna	96	1345	7.3	1
	Saprospiraceae_uncultured	100, 100	1340	7.3	15, 39
	PeM15	100, 100, 93	632	3.4	10, 60, 92
	SBR1031	100, 99, 100	561	3.0	37, 136, 127
	Chlorobium	97, 96, 97	510	2.8	27, 26, 52
	Flavobacteriales_unclassified	85, 99, 62	477	2.6	23, 49, 89
	Candidatus Rhodoluna	98	362	2.0	7
	Ectothiorhodospiraceae_uncultured	93	356	1.9	4
Inkwell bottom	Arcobacter	100, 100	4843	36.1	8, 243
	Desulforhopalus	97	1973	14.7	13
	Chlorobium	97, 96, 97	1344	10.0	27, 26, 52
	Bacteria_unclassified	100, 100, 100, 100, 100, 100, 94, 100, 100, 100	674	5.0	80, 84, 130, 167, 214, 250, 199, 310, 364, 389
	PRD18C08	100, 99	454	3.4	53, 173
	Spirochaeta	90, 100	363	2.7	44, 204
	Pseudoalteromonas	100	343	2.6	71
	Desulfofustis	99	280	2.1	62
	Sphingobacteriales_unclassified	100	232	1.7	43
	Desulfobacter	96	217	1.6	67

A



B

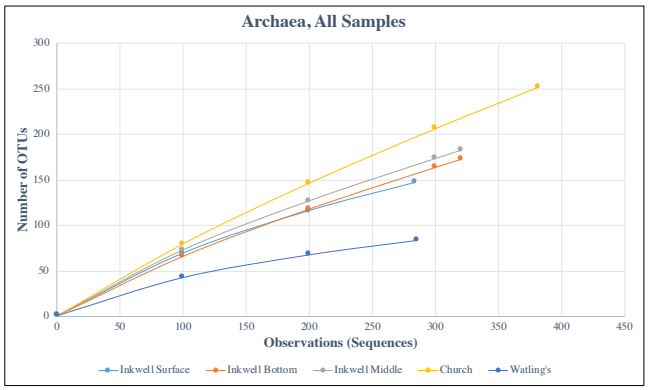


Figure 7: Rarefaction curves for A) bacterial, and B) archaeal biological samples.

(0.5%) in Watling's, photosynthesis appears to be primarily carried out by the two photoheterotrophs mentioned above (together accounting for 19.2%, relative abundance). Watling's hosted a higher percentage of organisms associated with marine or even hypersaline salinities than any of the other samples (25.3%). Further studies aimed at identifying Watling's conduit water's origin using oxygen and hydrogen stable isotope data, as well as additional genes for analyses, may help shed light on this pattern.

Inkwell has a depth similar to that of Watling's, but a much smaller surface area. Inkwell also hosts an active conduit, and the gradual nature of the changes in LDO and salinity over the 2-3-meter span of the halocline indicates there is some mixing occurring in the lower waters. The existence of a small fresh/brackish water spring supplying surface water has been postulated[21]. The relatively lush vegetation surrounding the hole has resulted in an input of tannins, which appear to remain suspended in the halocline while the fresher surface waters remain clear[23]. Visibility was at a minimum inside the halocline and increased in the saltier bottom waters. Photosynthetic communities in the surface layer and halocline were dominated by *Cyanobacteria* genera *Synechococcus* and *Cyanobium*. *Ectothiorhodospiraceae* (3.7%) and *Chlorobium* (0.2%) were present in smaller amounts. *Ectothiorhodospiraceae* account for 1.9% and 1.5% of the purple sulfur bacteria identified in the halocline and bottom, respectively, with the remaining 0.5% and 0.2% representing *Allochromatium* (also a member of the *Gammaproteobacteria*). The purple non-sulfur genus *Rhodobacter* was also identified in the surface (1.5%) and halocline (1.8%) communities but, due to their highly variable metabolic potential, they were not included in these functional analyses. The *Ectothiorhodospiraceae* decreased in relative abundance with depth as the green sulfur bacteria (primarily *Chlorobium*) increased. The *Chlorobia* are known

for their ability to outcompete other photosynthesizers in low light, tolerate higher sulfide concentrations than the PSB (purple sulfur bacteria) Family *Chromatiaceae*, and to accumulate underneath areas preferred by the purple sulfur bacteria, so the opposing distribution of these two groups in Inkwell is consistent with past research[24, 25]. *Chlorobi* were found to dominate photosynthetic communities near the chemoclines in Sawmill Sink (at 10 meter depth) and Cherokee Road Extension Blue Hole (at 33 meter depth), two deeper, stratified blue holes on Abaco and Andros islands, Bahamas [7, 16]. Alternatively, the *Chromatiaceae* have been found to position themselves near the top of vertical gradients containing sulfide, are tolerant of low oxygen concentrations and sensitive to sulfide concentrations over 2 mM[24]. Schwabe et al (2004) found a dense plate of *Allochromatium* and *Thiocapsa* (both members of the *Gammaproteobacteria* family *Chromatiaceae*) was responsible for the dark coloration of South Androw Black Hole[17]. These two genera belong to the same Order (*Chromatiales*) as the dominant PSB identified in this study, the *Ectothiorhodospiraceae*. They all utilize bacteriochlorophyll a and carotenoid spirilloxanthin accessory pigments (maximum absorption between 480 and 550 nm) while oxidizing sulfides, however the *Ectothiorhodospiraceae* store elemental sulfur in the extracellular globules, as opposed to the intracellular globules seen in the other two genera. The presence of *Ectothiorhodospiraceae* in all three layers of Inkwell and in Church suggests there is some sulfide present that is getting oxidized, although further testing would be needed to confirm phototrophic growth using sulfides. The presence of both purple and green sulfur bacteria may help explain the slight increase in temperature seen in the halocline, as these organisms may be dissipating excess light energy as heat[17].

A preliminary investigation of microbial metabolic potential sheds additional light on sulfur and nitrogen cycling in these three blue holes (**Table 6**). A corresponding list of identified genera assigned to each transformation is provided in **Supplemental Table 6A**. Metabolic reconstructions did offer some explanation of the sulfur chemistry in each hole. Sulfide oxidation in Watlings was most likely carried out primarily by *Cand. Thioglobus* and members represented by OTU22, identified in Silva as an unclassified member of family *Thioglobaceae* (98% identity) and in NCBI as an unclassified deep-sea mussel NZ3 thioautotrophic gill symbiont (95% identity). Both organisms are reliant on reduced sulfur species for growth, suggesting the presence of sulfide despite it not being detected in our water chemistry. *HIMB11*, *Thioprofundum* and *Thiosocius* are then able to oxidize intermediate-oxidation-state sulfur compounds to sulfate. OTU265, identified as an unclassified *Chromatiales* member with only 56% identity in Silva, had a 97% identity match in NCBI with the unclassified bacteria *Thiosocius teredinicola*, a gill symbiont of the giant shipworm *Kuphus polythalamius*. OTU22 and OTU265 sequence clusters, present only in Watling's, are both associated with sulfur oxidation and nitrogen reduction/fixation. *Cand. Thioglobus* is known to consume ammonium and produce nitrite, albiet under anaerobic conditions. Whether by biological assimilation or abiotic means, most ammonia appears to be getting removed from solution.

In Church, sulfide oxidation is inferred for the *Ectothiorhodospiraceae*. Organisms in this genus may be further oxidizing elemental sulfur (after storage) due to low sulfide availability, resulting in intermediate sulfur compounds that other sulfur oxidizers, such as *HIMB11*, can utilize. Small numbers of Desulfobacteraceae capable of reducing sulfate back to sulfide were identified (0.1%), but ultimately the source of sulfides maintaining the *Ectothiorhodospiraceae* community

are unknown. Nitrogen-reducting organisms were identified and are consistent with nitrate and nitrite levels. Ammonia is again below detectable limits in these waters.

A large community of sulfate-reducing bacteria was confirmed in Inkwell's bottom layer. The lack of sulfide in in these waters is most likely explained by the overwhelming abundance of *Arcobacter* (36.1%). This enigmatic genus comprises species involved with both sulfide oxidation and nitrogen fixation. Indeed, a role in reoxidizing sulfide formed by microbial sulfate or sulfur reduction has been proposed, which appears to be the case here[26]. *Chlorobium*, *Chlorobaculum*, *Sulfurimonas* and *Ectothiorhodospiraceae* are also likely oxidizing any available sulfide. Chemoautotrophic sulfide oxidation has also been observed near the oxic-anoxic interface of other stratified systems and thus cannot be discounted as a possibility here, as well[27]. Some *Arcobacter* may also be fixing nitrogen in the bottom waters, along with *Desulfocapsa*, *Gastranaerophilales* and *Sva048*, which may account for the relatively high levels of ammonia. A high incidence of uncultured organisms and genera with species-dependent metabolic potentials precludeed further analysis of microbial function. Further studies using multiple gene analyses and stable isotopes would help elucidate the biogeochemistry in Inkwell and potentially shed light on the nitrogen chemistry observed in this study. This study did not look at any microbial mats or films and surface-attached organisms may be playing a critical role in biogeochemical cycling. A comprehensive genomic study that included these structures as well as full nutrient data could provide much more insight into the biogeochemistry of these ecosystems.

Competition between oxygenic and anoxigenic phototrophs has been proposed as an explanation for the delay in the oxygenation of Earth's oceans during the Proterozoic[28]. The relatively shallow, stratified and dark nature of Inkwell make it a particularly unique and intriguing environment for studying photosynthesis, sulfur cycling dynamics and early Earth conditions. The success of the *Ectothiorhodospiraceae* in Church, and the competition between photosynthetic species in the dark waters of Inkwell, may warrant further study. Time-series analyses of photosynthetic communities that include fluorescence measurements and pigment analyses may shed light on the seasonal dynamics of competition between oxygenic and anoxigenic phototrophs.

Table 5: Relative abundances of selected functions in each community.

	Cyanobacteria	Purple sulfur bacteria	Green sulfur bacteria	Filamentous anoxygenic phototrophs	Photoheterotrophs	Fermentation	Organic material degradation	Marine/ Hypersaline
Inkwell Surface	35.0	3.7	0.2	0.0	15.1	0.9	6.7	9.8
Inkwell Halocline	31.7	2.4	2.8	1.9	11.4	3.2	12.2	9.9
Inkwell Bottom	0.5	1.7	10.3	0.0	16.7	26.3	8.1	3.5
Church	0.5	19.6	0.0	0.0	19.7	8.2	4.7	6.3
Watling's	5.0	0.2	0.0	0.0	25.7	0.7	13.8	25.3

Table 6: Sulfur and Nitrogen cycling capacity inferred for microbial communities in Watling's, Church, and Inkwell blue holes. Estimates are based on taxonomic identifications and provided as percent of sequences from a sample (relative abundance) associated with the given transformation and the corresponding number of sequences. Sulfur Oxidation, Sulfur Reduction, Nitrogen Reduction and Nitrogen Oxidation column values represent totals for each sample. Disproportionation/Other column includes elemental sulfur, sulfite and thiosulfate disproportionations and transformations involving thiocyanate and thiosulfide. DSR = dissimilatory sulfate reduction. DNRA = dissimilatory nitrate reduction to ammonia.

		Sulfur Oxidation	$HS^- \rightarrow S^0$	$S^0 \rightarrow SO_4^{2-}$	$SO_3^{2-} \rightarrow SO_4^{2-}$	$S_2O_3^{2-}$ Oxidation	$HS^- \rightarrow SO_4^{2-}$	Unknown	Disproportionation/Other	Sulfur Reduction	$S^0 \rightarrow HS^-$	$SO_3^{2-} \rightarrow HS^-$	$SO_4^{2-} \rightarrow SO_3^{2-} \rightarrow HS^-$ (DSR)	Nitrogen Reduction	$NO_3^- \rightarrow NO_2^-$	$NO_2^- \rightarrow N_2$	N_2 Fixation	DNRA	Nitrogen Oxidation	$NH_3 \rightarrow NO_2^-$	$NO_2^- \rightarrow NO_3^-$	$NH_3 \rightarrow NO_3^-$
Watling's	Relative Abundance (%)	9	0.8	0.6	4.1	4.5	3.6	0	0	0	0	0	0	17.2	13.5	3.2	3.7	3.6	0	0	0	0
	# Sequences	1608	149	122	725	808	635	0	0	0	0	0	0	3032	2374	560	658	635	0	0	0	0
Church	Relative Abundance (%)	20.3	19.8	0	0.5	0.5	0	0	0	0.1	0	0	0.1	4.7	4.5	0.9	0.4	0	0	0	0	0
	# Sequences	3762	3678	0	84	84	0	0	0	24	0	0	24	839	806	154	69	0	0	0	0	0
Inkwell Surface	Relative Abundance (%)	6.3	6	2.2	0	0.1	0	0.2	0	0	0	0	0	9.2	9	2.5	0.1	0	0.8	0.8	0.8	0
	# Sequences	1130	1077	406	0	23	0	30	0	0	0	0	0	1672	1642	442	26	0	149	149	149	0
Inkwell Halocline	Relative Abundance (%)	7.2	7.2	0.9	0.5	0	0	0	0.5	0.2	0	0	0.2	7.8	7.3	1.3	0.6	0	0.6	0.4	0.4	0.2
	# Sequences	1326	1326	170	100	0	0	0	91	46	0	0	46	1321	1235	240	113	0	101	67	67	34
Inkwell Bottom	Relative Abundance (%)	49.7	49.3	1.9	0	1.6	0	0	1	20.4	2.1	17.2	20.2	58.4	56.3	52.6	38	0	0	0	0	0
	# Sequences	6662	6617	249	0	207	0	0	134	2741	280	2304	2720	7823	7542	7054	5094	0	0	0	0	0

REFERENCES

1. Schwabe, S. and J. Carew, *Blue holes: an inappropriate moniker for water-filled caves in the Bahamas.* In: Davis RL, Gamble DW (Eds) Proceedings of 12th symposium on the geology of the Bahamas. Bahamian Field Station, San Salvador Island, Bahamas, pp 101–108, 2006.
2. Mylroie, J., J. Carew, and A. Moore, *Blue Holes: definition and genesis.* Carbonates and Evaporites, 1995. **10**(2): p. 225-233.
3. Larson, E. and J. Mylroie, *Diffuse versus conduit flow in coastal karst aquifers: the consequences of island area and perimeter relationships.* Geosciences, 2018. **8**(7).
4. Mylroie, J. and J. Carew, *Karst development on carbonate islands.* Speleogenesis and Evolution of Karst Aquifers, 2003. **1**(2).
5. Mylroie, J. and J. Carew, *Solution conduits as indicators of late quaternary sea level position.* Quaternary Science Reviews, 1988. **7**: p. 55-64.
6. Pace, M., J. Mylroie, and J. Carew, *Investigation and review of dissolution features on San Salvador Island, Bahamas.* In: White, B (Ed) Proceedings of 6th symposium on the geology of the Bahamas. Bahamian Field Station, San Salvador Island, Bahamas, pp 109–123, 1993.
7. Gonzalez, B., et al., *Microbial hotspots in anchialine blue holes: initial discoveries from the Bahamas.* Hydrobiologia, 2011. **677**(1): p. 149-156.
8. Davis, R. and C. Johnson, *Karst hydrology of San Salvador.* 1988.
9. Mylroie, J. and J. Carew, *Geology & karst geomorphology of San Salvador Island.* Carbonates and Evaporites, 1995. **10**(2): p. 193-206.
10. Mylroie, J. and J. Mylroie, *Development of the carbonate island karst model.* Journal of Cave and Karst Studies, 2007. **69**(1): p. 59-75.
11. Mylroie, J. and J. Mylroie, *Void development on carbonate coasts: creation of anchialine habitats.* Hydrobiologia, 2011. **677**(1): p. 15-32.
12. Schwabe, S., R. Herbert, and J. Carew, *A hypothesis for biogenic cave formation: a study conducted in the Bahamas.* In: Park LE, Freile D (Eds) Proceedings of the thirteenth symposium on the geology of the Bahamas and other carbonate regions. Gerace Research Center, San Salvador, pp 141–152, 2008.
13. McGee, D.K., et al., *Tracing groundwater geochemistry using δ13C on San Salvador Island (southeastern Bahamas): implications for carbonate island hydrogeology and dissolution.* Carbonates and Evaporites, 2010. **25**(2): p. 91-105.
14. Vermette, S. and R. Hudson, *Hydrology of Watling's Blue Hole: San Salvador, Bahamas.* Middle States Geographer, 2001. **34**: p. 55-62.
15. Smith, M.e.a., *Source of saline groundwater on tidally influenced blue holes from San Salvador Island, Bahamas.* submitted for publication.
16. Macalady, J.L.e.a., *Microbial biogeochemistry of a meromictic blue hole.* Geochimica et Cosmochimica Acta, 2010. **74**.
17. Schwabe, S. and R. Herbert, *Black Holes of the Bahamas: what they are and why they are black.* Quaternary International, 2004. **121**(1): p. 3-11.
18. Haas, S., et al., *Low-Light anoxygenic photosynthesis and Fe-S-biogeochemistry in a microbial mat.* Frontiers in Microbiology, 2018. **9**: p. 858.
19. Bottrell, S., et al., *Geochemistry and isotope systematics of sulphur in the mixing zone of Bahamian blue holes.* Applied Geochemistry, 1991. **6**: p. 97-103.

20. Schloss, P.D.e.a., *Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities.* . Applied and Environmental Microbiology, 2009. **75**(23): p. 7537-41.
21. Sampson, J. and K. Guilbeault, *Baseline physicochemical investigations on waters from three blue holes, San Salvador Island, Bahamas.* Studia Universitatis Babes-Bolyai, Geologia, 2013. **58**(1): p. 11-19.
22. Morris, R.e.a., *SAR11 clade dominate ocean surface bacterioplankton communities.* Nature, 2002. **420**: p. 806-10.
23. Mylroie, J. and J. Carew, *Field guide to geology & karst geomorphology of San Salvador Island, San Salvador, Bahamas. Gerace Research Center. 88 p.* .
24. Guerrero, R., et al., *Communities of phototrophic sulfur bacteria in lakes of the Spanish Mediterranean region.* Acta Academiae Aboensis, 1987. **47**(2): p. 125-151.
25. Vila, X. and C.A. Abella, *Effects of light quality on the physiology and the ecology of planktonic green sulfur bacteria in lakes.* Photosynthesis Research, 1994. **41**: p. 53-65.
26. Voordouw, G., et al., *Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria.* Applied Environmental Microbiology, 1996. **62**: p. 1623-29.
27. Hamilton, T.L., et al., *Coupled reductive and oxidative sulfur cycling in the phototrophic plate of a meromictic lake.* Geobiology, 2014. **12**(5): p. 451-68.
28. Johnston, D.T., et al., *Anoxygenic photosynthesis modulated Proterozoic oxygen and sustained Earth's middle age.* Proc Natl Acad Sci U S A, 2009. **106**(40): p. 16925-9.

APPENDICES

Appendix 1: Table 1A: Genera, Watling's Blue Hole

Table 1A: Genera present in Watling's Blue Hole. '% Identity': closest identity match in Silva and/or NCBI databases, 'Genus Seq. Total': total number of sequences bearing identification, 'Relative Abundance (%)': percent of analyzed sequences corresponding to genus, 'OTUs': OTUs corresponding to genus.

Genus	% Identity	Genus Seq. Total	Relative Abundance (%)	OTUs
Candidatus Aquiluna	97	1858	10.6	1
SAR11 Clade III	100	1540	8.8	2
Candidatus Rhodoluna	98	1518	8.6	7
Wenxinia	93	1270	7.2	9
Cellulophaga	95	1113	6.3	11
AEGEAN-169 marine group	100	1096	6.2	16
Congregibacter	96	909	5.2	18
Synechococcus	99, 99, 100	801	4.6	3, 166, 227
HIMB11	98	725	4.1	20
Deep-sea mussel NZ3 thioautotrophic gill symbiont	95	635	3.6	22
WHC2-2	98	505	2.9	17
Phaeocystidibacter	92	498	2.8	24
Roseibacillus	99	490	2.8	32
NS4 marine group	100	466	2.7	36
Litoricola	100, 100	450	2.6	19, 70
Yonghaparkia sp.	99	400	2.3	35
NS9 marine group	100, 98	353	2.0	50, 76
NS11-12 marine group	100	249	1.4	48
WHC7-12	93	234	1.3	51
Illumatobacter	95	191	1.1	29
SAR116 clade	100, 100, 100, 99	190	1.1	117, 137, 206, 208
Methylophilales bacterium	98	184	1.0	64
Luminiphilus	99, 98, 94	182	1.0	100, 59, 198
Sediminicola	94	175	1.0	46
MWH-UniP1 aquatic group	100	123	0.7	12
Balneola	100	110	0.6	86
Hydrogenophaga	98	106	0.6	85
Candidatus Thioglobus	97	104	0.6	98
Robiginitalea	100	98	0.6	83
Fulvivirga	95, 91, 96, 95	94	0.5	231, 203, 179, 235
Gleocapsa	100	81	0.5	88
Flexibacter	96	73	0.4	106
Acholeplasma	100	71	0.4	58
Thioprofundum	97	60	0.3	105
Owenweeksia	91	53	0.3	160
Erythrobacter	99	49	0.3	163
Proteobacteria_unclassified	86, 69	47	0.3	205, 285
Saprospiraceae_unclassified	100, 100	47	0.3	158, 39
Legionella	96	47	0.3	157
Lewinella	93, 93	43	0.2	170, 189
Thiohalocapsa	98	39	0.2	141
Candidatus Puniceispirillum	100	36	0.2	195
Salinibacter	100	32	0.2	114
Cetobacterium	100	28	0.2	140
Firmicutes_unclassified	91	28	0.2	14
SAR11 Clade Ia	100	27	0.2	238
Portibacter	90	26	0.1	158
Actibacter	99	25	0.1	239
Fuerstia	92	23	0.1	308
Thiosocius	97	23	0.1	265
Ectosymbiont of Cladonema	94	22	0.1	212
Gimesiaceae_unclassified	100	20	0.1	339
Spongiibacter	91	20	0.1	262

Appendix 2: Table 2A: Genera, Church Blue Hole

Table 2A: Genera present in Church Blue Hole. '% Identity': closest identity match in Silva and/or NCBI databases, 'Genus Seq. Total': total number of sequences bearing identification, 'Relative Abundance (%)': percent of analyzed sequences corresponding to genus, 'OTUs': OTUs corresponding to genus.

Genus	% Identity	Genus Seq. Total	Relative Abundance (%)	OTUs
SAR11 Clade III	100	4749	25.6	2
Ectothiorhodospiraceae_uncultured	93	3642	19.6	4
Candidatus Aquiluna	97	2238	12.1	1
Firmicutes_unclassified	91	1308	7.1	14
Candidatus Rhodoluna	98	1245	6.7	7
MWH-UniP1 aquatic group	100	1112	6.0	12
Wenxinia	93	1068	5.8	9
Litoricola	100, 100	622	3.4	19, 70
WHC2-2	98	437	2.4	17
Cellulophaga	95	330	1.8	11
Roseibacillus	94	231	1.2	66
Lewinella	91, 91, 93, 93	192	1.0	129, 132, 170, 241
Phaeocystidibacter	92	171	0.9	24
Luminiphilus	98	167	0.9	59
Acholeplasma	100	152	0.8	58
Yonghaparkia	99	103	0.6	35
Phaeodactylibacter	93, 100	89	0.5	154, 191
Sediminicola	94	87	0.5	46
HIMB11	98	84	0.5	20
Gleocapsa	100	60	0.3	88
Owenweeksia	91, 93	57	0.3	252, 133
Arcobacter	100	36	0.2	225
Portibacter	90	35	0.2	158
Cetobacterium	100	33	0.2	140
Leptolyngbya	97	33	0.2	178
Parahaliea	95	31	0.2	185
Fulvivirga	92	29	0.2	197
Salinibacter	100	29	0.2	114
Robiginitalea	100	28	0.2	83
Spongiibacter	94	26	0.1	269
Desulfobacteraceae_unclassified	98	24	0.1	192
Flexibacter	96	24	0.1	106
Alkanindiges	100	23	0.1	153
AEGEAN-169 marine group	100	21	0.1	16
Marivirga	92	20	0.1	232

Appendix 3: Table 3A: Genera, Inkwell Blue Hole, Surface

Table 3A: Genera present in Inkwell Blue Hole, Surface. '% Identity': closest identity match in Silva and/or NCBI databases, 'Genus Seq. Total': total number of sequences bearing identification, 'Relative Abundance (%)': percent of analyzed sequences corresponding to genus, 'OTUs': OTUs corresponding to genus.

Genus	% Identity	Genus Seq. Total	Relative Abundance (%)	OTUs
Synechococcus	98, 98, 99, 97	4632	25.6	143, 5, 3, 165
Candidatus Aquiluna	97	2156	11.9	1
Cyanobium	98, 99	1703	9.4	6, 40
PeM15	100, 100, 93, 94	1290	7.1	10, 60, 92, 131
Ectothiorhodospiraceae_uncultured	93	664	3.7	4
Flavobacteriales_unclassified	85, 99, 91	583	3.2	23, 49, 89
Candidatus Rhodoluna	98	528	2.9	7
Fluviicola	100, 100	488	2.7	28, 126
Phaeocystidibacter	92, 91, 92	396	2.2	56, 69, 91
Paracoccus	97	383	2.1	31
Robiginitalea	99	360	2.0	25
Citreimonas	98	352	1.9	34
Silvanigrella	92	342	1.9	30
MWH-UniP1 aquatic group	100, 100, 100	309	1.7	12, 115, 108
Chitinophaga	92	281	1.6	33
Rhodobacter	97, 97	264	1.5	102, 38
NS11-12 marine group	100, 100	262	1.4	45, 156
Saprospiraceae_uncultured	100, 100	246	1.4	39, 15
Illumatobacter	95	229	1.3	29
Ideonella	96	192	1.1	41
Roseibacterium	99	178	1.0	55
Candidatus Peregrinibacteria	99	167	0.9	42
Bacteria_unclassified	54, 95	165	0.9	54, 94
Pirellulaceae_uncultured	100	149	0.8	57
Luteolibacter	100	134	0.7	47
Pseudarcicella	100	129	0.7	72
NS3a marine group	100	128	0.7	68
Owenweeksia	93, 90, 93	100	0.6	81, 150, 99
Deltaproteobacteria_unclassified	85, 62	99	0.5	95, 109
Ilumatobacteraceae_unclassified	100	90	0.5	93
CL500-3	100, 100	89	0.5	73, 202
Labrys	94	82	0.5	101
Donghicola	99	80	0.4	96
Candidatus Intestinusbacter	96	79	0.4	97
Candidatus Megaira	100	68	0.4	61
Acidibacter	98	65	0.4	75
Haematobacter	98	63	0.3	111
Gracilimonas	92	61	0.3	103
Blastomonas	98	56	0.3	112
Erythrobacter	98	51	0.3	116
Roseicyclus	99	50	0.3	79
EF100-94H03	100	35	0.2	151
Chlorobium	98	30	0.2	52
SAR324 clade(Marine group B)	100	30	0.2	190
Cytophaga	90	29	0.2	135
Mucilaginibacter	90	29	0.2	118
Phaeodactylibacter	93	29	0.2	124
Ruficoccus	93	26	0.1	152
Cellulophaga	95	26	0.1	11
VC2.1 Bac22	93	25	0.1	74
Thioprofundum	97	23	0.1	105
Thalassobaculum	100	23	0.1	147
Marinobacterium	92	21	0.1	122
NS9 marine group	98	20	0.1	76
Pseudorhodobacter	99	20	0.1	219

Appendix 4: Table 4A: Genera, Inkwell Blue Hole, Halocline

Table 4A: Genera present in Inkwell Blue Hole, Halocline. '% Identity': closest identity match in Silva and/or NCBI databases, 'Genus Seq. Total': total number of sequences bearing identification, 'Relative Abundance (%)': percent of analyzed sequences corresponding to genus, 'OTUs': OTUs corresponding to genus.

Genus	% Identity	Genus Seq. Total	Relative Abundance (%)	OTUs
Cyanobium	95, 97, 99, 99	3489	18.9	6, 5, 40, 146
Synechococcus	97, 98	2356	12.8	165, 3
Candidatus Aquiluna	96	1345	7.3	1
Saprospiraceae_uncultured	100, 100	1340	7.3	15, 39
PeM15 ge	100, 100, 93	632	3.4	10, 60, 92
SBR1031 ge	100, 99, 100	561	3.0	37, 136, 127
Chlorobium	97, 96, 97	510	2.8	27, 26, 52
Flavobacteriales_unclassified	85, 99, 62	477	2.6	23, 49, 89
Candidatus Rhodoluna limnophila	98	362	2.0	7
Ectothiorhodospiraceae_uncultured	93	356	1.9	4
Rhodobacter	97, 99, 97	331	1.8	38, 175, 102
Bacteria unclassified	54, 100, 95, 100, 94	301	1.6	54, 110, 94, 84, 199
Robiginitalea	99	247	1.3	25
Candidatus Chloroploca	93, 100	225	1.2	87, 90
MWH-UniP1 aquatic group	100, 100, 100	213	1.2	12, 108, 115
Chitinophaga	92	204	1.1	33
Owenweeksia	93, 90, 93, 93	201	1.1	81, 150, 99, 133
Sphingobacteriales_unclassified	100	192	1.0	43
Ideonella	96	184	1.0	41
Candidatus Peregrinibacteria	99	178	1.0	42
Silvanigrella	92	176	1.0	30
Maribellus luteus	96	166	0.9	63
Niveispirillum	94	155	0.8	77
Anaerolineaceae_unclassified	96, 71	154	0.8	104, 121
Phaeocystidibacter	91, 92, 92	141	0.8	69, 56, 91
Luteolibacter	100	139	0.8	47
Fluviicola	100, 100	137	0.7	28, 126
Citreimonas	98	132	0.7	34
Skermanella	94	125	0.7	82
VC2.1 Bac22	93	122	0.7	74
Saccharomonospora	97	118	0.6	31
Illumatobacter	95	109	0.6	29
Candidatus Megaira	100	101	0.5	61
Deltaproteobacteria_unclassified	85, 62	101	0.5	95, 109
Absconditabacteriales (SR1)	100	100	0.5	119
Allochromatium	100	100	0.5	21
Acidibacter	98	98	0.5	75
Acidothermus	95	95	0.5	131
CL500-3	100	93	0.5	73
Roseicyclus	99	90	0.5	79
cvE6	100	89	0.5	65
NS11-12 marine group	100	76	0.4	45
Thioalkalivibrio	91	70	0.4	123
Spirochaeta	90	69	0.4	44
Pirellulaceae_uncultured	100	67	0.4	57
Oscillochloris	91	65	0.4	125
Marinobacterium	92	64	0.3	122
Chloronema	93	63	0.3	120
NS3a marine group	100	53	0.3	68
Phaeodactylibacter	93	53	0.3	124
Mucilaginibacter	90	50	0.3	118
Roseibacterium	99	47	0.3	55
Gracilimonas	92	45	0.2	103
Paracaedibacteraceae_uncultured	100	45	0.2	149
Pseudooceanicola	98	45	0.2	113
Terrimicrobium	100	45	0.2	159
Lentimicrobiaceae	100	44	0.2	78
OM190	100	41	0.2	148
Cytophaga	90	39	0.2	135

Table 4A. (Continued)

Thalassobaculum	100	36	0.2	147
Leisingera	97	36	0.2	142
Bordetella	98	35	0.2	180
Candidatus Scalindua marina	100	34	0.2	93
Bacteroidia_unclassified	99	33	0.2	169
Donghicola	99	32	0.2	96
Sinirhodobacter	97	32	0.2	177
Pseudarcicella	100	31	0.2	72
Candidatus Intestinusbacter	96	28	0.2	97
Pedosphaeraceae_unclassified	100	28	0.2	164
Candidatus Moranbacteria	87	27	0.1	193
Ruficoccus	93	27	0.1	152
Candidatus Uhrbacteria	90	26	0.1	196
Jhaorihella	91	26	0.1	194
Rhodothermus	92	26	0.1	181
Erythrobacter	98	26	0.1	116
NS9 marine group	98	26	0.1	76
Blastomonas	98	26	0.1	112
Saccharicrinis	92	25	0.1	210
Desulfopila	93	24	0.1	139
PB19	87	23	0.1	200
Proteobacteria_unclassified	79	23	0.1	213
Desulfobacter	96	22	0.1	67
Haematobacter	98	22	0.1	111
Desulfocapsa	95	21	0.1	107
Desulfomonile	88	20	0.1	223

Appendix 5: Table 5A: Genera, Inkwell Blue Hole, Bottom

Table 5A: Genera present in Inkwell Blue Hole, Bottom. '% Identity': closest identity match in Silva and/or NCBI databases, 'Genus Seq. Total': total number of sequences bearing identification, 'Relative Abundance (%)': percent of analyzed sequences corresponding to genus, 'OTUs': OTUs corresponding to genus.

Genus	% Identity	Genus Seq. Total	Relative Abundance (%)	OTUs
Arcobacter	100, 100	4843	36.1	8, 243
Desulforhopalus	97	1973	14.7	13
Chlorobium	97, 96, 97	1344	10.0	27, 26, 52
Bacteria_unclassified	100, 100, 100, 100, 100, 100, 94, 100, 100, 100	674	5.0	80, 84, 130, 167, 214, 250, 199, 310, 364, 389
PRD18C08	100, 99	454	3.4	53, 173
Spirochaeta	90, 100	363	2.7	44, 204
Pseudoalteromonas	100	343	2.6	71
Desulfofustis	99	280	2.1	62
Sphingobacteriales_unclassified	100	232	1.7	43
Desulfobacter	96	217	1.6	67
Lentimicrobium	90, 92	205	1.5	145, 78
Ectothiorhodospiraceae_uncultured	93	202	1.5	4
Sulfurimonas	96, 100, 95	183	1.4	134, 182, 268
Desulfocapsa	95, 92	134	1.0	107, 226
cvE6	100	130	1.0	65
Victivallales_unclassified	52, 72	128	1.0	144, 248
Vibrio	99, 98	123	0.9	155, 174
Desulfopila	93, 97	79	0.6	139, 171
Gastranaerophilales	92	76	0.6	138
Candidatus Megaira	100	76	0.6	61
Absconditabacteriales (SR1)	100	69	0.5	119
Candidatus Komeilibacteria	100	63	0.5	128
Deltaproteobacteria_unclassified	99	60	0.4	183
SBR1031	100, 100	60	0.4	37, 309
Latescibacteria	84, 100	55	0.4	249, 186
Grimontia	98	52	0.4	201
CMW-169	100	51	0.4	176
Maribellus	96	50	0.4	63
Desulfotignum	97	47	0.3	228
Chlorobaculum	96	45	0.3	172
Anaerolinea_uncultured	100, 100	44	0.3	161, 272
Candidatus Peregrinibacteria	100, 100	43	0.3	211, 302
Sva0485	95	41	0.3	184
Synechococcus	98	41	0.3	3
Desulfococcus	97	39	0.3	215
Simkania	94	39	0.3	207
Desulfatiglans	100	38	0.3	234
Hungateiclostridiaceae_unclassified	92	34	0.3	358
Omnitrophicaeota	100	33	0.2	162
SAR116 clade	100	32	0.2	217
Acinetobacter	100	31	0.2	168
Anaerolineaceae_unclassified	96	31	0.2	104
Desulfosarcina	100	30	0.2	187
Sva0081 sediment group	97	28	0.2	254
Desulfobacteraceae_unclassified	100	27	0.2	266
VC2.1 Bac22	93	26	0.2	74
Neisseria	95	25	0.2	278
Thiohalobacter	94	24	0.2	261
Algidimarina	96	24	0.2	281
MSBL3	100	24	0.2	355
Saprospiraceae_uncultured	100	24	0.2	15
Proteobacteria_unclassified	63	23	0.2	229
Owenweeksia	93	21	0.2	411
Allochromatium	100	21	0.2	21
Candidatus Uhrbacteria	100	20	0.2	263
Psychrobacter	100	20	0.2	382
Cyanobium	95	20	0.1	6
MidBa8	100	20	0.1	381

Appendix 6: Table 6A: Genera Associated with N and S Transformations

Table 6A: Genera present in each sample with member species capable of various Sulfur and Nitrogen transformations.

	$HS^- \rightarrow S^0$	$S^0 \rightarrow SO_4^{2-}$	$SO_3^{2-} \rightarrow SO_4^{2-}$	$S_2O_3^{2-}$ Oxidation	$HS^- \rightarrow SO_4^{2-}$	Unknown	Disproportionation/Other	$S^0 \rightarrow HS^-$	$SO_3^{2-} \rightarrow HS^-$	$SO_4^{2-} \rightarrow SO_3^{2-} \rightarrow HS^-$ (DSR)	$NO_3^- \rightarrow NO_2^-$	$NO_2^- \rightarrow N_2$	N_2 Fixation	DNRA	$NH_3 \rightarrow NO_2^-$	$NO_2^- \rightarrow NO_3^-$	$NH_3 \rightarrow NO_3^-$
Watling's	0.8 C. Thioglobus (Otu98), Ectosymbiont of Cladonema (Otu212), Thiosocius (Otu265)	0.6 Thioprofundum (Otu105), Thiohalocapsa (Otu141), Thiosocius (Otu265)	4.1 HIMB11 (Otu20)	4.5 Thioprofundum (Otu105), Thiosocius (Otu265), HIMB11 (Otu20)	3.6 NZ3 gill symbiont (Otu22)						Cellulophaga (Otu11), Phaeocystidibacter (Otu24), 13.5 NS4 marine group (Otu36), Sediminicola (Otu46), Fulvivirga (Otu231, 203, 179, 235), Cetobacterium (Otu140)	3.2 NS4 marine group (Otu36), Fulvivirga (Otu231, 203, 179, 235)	3.7 NZ3 gill symbiont (Otu22), Thiosocius (Otu265)	3.6 NZ3 gill symbiont (Otu22)			
Church	19.8 Ectothiorhodospiraceae (Otu4), Arcobacter (Otu225)		0.5 HIMB11 (Otu20)	0.5 HIMB11 (Otu20)						0.1 Desulfobacteraceae (Otu192)	Cellulophaga (Otu11), Phaeocystiidibacter (Otu24), 4.5 Sediminicola (Otu46), Phaeodactylibacter (Otu154, 191), Arcobacter (Otu225), Cetobacterium (Otu140), Parahaliea (Otu185), Fulvivirga (Otu197)	0.9 Phaeodactylibacter (Otu154, 191), Arcobacter (Otu225), Fulvivirga (Otu197)	0.4 Arcobacter (Otu225), Leptolyngbya (Otu178)				
Inkwell Surface	6.0 Ectothiorhodospiraceae (Otu4), Paracoccus (Otu31)	2.2 Paracoccus (Otu31), Thioprofundum (Otu105)		0.1 Thioprofundum (Otu105)		0.2 SAR324 (Otu190)					Citreimonas (Otu34), 9.0 Ideonella (Otu41), Phaeocystidibacter (Otu56, 69, 91), Luteolitbacter (Otu47), Donghicola (Otu96), Roseicyclus (Otu79), Phaeodactylibacter (Otu124), Ruficoccus (Otu152), Paracoccus (Otu31)	2.5 Paracoccus (Otu31), SAR324 (Otu190), Phaeodactylibacter (Otu124)	0.1 Ruficoccus (Otu152)		0.8 Pirellulaceae (Otu57)	0.8 Pirellulaceae (Otu57)	
Inkwell Halocline	7.2 Thioalkylvibrio (Otu123), Oscillochloris (Otu125), Ectothiorhodospiraceae (Otu4), Allochromatium (Otu21), Chlorobium (Otu27, 26, 52), C. Chloroploca (Otu87, 90)	0.9 Thioalkylvibrio (Otu123), Allochromatium (Otu21)	0.5 Allochromatium (Otu21)				0.5 Desulfocapsa (Otu107), Thioalkylvibrio (Otu123)			0.2 Desulfobacter (Otu67), Desulfopila (Otu139)	Ideonella (Otu41), Niveispirillum (Otu77), Luteolibacter (Otu47), Phaeocystidibacter (Otu69, 56, 91), 7.3 Citreimonas (Otu34), Saccharomonospora (Otu31), Illumatobacter (Otu29), Roseicyclus (Otu79), Thioalkylvibrio (Otu123), NS3a marine group (Otu68), Phaeodactylibacter (Otu124), Donghicola (Otu96), Sinirhodobacter (Otu177), Ruficoccus (Otu152)	1.3 Niveispirillum (Otu77), Phaeodactylibacter (Otu124), Sinirhodobacter (Otu177)	0.6 Oscillochloris (Otu125), Ruficoccus (Otu152), Desulfocapsa (Otu107)		0.4 Pirellulaceae (Otu57)	0.4 Pirellulaceae (Otu57)	0.2 C. Scalindua (Otu93)
Inkwell Bottom	49.3 Arcobacter (Otu8, 243), Chlorobium (Otu27, 26, 52), Chlorobaculum (Otu172), Ectothiorhodospiraceae (Otu4), Sulfurimonas (Otu134, 182, 268)	1.9 Sulfurimonas (Otu134, 182, 268), Chlorobaculum (Otu172), Allochromatium (Otu21)		1.6 Sulfurimonas (Otu134, 182, 268), Thiohalobacter (Otu261)			1.0 Desulfocapsa (Otu107, 226)	2.1 Desulfofustis (Otu62)	17.2 Desulforhopalus (Otu13), Desulfofustis (Otu62), Desulfosarcina (Otu187), Allochromatium (Otu21)	Desulforhopalus (Otu13), 20.2 Desulfofustis (Otu62), Desulfobacter (Otu67), Desulfotignum (Otu228), Sva0485 (Otu184), Desulfococcus (Otu215), Desulfatiglans (Otu234), Desulfosarcina (Otu187), Desulfobacteraceae (Otu266), Sva0081 (Otu254)	56.3 Arcobacter (Otu8, 243), Desulforhopalus (Otu13), Pseudoalteromonas (Otu71), Sulfurimonas (Otu134, 182, 268), Vibrio (Otu155, 174), Grimontia (Otu201), Neisseria (Otu278)	52.6 Arcobacter (Otu8, 243), Desulforhopalus (Otu13), Sulfurimonas (Otu134, 182, 268), Neisseria (Otu278), Desulfosarcina (Otu187)	38.0 Arcobacter (Otu8, 243), Desulfocapsa (Otu107, 226), Gastranaerophilales (Otu138), Sva0485 (Otu184)				