

Microbial hotspots in anchialine blue holes: initial discoveries from the Bahamas

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Abstract Inland blue holes of the Bahamas are anchialine ecosystems with distinct fresh and salt water layers, and anoxic or microoxic conditions at depth. Scientific cave diving and geomicrobiology exploration of blue holes are providing a first glimpse of the geochemistry and microbial life in these vertically stratified karst features. We hypothesized that two geographically adjacent, sunlit blue holes on Abaco Island would have comparable biogeochemistry and microbial life. Water samples were analyzed using in situ multiparameter dataloggers and field and laboratory tests, and diver-retrieved microbial samples were analyzed using nucleic acid analysis. Microbial 16S rRNA genes were dominated by members of the anoxygenic phototroph clade Chlorobi, with smaller numbers of Deltaproteobacteria, in both blue holes.

However, spatial distributions of microbial biomass and species present within these major clades were significantly different. We also found that differences in the intensity of solar insolation, terrestrial and marine inputs, water residence time, depth to the halo/chemocline, and cave passage geometry strongly influence geochemical changes with depth. The biogeochemical diversity of inland blue holes in the Bahamas make them valuable as natural laboratories, repositories of microbial diversity, and analogs for stratified and sulfidic oceans present early in Earth's history.

Keywords Blue holes · Anchialine caves · 16S rRNA clone libraries · Microbial ecology · Bahamas

Introduction

Anchialine caves characteristically contain vertically stratified fresh and salt water under tidal influence (Humphreys, 1999; Pohlman et al., 2000; Seymour et al., 2007). Vertical stratification results from salinity gradients and the absence of strong currents and wind- or wave-induced mixing (Iliffe, 2000). Variability in geological, hydrological, and chemical characteristics such as passage configuration, tidal cycles, and salinity stratification has yielded a great diversity of anchialine cave systems globally. These systems are isolated natural laboratories that have the potential to reveal fundamental relationships between biota and

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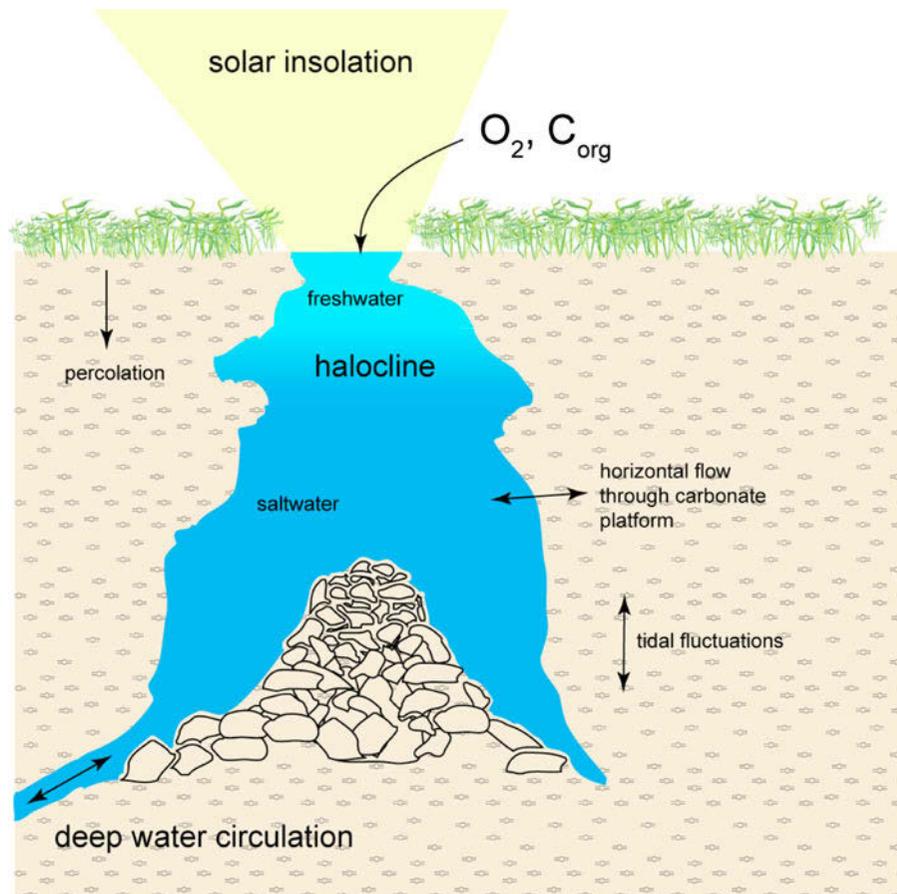
biogeochemistry, both in the modern day and in their role as analogs for stratified ocean conditions prevalent earlier in Earth's history.

Inland blue holes and associated networks of submerged passageways (Fig. 1) are prominent karst features found throughout the Bahamas (Whitaker & Smart, 1997). These water-filled sinkholes, which formed by dissolution of limestone during periods of low sea level, contain tidally influenced layers of fresh or brackish and marine waters separated by a halocline. Cave development may continue under flooded conditions in the halocline, where mixing corrosion causes limestone dissolution to occur (Back et al., 1986). Given the subsurface connectivity of blue holes with the sea, one might expect to find water chemistry dominated by that of the surrounding ocean. However, water chemistry profiles collected from diver-carried water quality analyzers in blue holes throughout Andros and Abaco Islands bear little resemblance to ocean conditions.

Blue holes differ from other marine habitats in that they have little or no photosynthetic oxygen production and restricted vertical mixing. These two factors contribute to anoxic or microoxic conditions and biogeochemical cycling that differs strongly from that taking place in marine environments that may be less than 1 km away. Blue holes may contain multiple pycnoclines, each associated with complex physico-chemical profiles (Stock et al., 1986; Bishop et al., 2004; Seymour et al., 2007). Rock permeability and distance from the coastline influence mixing and have a direct relationship to the overall thickness and depth of the halocline/pycnocline (Ilfie, 2000).

Unlike most cave ecosystems, which are energy-limited and reliant on small inputs of surface-derived organic matter (Dickson, 1975), food webs in anchialine caves may be based partially or wholly on indigenous bacterial primary production (Pohlman et al., 1997; Koch, 2001). Isotopic data obtained by Pohlman et al. (1997) from anchialine Yucatan cenotes

Fig. 1 Characteristic sinkhole-type Bahamian inland blue hole



show that lithoautotrophic bacteria may provide enough organic matter to support two to two and a half trophic levels. Autochthonous food webs are also present in caves such as Movile in Romania (Sarbu et al., 1996) and the Frasassi cave system in Italy (Macalady et al., 2008), both of which have ecosystems based on sulfide-oxidizing and other lithoautotrophic bacteria. In these examples, bacterial activity is concentrated at redox gradients where cave air or oxygenated sea water meet reduced geothermal or other deep water inputs.

Bahamian blue holes are not generally known to be associated with geothermal (Epp et al., 1970) or deep water sources that could supply reduced chemicals for microbial primary production. Therefore, redox stratification providing energy for microbial carbon fixation must arise by other means. Hydrogen sulfide is produced in blue holes when organic matter is supplied to the halocline faster than oxygen. Under these conditions, sulfate-reducing bacteria degrade organic matter using marine sulfate, with sulfide as a waste product. Inputs of organic carbon may derive from blue hole entrances, which collect surface runoff and/or provide sunlit habitats for microbial phototrophs, algae, and plants. Organic carbon inputs from overlying soils may also be significant (Pohlman et al., 2000). For example, research done in Yucatán cenotes (Pohlman et al., 1997) showed that dissolved and particulate organic carbon from overlying soils and detritus percolates in with rainwater, contributing to the overall carbon budget.

Methods

Here, we present a first look at microbial diversity and its relationship to biogeochemical processes within the inland blue holes of Andros and Abaco Islands. Geochemical data were analyzed with depth on Abaco and Andros Islands using diver-carried multiparameter data loggers. Sulfide concentration profiles were obtained from diver-collected water samples analyzed at the surface using a portable spectrophotometer and sulfide tests (methylene blue method) carried out according to the manufacturer's instructions (Hach Co., Loveland, CO, USA). Planktonic cells were collected using a custom-made pump and in-line filtration system loaded with ashed, 0.4 µm pore size glass filters (Whatman A/E) appropriate for both

nucleic acid and pigment analyses. Both filters and microbial biofilms were preserved immediately after collection in 4:1 (v:v) RNAlater (Ambion, Inc.) and stored on ice for transport to the laboratory.

Environmental DNA was extracted from both filter-collected microbial cells and cave wall microbial mats using a previously described chloroform–phenol extraction protocol (Macalady et al., 2008). The Cherokee Road Extension Blue Hole (Abaco Island) sample was collected at a depth of 33.2 m, which is the depth where oxygen and sulfide gradients overlap (Fig. 2). The Sawmill Sink (Abaco Island) filter samples were collected at 10 m depth, which is the depth of maximum turbidity in the microbial plate. Environmental DNA was used as a template for 16S rRNA PCR amplification, cloning, sequencing, and analysis as previously described (Macalady et al., 2008). Sequences from Sawmill Sink and Cherokee Road Extension Blue Hole were added to existing alignments containing >200,000 full-length bacterial 16S rRNA gene sequences in the software program ARB (Ludwig et al., 2004) in order to determine their taxonomic identity.

Results

The three geochemical profiles examined provide only a glance at the true complexity of the vertical stratification within the Bahamas blue holes and anchialine systems altogether.

All three blue holes, Sawmill Sink, Cherokee Road Extension Blue Hole, and Sanctuary Blue Hole, have fresh or slightly brackish surface pools and increasing salinities with depth, transitioning to nearly marine waters below the halocline. Temperature in all three blue holes increases across the halocline. Cooling is present in the deeper marine waters except for Cherokee Road Extension Blue Hole which maintains a warming trend throughout.

Oxidative conditions prevailed in the surface waters of all three blue holes, while a reducing environment existed below the halocline in deeper waters. These reducing conditions are further exemplified by shifts to lower pH values at depth. Dissolved oxygen concentrations reached nearly anoxic levels at the halocline in all three systems with a slight rebound present in Sawmill Sink and Sanctuary Blue Hole. Sulfide concentrations reached maximums at the

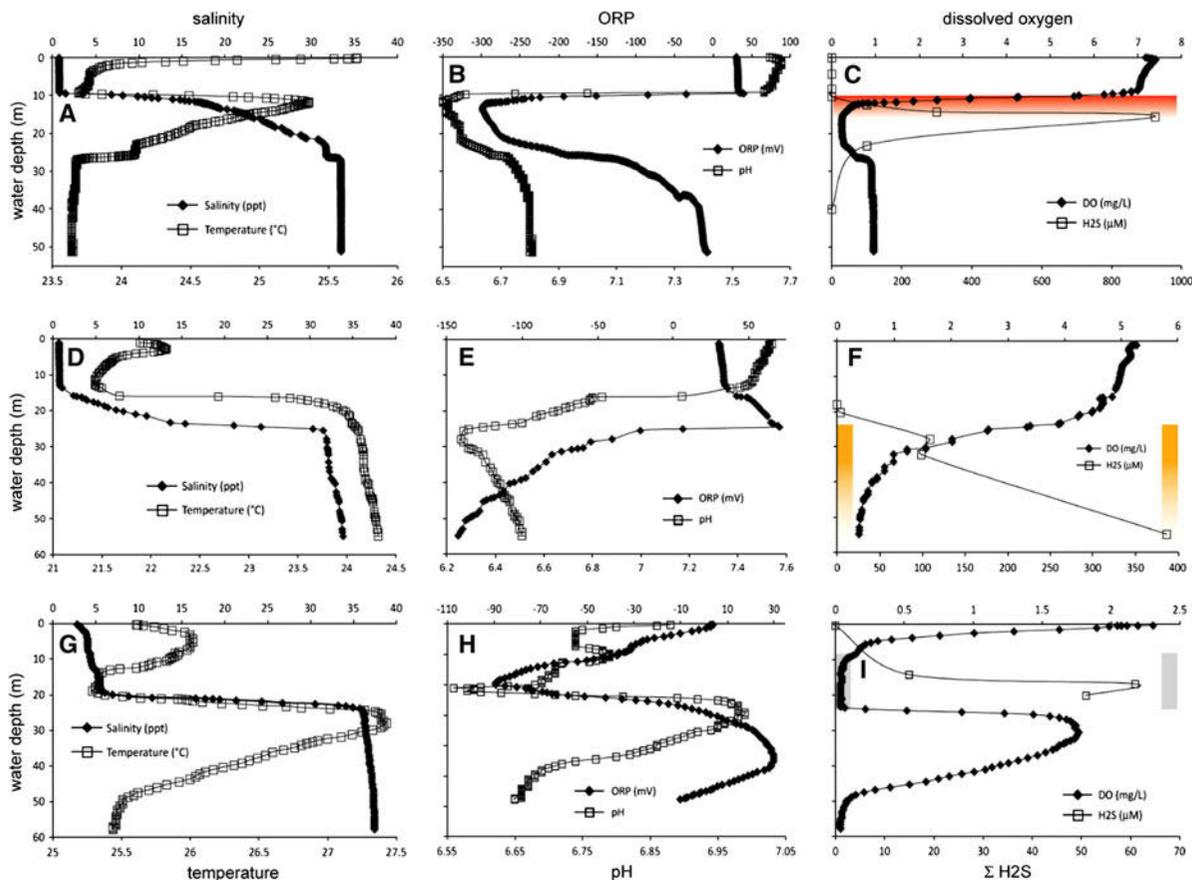


Fig. 2 Physico-chemical profiles measured using a diver-carried YSI 600XLM sonde in blue holes on Andros and Abaco Islands. **A–C** Sawmill Sink, Abaco; **D–F** Cherokee Road Extension Blue Hole, Abaco; **G–I** Sanctuary Blue Hole, Andros.

haloclines in Sawmill Sink and Sanctuary Blue Hole, but continued to increase with depth in Cherokee Road Extension Blue Hole.

Spatial distribution, color, and morphology of microbial plates varied at each blue hole, but highest densities were present at or just below the halocline. Sawmill Sink has a densely stratified purple to brown microbial plate which lies at the halocline and existing throughout the year. Cherokee Road Extension Blue Hole has orange biofilms initiating at the halocline and extending downward in lighted regions of the cave. Small vertical protrusions are scattered throughout the biofilm. Biofilms present in Sanctuary Blue Hole are constrained to the halocline and gray in color.

Extractions yielded nearly full-length 16S rRNA sequences from Sawmill Sink ($n = 93$) and Cherokee Road Extension Blue Hole ($n = 93$). Over 15 bacterial

Data were collected at 2 s intervals (except for H_2S) between the surface and maximum depth. The spatial location and color of microbial plates (**C**) and wall biofilms (**F** and **I**) with respect to the profiles are shown using *shaded rectangles*

lineages were present in each of the clone libraries from Sawmill Sink and Cherokee Road Extension Blue Hole. *Chlorobi*, *Deltaproteobacteria*, and *Gammaproteobacteria* were the most prevalent of the 16S rRNA clones from Sawmill Sink. 16S rRNA clones from Cherokee Road Extension Blue Hole were *Chlorobi*, *Deltaproteobacteria*, and *GN1* dominant.

Discussion

Representative geochemical profiles for three inland blue holes are shown (Fig. 2). The salinity profiles in the left hand column illustrate the recurring theme of freshwater overlying water with marine salinity, the principle hallmark of anchialine systems, and a feature shared among all of the inland blue holes investigated.

Water temperatures in the upper, freshwater layer may have complex relationships with depth and/or multiple modes due to temporal variations in rainwater and surface water inputs, seasonal oscillations in air temperature, and variations in the intensity of solar insolation (if present) where the profiles were collected. Temperatures measured at the maximum depths on Abaco Island appear to be cooler than the mean annual air temperature of the Bahamas (25.5°C per IABACOEL1 Weather Station). A possible explanation for this phenomenon is active circulation of deep ocean water within the Bahamas carbonate platform, a process referred to as endo-upwelling (Whitaker & Smart, 1997). Such deepwater circulation may be the driving force behind microbial populations being dominated by anoxygenic phototrophs, where nutrients from ocean environments below the photic zone are exchanging with blue hole microbial assemblages.

Redox stratification in solutional blue holes may be strong as in Sawmill Sink (Fig. 2B) or weak as in Cherokee Road Extension Blue Hole (Fig. 2E), but sharp changes in redox potential (mirrored by oxygen concentrations) with depth are consistently present in the water column of all inland blue holes examined to date (this study; Schwabe & Herbert, 2004; Macalady et al., 2010). In solutional blue holes without significant horizontal passage development, a sharp drop in redox potential is commonly observed at the top of the halocline (Fig. 2B, E). At depth, redox potential and oxygen concentrations may recover (Fig. 2B), indicating recharge of oxygenated seawater from deep below (Iliffe & Kornicker, 2009). In fracture-type blue holes (e.g., Sanctuary Blue Hole) (Myroie et al., 1995), horizontal water flow through the carbonate platform may result in more complex relationships between redox potential and depth (Fig. 2H). Since fracture systems run parallel to platform margins, they may have multiple sites for water exchange. Hydrological data from all three blue holes as reported here and from that of previous investigations (e.g., Whitaker & Smart, 1993; Kornicker et al., 2002) suggest that using redox potentials along with temperature plots, is a useful tool for mapping water flow patterns throughout the carbonate platforms of the Bahamas.

We are particularly interested in Bahamian sinkholes as analogs for Proterozoic oceans, in which anoxic water may have been present at depths shallow enough to intersect the photic zone (Anbar & Knoll,

2002). We therefore chose two sinkholes, Sawmill Sink and Cherokee Road Extension Blue Hole, with conspicuous phototrophic communities and contrasting geochemistry for further investigation. These communities were observed in situ by scientific cave divers in December 2008 and in July and December 2009. Geochemical profiles collected over this period and up to several years previously were nearly identical for each sinkhole, and are therefore well represented by the profiles shown (Fig. 2).

Sawmill Sink hosts a dense microbial plate present throughout the year at the top of the halocline (10 m depth). The color of the microbial plate ranges from purple-brown (July) to dark reddish-brown (December) (Fig. 3A). Based on the color of the July sample, we developed an initial hypothesis that summer phototrophy is dominated by members of the purple sulfur bacteria, a subgroup of *Gammaproteobacteria*. Anoxygenic phototroph members of the *Gammaproteobacteria* appear to dominate the purple-pink microbial plate present in a stratified karst lake on Andros Island (Schwabe & Herbert, 2004). However, the great majority of 16S rRNA clones in the Sawmill Sink microbial plate affiliate with *Chlorobi* (Fig. 3C, left panel), a lineage of obligate bacterial photoautotrophs unrelated to the *Gammaproteobacteria*. The dominance of *Chlorobi* in the Sawmill Sink chemocline is consistent with quantitative analyses of photosynthetic pigments extracted from filters collected at the same depth as those used for DNA extraction. The pigment analyses showed that that diagnostic *Chlorobi* chlorophyll (Bchl_c) is ten times more abundant than all other chlorophylls combined (Macalady et al., 2010). Although neither 16S rRNA clone frequencies nor pigment concentrations can be translated directly to population densities, the evidence presented here strongly suggests that *Chlorobi* are the most ecologically successful phototrophs in the Sawmill Sink chemocline.

The geochemical profile of the Cherokee Road Extension Blue Hole strongly contrasts with Sawmill Sink (Fig. 2). The primary redox boundary (redoxcline) corresponds to the bottom of the halocline rather than to the top of the halocline as in Sawmill Sink. The halocline at Cherokee Road Extension Blue Hole is deep (15–20 m) and non-sulfidic, although sulfide is present below the redoxcline at depths >25 m. These physical and chemical factors contribute to the lack of a conspicuous microbial plate in the water column at

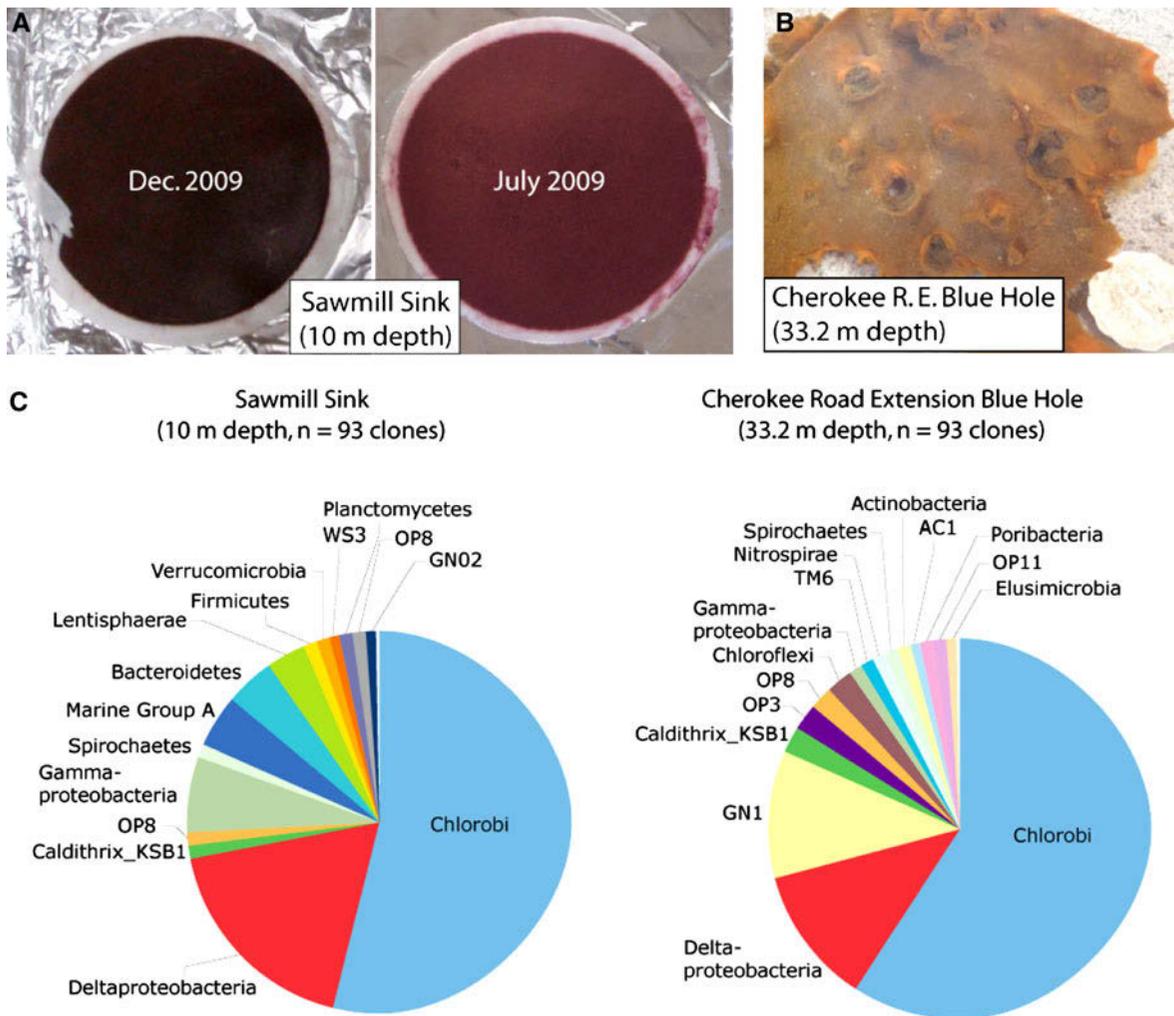


Fig. 3 Physical appearances (A, B) and population structures (C) of two inland blue hole microbial communities based on bacterial 16S rRNA cloning. Geochemical data for these

samples are shown in Fig. 2A–C (Sawmill Sink) and Fig. 2D–F (Cherokee Road Extension Blue Hole)

Cherokee Road Extension Blue Hole. Nonetheless, a highly pigmented microbial biofilm is present on cave wall surfaces oriented toward the sinkhole entrance in micro-oxic and anoxic water. The biofilm is thickest (1–2 cm thick) at the top of the redoxcline, and becomes thinner and more sparsely distributed at deeper depths. Together these observations suggest that the biofilm is based on anoxygenic photoautotrophy, a hypothesis supported by the results of 16S rRNA cloning. Members of the anoxygenic phototroph *Chlorobi* lineage are the most abundant clones in 16S rRNA libraries constructed from samples collected across the depth range where the wall biofilm occurs (Fig. 3C, right panel, and data not shown).

Chlorobi are known for their ability to outcompete other phototrophs at low light intensities, and to oxidize reduced sulfur compounds such as hydrogen sulfide during photosynthesis (Tabita & Hanson, 2004). Their prevalence in inland blue holes such as Sawmill Sink and Cherokee Road Extension Blue Hole is a consequence of the light quantity and quality available in these dimly lit caves. Sulfide fueling the growth of *Chlorobi* in these systems comes from seawater sulfate that is reduced by other microbial groups. Sulfate-reducing *Deltaproteobacteria* are abundant in 16S rRNA libraries from both Sawmill Sink and Cherokee Road Extension Blue Hole (Fig. 3C), suggesting that significant sulfur reduction

may occur within their phototrophic microbial communities, as well as deeper in the water column and outside the photic zone of the caves.

In addition to *Chlorobi* and *Deltaproteobacteria*, Sawmill Sink clones include relatives of *Allochromatium* spp., members of a clade of Gammaproteobacterial anoxygenic phototrophs with characteristic carotenoid pigments (purple sulfur bacteria). This result is consistent with the idea that phototroph populations in the Sawmill Sink microbial plate may oscillate seasonally (Fig. 3A) in response to light and/or water column geochemistry. A few clones representing bacterial lineages typically carrying out the anaerobic degradation of organic matter were retrieved in both clone libraries (*Spirochaetes*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Planctomycetes*). However, the majority of remaining clones in both libraries affiliate with unknown or uncultivated lineages, with the result that no inferences about their metabolism or physiology can be made based on taxonomy. Clones representing members of the enigmatic GN1 clade are particularly abundant in the Cherokee Road Extension Blue Hole library. If their populations numbers prove to be significant based on future study, then this environment will provide an excellent opportunity to investigate the activities of this novel group.

Many aspects of the microbial ecology and biogeochemistry of inland blue holes remain to be investigated in future study. Based on our initial survey, Bahamian inland blue holes have widely varying biogeochemistry that partially overprints their common anchialine nature. This variation makes them valuable natural experiments that reveal constraints on the biogeochemistry of stratified ocean waters prevalent in early Earth history (Reinhard et al., 2009) and emerging in the modern day as oxygen minimum zones expand due to anthropogenic global change (Diaz & Rosenberg, 2008). Biogeochemical contrasts are apparent even in closely spaced blue holes on the same island (i.e., Fig. 2, Sawmill Sink and Cherokee Road Extension Blue Hole), and appear to be sensitive to environmental factors such as solar insolation, water flow velocities, and organic matter inputs. Relatively subtle changes in these characteristics may strongly affect water quality in Bahamian island aquifers, as well as the spatial and taxonomic distributions of indigenous microbial communities and the higher trophic levels dependent on them in blue hole food webs.

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