

# Cyanobacterial Diversity and Halotolerance in a Variable Hypersaline Environment

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**Abstract** The Great Salt Plains (GSP) in north-central Oklahoma, USA is an expansive salt flat (~65 km<sup>2</sup>) that is part of the federally protected Salt Plains National Wildlife Refuge. The GSP serves as an ideal environment to study the microbial diversity of a terrestrial, hypersaline system that experiences wide fluctuations in freshwater influx and diel temperature. Our study assessed cyanobacterial diversity at the GSP by focusing on the taxonomic and physiological diversity of GSP isolates, and the 16S rRNA phylogenetic diversity of isolates and environmental clones from three sites (north, central, and south). Taxonomic diversity of isolates was limited to a few genera (mostly *Phormidium* and *Geitlerinema*), but physiological diversity based on halotolerance ranges was strikingly more diverse,

even between strains of the same phylotype. The phylogenetic tree revealed diversity that spanned a number of cyanobacterial lineages, although diversity at each site was dominated by only a few phylotypes. Unlike other hypersaline systems, a number of environmental clones from the GSP were members of the heterocystous lineage. Although a number of cyanobacterial isolates were close matches with prevalent environmental clones, it is not certain if these clones reflect the same halotolerance ranges of their matching isolates. This caveat is based on the notable disparities we found between strains of the same phylotype and their inherent halotolerance. Our findings support the hypothesis that variable or poikilotrophic environments promote diversification, and in particular, select for variation in ecotype more than phylotype.

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## Introduction

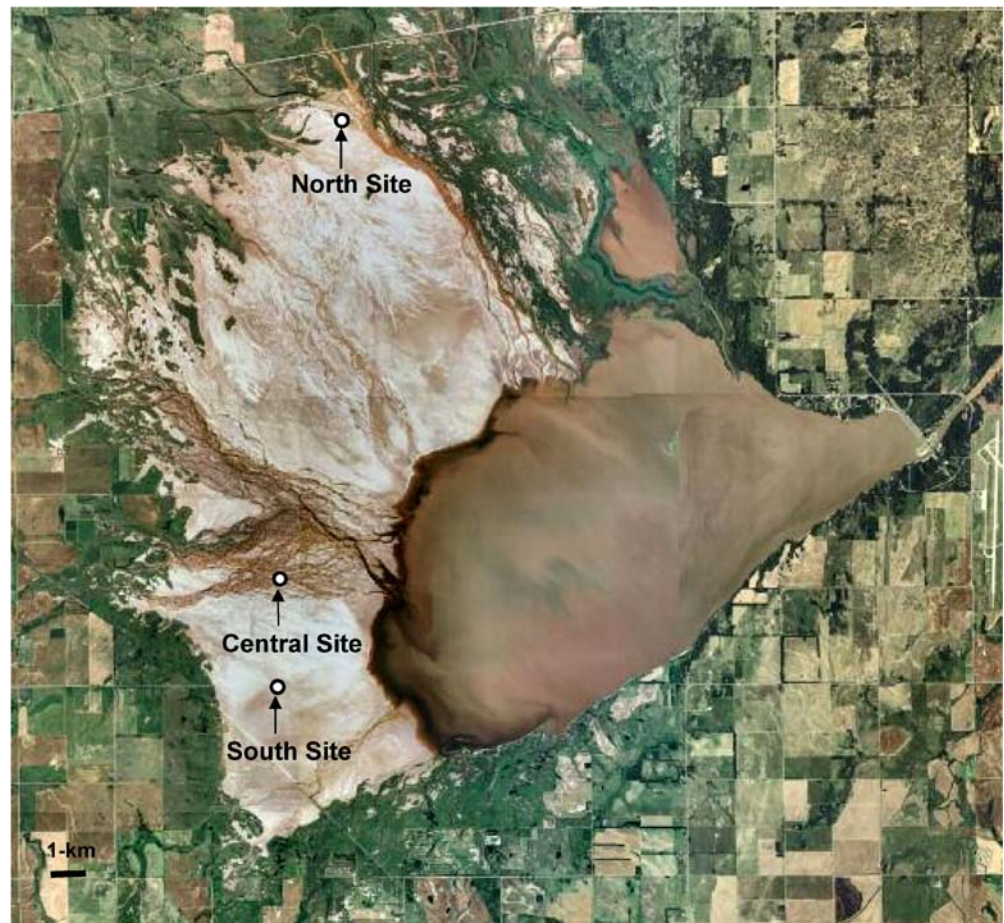
The cyanobacteria are a diverse group of microorganisms that are found in an array of habitats, which vary from aquatic to terrestrial, ultraoligotrophic to hypereutrophic. They are also found in habitats that are considered to be extreme, such as hot springs and hypersaline lakes. Much work has been done to characterize the cyanobacterial communities in aquatic hypersaline (>50 ppt total dissolved solids [TDS]) systems, particularly benthic microbial mats (some examples: [5, 8, 12, 14–17, 32]). Only a few studies have looked at cyanobacterial communities from terrestrial hypersaline systems such as thalassic salt flats [44] and evaporite crusts [40, 41], and one has documented cyanobacteria from an athalassic salt flat in the Atacama desert of northern Chile [18]. For a good review of cyanobacterial ecology in hypersaline systems, see Oren (2000) [36].

The most common cyanobacterial taxa found in hypersaline environments include *Aphanothece halophytica* and a variety of *Lyngbya*, *Microcoleus*, *Phormidium*, *Spirulina*, and Synechococcal-like species. Conspicuously absent are the heterocystous-forming taxa. The vast majority of studies characterizing cyanobacterial communities in hypersaline environments have been performed in relatively stable aquatic systems. Unlike year-round solar salterns and meromictic hypersaline lakes, terrestrial hypersaline systems are less able to buffer the effects of direct solar radiation and precipitation. As such, terrestrial salt flats that are not in arid climes can experience frequent variations in diel surface temperature and pore water salinity after rain events [27]. These dramatic shifts in soil temperature and osmotic/ionic potential create a highly variable or “poikilothermic” [22] environment. Such an environment is considered to be truly extreme because resident organisms must contend not only with the high salt and desiccating conditions of a terrestrial hypersaline habitat, but also the rapid changes in salinity, water availability, and temperature that can occur in a matter of seconds when rain and/or flooding occurs.

Terrestrial saline environments are found extensively throughout North America, particularly in the Great Plains region. However, there are no documented studies of cyanobacterial diversity in these salty habitats. The Great Salt Plains (GSP) in north-central Oklahoma is one of the largest salt flats (~65 km<sup>2</sup>) in the Great Plains region, and is part of the Salt Plains National Wildlife Refuge. The GSP is rarely completely dry at the soil surface because of the upward wicking of near-surface groundwater brine, but can experience rapid shifts in salinity, surface temperature, and water availability when rain and flooding events occur [27, 38]. The surficial features of the GSP vary from large tracts of flat, salt encrusted topography to undulating, channelized features that have heterogeneous salt patches. In certain areas, supersaturated brine pools develop and then disappear after a scouring flood event. On the northwestern fringe of the GSP, freshwater inputs from nearby creek overflows intermittently sustain dozens of ephemeral pools that have conspicuous benthic microbial mats.

The GSP is part of a network of microbial observatories funded by the National Science Foundation, and has been designated the Salt Plains Microbial Observatory

**Figure 1** Aerial image of the Great Salt Plains showing the north, central, and south study sites. Source: 2003 NAIP aerial image database



(SPMO). As part of a larger collaborative effort to characterize the microbial community found at the GSP, we focused this study on the cyanobacterial communities from a variety of habitat types at the GSP. To assess cyanobacterial diversity at the GSP, we examined both the phylogenetic diversity of isolates and environmental clones, as well as the halotolerance ranges of isolated strains. By comparing our phylogenetic and physiological diversity results with the environmental conditions at the GSP, we can improve our understanding of cyanobacterial growth, adaptation, and distribution in terrestrial hypersaline systems.

## Materials and Methods

### Study Site

The GSP is located in Alfalfa County, north-central Oklahoma and is surrounded by mixed agriculture and a reservoir along most of its eastern boundary (Fig. 1). The GSP consists of loose Quaternary deposits that are saturated with Permian brine that varies spatially, but is typically greater than 100 ppt TDS [26]. The southern end of the GSP commonly has brine salinities exceeding 240 ppt TDS [49]. During dry periods, the brine becomes supersaturated at the surface and forms an evaporitic salt crust. The thickness of the salt crust varies, but is usually less than a centimeter thick. Brine salts are mainly composed of sodium chloride (>99%) [26, 49].

The south (N36°42'26" W98°15'36") and central (N36°43'51" W98°15'33") sampling sites are approximately 3.25 km apart and the north site (N36°47'79" W98°14'78") is approximately 10 km from the central site (Fig. 1). The sites were selected based on notable differences in surface feature, proximity to a vegetation boundary, and the frequency of freshwater influx. The south site is flat and featureless, lacking any surficial channels or ephemeral creek beds. In contrast, the central site has an undulating, dynamic surface that becomes channelized during heavy rain events. The north site resembles a coastal mudflat, where intermittent creek overflows sustain dozens of ephemeral pools. The south site is approximately 1 km from the nearest vegetation boundary, whereas the central and north sites are less than 100 m from a vegetation line (Fig. 1). Compared to the south site, the central and north sites experience increased inputs of freshwater during rain events. This is because of the combined effects of nearby creek overflow and direct inflow along surface channels. Other than differences in freshwater input, the sites share a similar sandy-loam soil composition.

### Soil and Pool Sampling

Soil and water samples for physical and pigment analyses were collected in parallel at all three sites over approximately monthly intervals from the end of April to mid-September in 2003. Using the bottom of a sterile polystyrene petri dish (8.5-cm diameter, 1-cm deep), 10 random soil or pool-sediment cores within a 2-m<sup>2</sup> quadrat were taken and mixed together in a sterile plastic bag. In total, we collected samples from three quadrats at each site, on each date. Soil and sediment from each bag was then aliquoted into sterile 50-mL screw-cap polyethylene tubes and placed on dry ice in the field. These tubes were taken to the laboratory within hours of sampling and stored at -80°C until further analyses. We also collected 90 soil and pool-sediment samples for algal isolations in sterile 2-oz Whirlpak™ bags in the summer of 2002 (July and August) and in the spring of 2003 (March and April). These samples were placed on ice in the field and stored at 4°C for no more than 24 h before algal isolation.

### Soil and Water Analyses

Soil-pore water and pool-water salinities were measured using a handheld salinity refractometer with automatic temperature compensation. Dilutions (10<sup>-1</sup>) with deionized water were performed when sample salinity values exceeded 100 ppt. When soil samples were too dry to obtain pore water directly, soil was compressed in a 5-mL syringe to produce enough pore water (20–100 µL) for salinity measurements. Soil pH was measured with an HI 9023 pH meter (Hanna Instruments, Woonsocket, RI, USA) following a standard potentiometric protocol [1].

### Pigment Analyses

Frozen soil samples were thawed and mixed in a 1:1 v/v ratio with dimethyl formamide in 20-mL glass scintillation vials for chlorophyll (Chl) extraction. Vials were tightly capped, sealed with Parafilm, and vortexed at high speed for 1 min. All vials were placed in the dark at room temperature for 48 h to ensure maximum extraction efficiency. Two milliliters of extractant from each sample vial was clarified by centrifugation before scanning absorbance at 400–750 nm using a UV160U spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). All Chl *a* values were corrected by subtracting absorbance measured at A750 and then calculated using the formulas of Jeffrey and Humphrey (1975) [25]. Phycocyanin was extracted with phosphate buffer solution (pH 6.8) under low light conditions. Frozen soil samples were thawed and mixed in a 2:1 v/v ratio of soil



to phosphate buffer for a total volume of 30 mL in 50-mL polyethylene tubes. Each sample was homogenized with a tissue homogenizer for 5 min and allowed to settle for 2–3 min. Subsamples (1.5 mL) of cleared homogenate were microcentrifuged at 11,000 $\times$ *g*. For further removal of any suspended particulates, the supernatant of each sample was clarified using Acrodisc™ syringe filters (0.22- $\mu$ m pore size) before spectrophotometric measurements (400–750 nm). Phycocyanin concentrations were calculated using the equation of Beer and Eshel (1985) [6].

### Cyanobacterial Isolation and Identification

Parallel subsamples of soil (10 g) and pool sediment (10 mL) were aseptically suspended in 75 mL of sterile liquid medium or directly plated (~1 g soil or benthic mat, 1 mL pool-water) onto 1% agar plates (Bacto™) with the same medium composed of GSP salts and f/2 medium [23]. To maximize the diversity of cyanobacteria isolated, we used three medium salinities: 10, 50, and 100 ppt TDS. Liquid and plate media with soil inocula were incubated under a cool-white light (2' 20-W bulbs and 4' 40-W bulbs) of 60  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, 14:10 L/D at 25–28°C. Once algal growth became visible (1–5 days), we repeated streak-plating to obtain unialgal cultures. Filamentous cyanobacteria were isolated using a phototactic purification method [42]. Using a Nikon E400 phase-contrast microscope, all cyanobacteria were identified from live material to genus and when possible, species, under oil-immersion at 1,000 $\times$  magnification. For the purposes of consistency, cyanobacterial strains were identified using the taxonomic keys of Komarek and Anagnostidis [3] and Anagnostidis and Komarek [28]. Cyanobacterial strains deposited in the UTEX and/or CCMP culture collections are listed in Table 2.

### Halotolerance Assays

Each cyanobacterial isolate was inoculated into 125-mL flasks containing 20 mL of AS-100 medium [43] at six salinities (0, 10, 50, 100, 150, and 175 ppt NaCl). Triplicate cultures at each salinity were grown under a cool white light (2' 20-W bulbs and 4' 40-W bulbs) of 60  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, 14:10 L/D at 25°C for 5 days. Aliquots of 10  $\mu$ L from each acclimated culture were pipetted onto 1 mL of 1% agar (AS100 medium) at the corresponding salinity, in triplicate wells of 24-well plates. Each plate was sealed with cellophane tape to minimize desiccation and incubated at 60  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, 14:10 L/D at 25°C. Chl biomass was measured daily using a custom-built fiber optic fluorometer [23] modified with a volt meter for readout. During readings, 24-well plates were placed in a

customized black box. A hole below each well accommodated the fluorometer's fiber optic cable, allowing measurement of Chl *a* fluorescence in darkness (except for the blue excitation light) through the agar. Cyanobacterial growth rates ( $d^{-1}$ ) at each salinity were calculated as the regression slopes of ln-transformed exponential growth curves.

### Environmental Clone Libraries

Genomic DNA was extracted from thawed soil (south and central sites) and pool sediment (north site) samples of the April 26, 2003 sampling date, using the protocol of the MO BIO Ultra Clean Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Once pure genomic DNA was obtained, paired cyanobacterial-specific (although not exclusive) primers CYA359F, CYA781R, and CYA106F [33] were used to target partial (~700 bp) 16S rDNA for symmetric amplifications. Amplifications were conducted as described previously [8–11]. Amplified rDNA products were then purified using 1.2% transcatheter arterial embolization (TAE) agarose gel and a nebulizing purification kit (Ultrafree®-DA, Millipore Corp., Bedford, MA USA). Cloning of the amplified rDNA products was performed using TOPO TA Cloning Kits with the One Shot® electroporation protocol for TOP10 competent cells (Invitrogen Corp., Carlsbad, CA, USA). Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) were then used to prepare targeted DNA for sequencing.

### Sequence Data

One hundred and forty clones and 70 GSP isolates were included in the analyses of 16S rRNA genes. Cultures were grown, harvested, and DNA extracted as described previously [9–11]. Growth media (VM; McCracken) were supplemented (1:1) with either Seawater Alga Gro (Carolina Biological Supply) alone or in combination with supplemental NaCl (5% final concentration). Paired cyanobacterial-specific (although not exclusive) primers [33] were used to target partial 16S rRNA genes (~700 bp) for symmetric amplifications. Amplifications were conducted as described above. Sequencing reactions that generated overlapping data from both strands of a target were conducted as described previously [9, 11] for both environmental clones and isolates. Sequences were determined by an ABI 373 (PerkinElmer, Foster City, CA, USA) automated sequencer using the PRISM reagents according to the manufacturer's protocols. Sequencher 4.1.1 (GeneCodes, Inc. Ann Arbor, MI, USA) was used to edit all nucleotide data.

## Alignment and Phylogenetic Analysis

All alignments were assembled manually using MacClade v. 4.08 [29]. Both culture-based and cloned sequences were included in the 16S rRNA alignments. Contextual sequences were selected by using BLAST [2] to identify named sequences from the NCBI database that showed relatively high similarity ( $e < 0.1$ ) to the experimental data. The global alignment contained 296 sequences. Of the 296 sequences in the global alignment, 91 experimental sequences were excluded because of identity or near identity (>99%) with at least one other experimental sequence. From a total of 748 sites, 81 sites were excluded from phylogenetic analysis because (1) assumptions of homology could not be justified because of base substitution coupled with length heterogeneity and/or (2) corresponding sequences on the 5' end or on the 3' end were missing for some of the taxa. Modeltest v 3.5 [37] and PAUP\* v. 4.0b10 [45] were used in tandem to identify the model that best fit the data for use in molecular phylogenetic analysis (Base=[0.2497 0.2025 0.3231]; Nst=6 Rmat=[0.8791 2.2998 1.4526 0.4358 3.9838]; Rates=-gamma; Shape=0.5543; Pinvar=0.3557). A maximum likelihood (ML) tree was inferred from the truncated alignment after analysis in PAUP\* 4.0b10 [45]. The initial tree for the ML analysis was obtained by the neighbor-joining method. Tree space during the ML analysis was searched heuristically using the TBR algorithm in PAUP\*. Bootstrap support for the nodes in the optimal tree was determined using 100 replicates of analyses in which all initial trees were obtained by the neighbor-joining method. Heuristic searches of tree space for each bootstrap replicate were conducted using the NNI algorithm in PAUP\*.

## Results

All chemical and biological variables measured at each site were found to be significantly different in site-to-site comparisons (Table 1). Average salinity was notably highest at the south site, and lowest at the central site. The north site had a higher mean salinity than the central site, but it also had the largest standard error value, which indicates a higher degree of salinity variation over the sampling season. The north site also had a higher degree of variation in pH, and tended to be less alkaline than the other two sites. Chl *a*, which represents the biomass of the total algal community, was an order of magnitude higher at the north site compared to the south and central sites. Mean phycocyanin, which is a biomarker for the presence of cyanobacteria, was two orders of magnitude higher at the north site compared to the other two sites.

The large majority of cyanobacterial isolates were nonheterocystous, filamentous forms mainly represented by *Phormidium* and *Geitlerinema* species (Table 2). All Chroococcales (*Aphanocapsa* sp. and *Aphanothece* sp.) taxa and a Nostoclean strain (*Chlorogleopsis* sp.) were colonial forms. Only one filamentous, heterocystous strain (*Nodularia* sp.) was successfully isolated. Most isolates were found to be halotolerant up to 5% NaCl, but a few could grow in media with up to 15% NaCl. Far fewer strains were considered to be halophilic (i.e., requiring at least 1% NaCl), and most of these strains had a relatively narrow range of salinities that they could grow in. The exception was *Geitlerinema* sp. 107-2, which could grow in 1–15% NaCl. There were only four halointolerant strains, one of which was the heterocyst-forming *Chlorogleopsis* sp. No correlation between taxonomic ID or halotolerance category with sample type was detected (Table 2).

Pairwise comparisons demonstrated that a number of GSP isolates were identical with respect to their partial 16S rRNA sequences. The growth rates of these corresponding phylotypes at various salinity concentrations are presented in Fig. 2. Only one phylotype (*Geitlerinema* Phylotype 2) had a few strains with similar growth rates at each salinity tested. All of the other strains within each phylotype, including those within the *Geitlerinema* Phylotype 2 group, had notably different growth rates and halotolerance ranges (Fig. 2). Four out of the six phylotype groups had both halotolerant and halophilic strains.

Cyanobacterial isolates and environmental clones from the GSP exhibit a broad spectrum of phylogenetic diversity based on partial 16S rRNA analyses (Fig. 3a–c). The highest diversity of clones was found at the north site. The plurality of isolates and environmental clones clustered with other *Geitlerinema* (Fig. 3a) and *Phormidium* (Fig. 3b) sequences from GenBank. A cluster of related GSP isolates and clones in the *Geitlerinema* and Chroococcalean (i.e., *Cyanothece*, *Euhalothece*, and *Aphanothece*) lineages (Fig. 3a) form relatively deep branches that exclude non-GSP taxa. There were also deeply branched clusters of unique GSP strains and clones that are distantly (<80% sequence similarity) related to *Planktothrix* and *Prochlorothrix* species (Fig. 3c), although these GSP strains were taxonomically identified as *Phormidium* or *Pseudanabaena* species. There were notable examples of site-specific clone distribution, including the restriction of the heterocystous-lineage clones to the north site (Fig. 3b), and *Komvophoron*-related strains and clones (Fig. 3a) restricted to the south site. The *Komvophoron* alliance of clones that includes the WP62 isolate is a distinctive clade that has no close allies among the contextual sequences, save for a very distantly related *Halospirulina* (<80% sequence similarity).

**Table 1** Overall means and (standard errors) of environmental variables from the south, central, and north study sites at the GSP

Variable	South	Central	North	<i>p</i>
Salinity (ppt)	310 (5)	159 (31)†	182 (45)†	0.002
pH	7.97 (0.08)*	8.75 (0.19)	7.50 (0.25)*	<0.001
Chl <i>a</i> (µg cm <sup>-3</sup> )	0.56 (0.09)	0.49 (0.10)	4.84 (0.98)*†	<0.001
Phycocyanin (µg cm <sup>-3</sup> )	0.02 (0.01)	0.01 (0.006)	2.23 (1.33)*†	0.020

Means were based on soil and/or brine pool samples ( $n=12$ ) taken at approximate monthly intervals from late April to mid-September, 2003. A repeated-measures ANOVA was performed on each dataset to detect statistically significant differences between sites ( $\alpha=0.05$ ). Tukey post hoc tests between treatment comparisons are distinguished as those sites that were significantly different ( $p<0.05$ ) from the central site (\*) and sites that were significantly different ( $p<0.05$ ) from the south site (†).

**Table 2** Summary of halotolerance ranges and salinity optima ( $\mu_{\max}$ ) determined for a subset of cyanobacterial taxa isolated from the GSP

CCMP No. UTEX No.	Strain ID	Taxonomic ID	Morphotype	Sample type	Halotolerance category	Salinity range and ( $\mu_{\max}$ ) % NaCl
CCMP 2524 UTEX SP23	173-4	<i>Aphanocapsa</i> sp.	Colonial, coccoid cells	Soil (dry algal mat)	Halotolerant	0–5 (0)
CCMP 2529 UTEX SP24	132-4	<i>Aphanothece</i> sp.	Colonial, ovoid cells	Pool	Halotolerant	0–10 (1–5)
CCMP 2555 UTEX SP25	173-2	<i>Aphanothece</i> sp.	Colonial, ovoid cells	Soil (dry algal mat)	Halotolerant	0–5 (0–1)
NIC UTEX SP26	101-1	<i>Cyanodictyon</i> sp.	Colonial (net-like), ovoid cells in filamentous chains	Soil	Halophilic	5–15 (15)
NIC NIC	606-1	<i>Chlorogleopsis</i> sp.	Heterocystous, amorphous mass of cells form thallus	Soil (dry algal mat)	Halointolerant	0–1 (0)
NIC NIC	107-2	<i>Geitlerinema</i> sp.	Non-heterocystous, long, thin filaments	Soil	Halophilic	1–15 (1)
NIC NIC	108-2	<i>Geitlerinema</i> sp.	Non-heterocystous, long, thin filaments	Soil	Halophilic	1–10 (1–10)
CCMP 2535 UTEX SP31	133-1	<i>Geitlerinema</i> sp.	non-heterocystous, long, thin filaments	Soil (wet algal mat)	Halotolerant	0–10 (5)
NIC NIC	175-1	<i>Geitlerinema</i> sp.	Non-heterocystous, long, thin filaments	Pool (sediment)	Halointolerant	0–1 (1)
CCMP 2528 NIC	137-3	<i>Geitlerinema amphibium</i>	Non-heterocystous, long, thin filaments	Pool (sediment)	Halotolerant	0–10 (5)
CCMP 2534 UTEX SP28	167-2	<i>Geitlerinema carotinosum</i>	Non-heterocystous, long, thin filaments	Pool (sediment)	Halotolerant	0–5 (1)
CCMP 2572 UTEX SP29	174-1	<i>Geitlerinema earlei</i>	Non-heterocystous, long, thin filaments, pointed apical cell	Pool	Halotolerant	0–5 (1)
CCMP 2551 UTEX SP30	166-2	<i>Geitlerinema exile</i>	Non-heterocystous, long, thin filaments, hooked apical cell	Pool (sediment)	Halointolerant	0–1 (1)
CCMP 2551 UTEX SP30	210-1	<i>Halomiconema</i> sp.	Non-heterocystous, long, thin filaments	Soil (wet algal mat)	Halotolerant	0–15 (1)
NIC UTEX SP33	300-7	<i>Komvophoron</i> sp.	Short filaments with barrel-shaped cells, apical cell acuminate	Soil	Halotolerant	0–5 (1)
NIC NIC	WP62	<i>Komvophoron</i> sp.	Short filaments with barrel-shaped cells, apical cell acuminate	Soil	Halotolerant	0–5 (1)
NIC UTEX SP34	115-1	<i>Leptolyngbya</i> sp.	Non-heterocystous, long filaments, facultative sheath	Soil	Halointolerant	0–1 (0–1)
CCMP 2520 UTEX SP35	165-1	<i>Lyngbya</i> sp.	Non-heterocystous, long filaments, facultative sheath	Pool (sediment)	Halotolerant	0–5 (1)

**Table 2** (continued)

CCMP No. UTEX No.	Strain ID	Taxonomic ID	Morphotype	Sample type	Halotolerance category	Salinity range and ( $\mu_{\max}$ ) % NaCl
CCMP 2569 UTEX SP39	157-1	<i>Nodularia</i> sp. (formerly <i>P. laetivirens</i> )	Heterocystous, long filaments, acuminate apical cell	Soil (dry algal mat)	Halotolerant	0–5 (1)
NIC NIC	164-1	<i>Phormidium</i> sp.	Non-heterocystous, long filaments	Pool (sediment)	Halotolerant	0–5 (0)
CCMP 2554 NIC	172-1	<i>Phormidium</i> sp.	Non-heterocystous, long filaments	Soil	Halotolerant	0–5 (0)
CCMP 2523 UTEX SP42	213-5	<i>Phormidium</i> sp.	Non-heterocystous, long filaments	Soil	Halotolerant	0–15 (1)
CCMP 2521 UTEX SP43	214-3	<i>Phormidium</i> sp.	Non-heterocystous, long filaments	Soil	Halophilic	1–5 (1)
CCMP 2532 UTEX SP32	210-4	<i>Phormidium breve</i>	Non-heterocystous, long filaments, acuminate apical cell	Soil	Halophilic	1–5 (1)
NIC UTEX SP37	204-3	<i>Phormidium janthiphorum</i>	Non-heterocystous, long filaments, acuminate apical cell	Soil (wet algal mat)	Halotolerant	0–5 (0–1)
NIC UTEX SP38	218-2	<i>Phormidium keutzingianum</i>	Non-heterocystous, long filaments	Soil	Halotolerant	0–15 (1)
CCMP 2574 UTEX SP40	177-1	<i>Phormidium okenii</i>	Non-heterocystous, long filaments, acuminate apical cell	Soil (dry algal mat)	Halotolerant	0–5 (1)
NIC UTEX SP45	112-5	<i>Pseudanabaena</i> sp.	Non-heterocystous, long filaments with barrel-shaped cells	Soil	Halotolerant	0–5 (1)
NIC UTEX SP46	132-2	<i>Pseudanabaena</i> sp.	Non-heterocystous, long filaments with barrel-shaped cells	Pool	Halotolerant	0–5 (1)
NIC UTEX SP47	150-2	<i>Pseudanabaena</i> sp.	Non-heterocystous, long filaments with barrel-shaped cells	Pool (sediment)	Halotolerant	0–5 (1)
CCMP 2567 UTEX SP48	202-5	<i>Pseudanabaena</i> sp.	Non-heterocystous, long filaments with barrel-shaped cells brine	Pool	Halotolerant	0–17.5 (1)
CCMP 2552 UTEX SP44	136-2	<i>Pseudanabaena galeata</i>	Non-heterocystous, long filaments with barrel-shaped cells	Pool (sediment)	Halophilic	1–5 (1)
CCMP 2553 UTEX SP49	159-1	<i>Spirulina</i> sp.	Non-heterocystous, long, helical filaments	Pool (sediment)	Halotolerant	0–5 (0–1)
NIC UTEX SP50	176-3	<i>Tychonema bornetii</i>	Non-heterocystous, long filaments, disc-shaped cells	Soil (dry algal mat)	Halotolerant	0–5 (1)

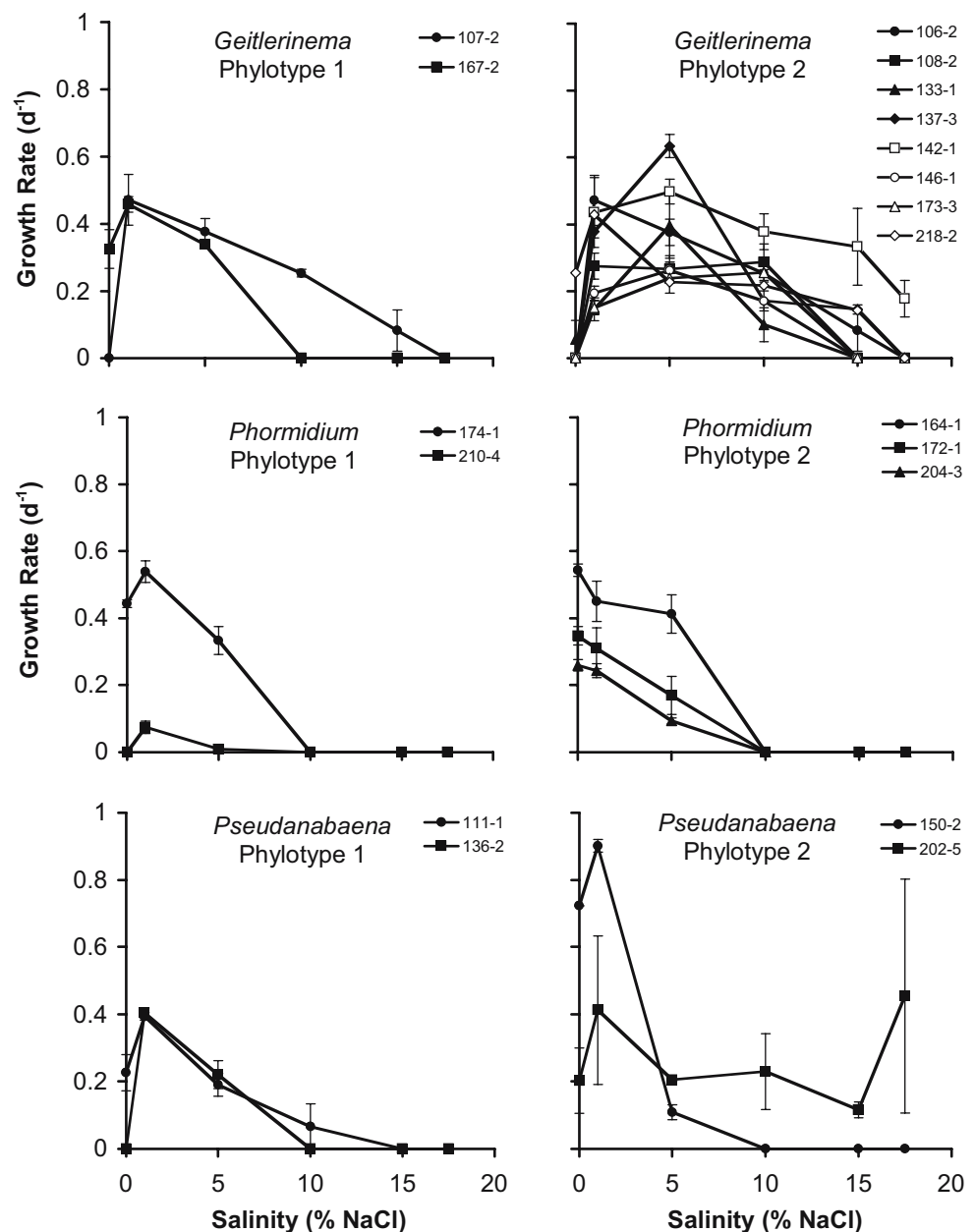
CCMP and UTEX culture collection strain numbers are provided in addition to our original strain numbers for each isolate. Halotolerance categories represent an ability to grow in a specific salinity range: halointolerant (freshwater to brackish), halotolerant (freshwater to hypersaline), halophilic (requires  $\geq 1\%$  NaCl).

NIC = not in collection

The majority of clones, however, had the same or similar 16S rRNA sequences to other GSP clones and isolates. A number of cyanobacterial isolates (mostly *Phormidium* strains) had no corresponding environmental clones, where-

as most clones had a close relative from the isolate collection. The only exceptions to this general finding were the heterocystous-lineage clones (i.e., only two heterocystous strains were isolated) and two C.Y.S clones that were

**Figure 2** Growth rates ( $d^{-1}$ ) at different salinities of individual GSP strains that belong to six different phylotypes determined from 16S rRNA analyses (see Fig 3). Note that taxonomic IDs of strains in Table 2 do not necessarily reflect generic phylotype assignments



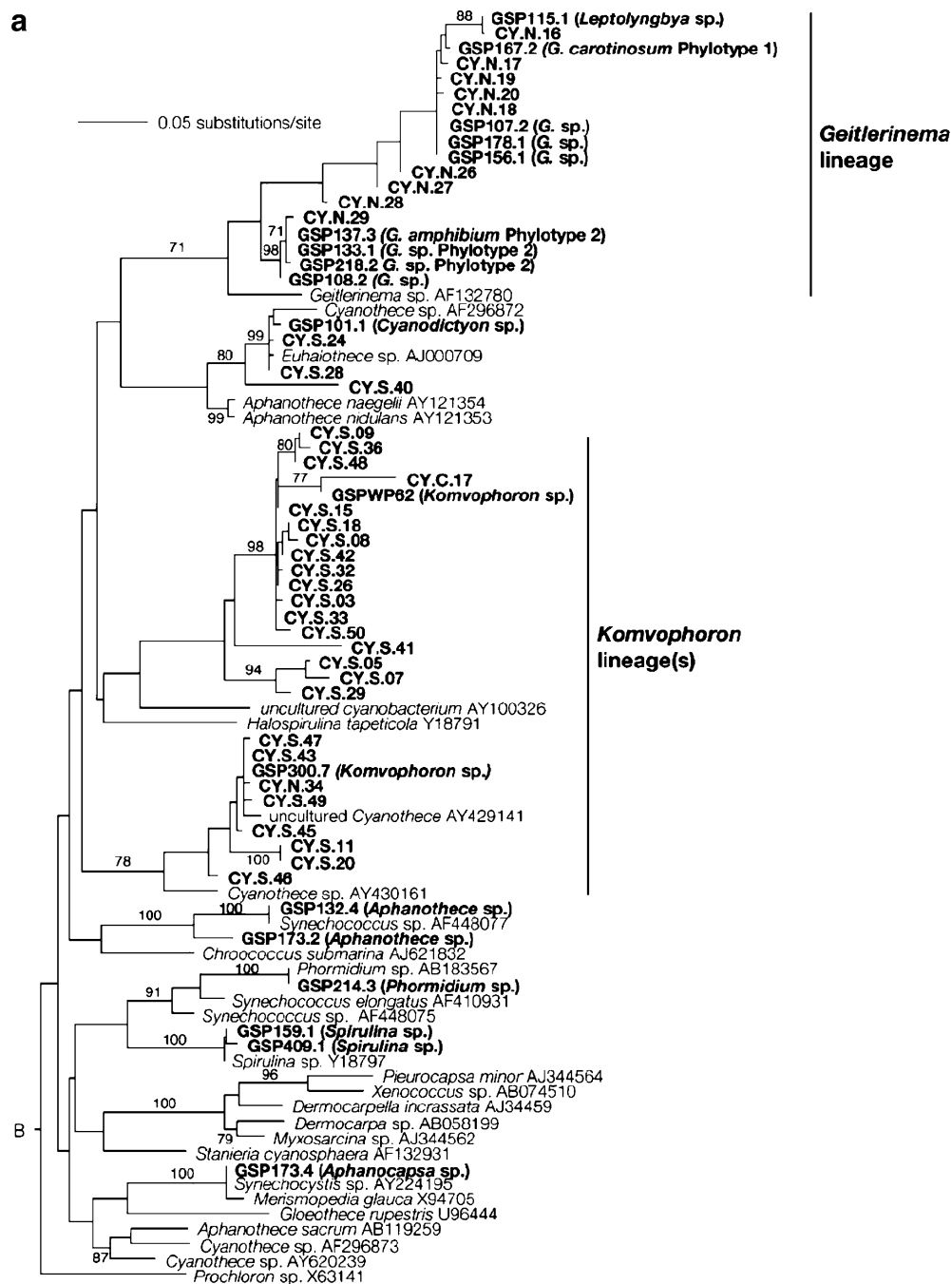
closely related to *Microcoleus chthonoplastes* (Fig. 3b), a taxon that was not isolated at the GSP. Not surprisingly, the primers used in our study of environmental clones picked up plastid 16S rRNA from a variety of diatoms, and also amplified DNA attributed to the enigmatic Verrucomicrobia. These data will be reported elsewhere.

## Discussion

Contrary to the expectation that extreme environments are low-diversity habitats, we found a remarkable array of

cyanobacterial taxa that were both phylogenetically and physiologically diverse. Whereas taxonomic diversity based on the morpho-species concept was comparatively low (mostly *Phormidium* and *Geitlerinema*), both GSP isolates and environmental clones were found to span a number of phylogenetic lineages based on 16S rRNA sequences. A notable proportion of GSP strains and clones belonging to the *Geitlerinema* and *Cyanothece* lineages represent novel taxa that have yet to be described. The GSP *Komvophoron* strains (300-7 and WP62) and related clones are one such group of unique organisms that fall into the *Cyanothece* lineage, yet morphologically are quite different from typical, unicellular *Cyanothece* spp.





**Figure 3** ML tree showing the distribution of both GSP isolates (“GSP” labels) and environmental clones (“CY” labels). Sample sites where each clone originated are denoted in clone labels (N=North, C=Central, S=South). The tree is separated into three parts (A–C), with

connections to each denoted with the appropriate corresponding letter. Branch lengths are drawn proportional to inferred change (see *scale* on each). Bootstrap values ( $\geq 70$ ) from 100 replicates are mapped to the appropriate internodes

When comparing the partial 16S rRNA sequences of the GSP isolates to sequences in GenBank, the majority (i.e.,  $>80\%$ ) of isolates and clones had less than 95% sequence similarity with other GenBank taxa. A number of close relatives ( $\geq 99\%$  sequence similarity) were morphologically dissimilar to GSP isolates, and originated from a variety of

habitats including freshwater, marine, and hypersaline [4, 13, 20, 34, 39]. Little concordance was observed between the taxonomic identity of GSP isolates and their closest relatives from published data. Only two of the GSP isolates had the same genus names as their closest relatives, and all closest relatives of the colonial GSP strains were unicellular.

b

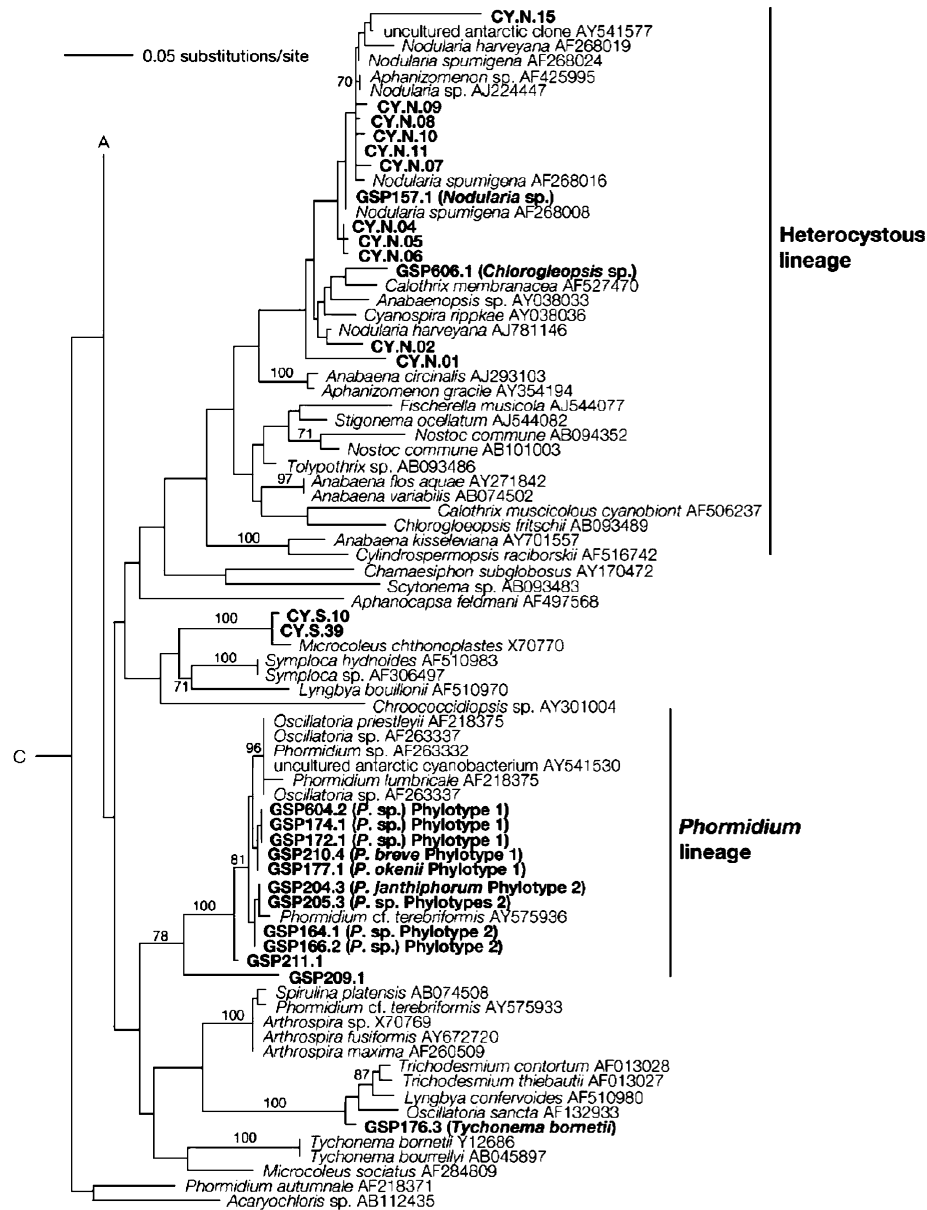
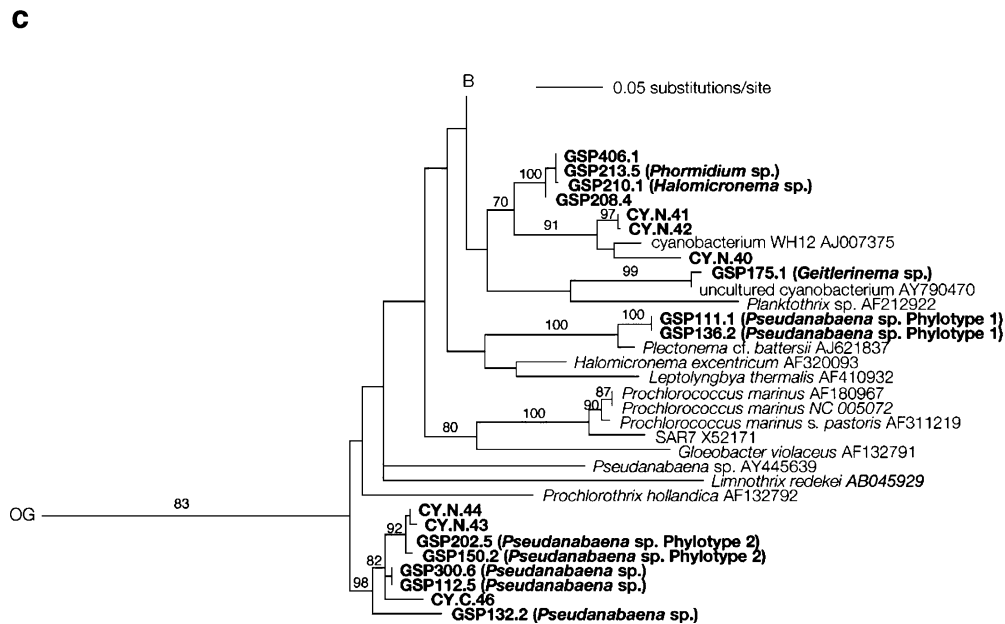


Figure 3 (Continued)

In fact, all GSP isolates were of the colonial or filamentous-mat-forming variety, although many were phylogenetically related to unicellular forms. Colonial and mat growth forms can mediate or protect against changing environmental conditions (e.g., temperature, salinity, desiccation), which is probably why they are the dominant growth forms at the GSP. We also resolved a moderately diverse and robustly supported basal clade (bootstrap value of 98%) comprising only GSP isolates and environmental clones. The presence of several unique isolates and environmental clone sequences from both the north and central sites and the group's exclusivity to the GSP

indicates that this is likely a common, possibly endemic group of cyanobacteria at the GSP. These isolates were tentatively identified as *Pseudanabaena* based on morphology, but their possible basal position suggests that further characterization is well justified.

Unlike typical bacterial clone libraries from soil, there were multiple clones of the same or highly similar 16S rRNA sequence from all sites at the GSP, but each site typically had unique sequences. If we assume that any potential polymerase chain reaction (PCR) bias is the same for all sites, these data show that each site at the GSP has a unique community of cyanobacteria, in addition to the significant differences in



**Figure 3** (Continued)

biomass noted previously. The high proportion of similar clones at each site suggests that the cyanobacterial taxa represented by these clones are the most abundant at these sites. Therefore, *Komvophoron*-related taxa are prevalent at the south site, and *Geitlerinema* and heterocystous cyanobacteria dominate the north site. Most interestingly, the majority of clones from the central site were characterized as plastid 16S rRNA from diatoms. In this instance, there may not have been enough cyanobacterial rRNA gene copies to compete for primer sites, and as such, the more abundant diatom plastid DNA was amplified. This interpretation is consistent with our current and previous [27] findings, which show cyanobacterial biomass to be very low relative to diatom biomass at the central site.

Many of the GSP clones had similar or matching 16S rRNA sequences to isolated strains. However, a number of strains (mostly *Phormidium* spp.) did not closely match any environmental clones. Therefore, it is less likely that these strains represent important members of the cyanobacterial community at the GSP, at least during the period we sampled or at the specific patches where the clone libraries were sampled. Also, all of these *Phormidium* strains preferred either fresh or brackish water, which indicates that these taxa were not actively growing at any of the sites at the GSP (i.e., average salinities at all sites  $\gg 10$  ppt TDS). Conversely, *Geitlerinema* strains that had several matching clones tended to be halophilic and could grow in excess of 10% NaCl (100 ppt TDS). Although we found incongruity between phylotype and halotolerance, there remains an obvious absence of phylotypes associated with “freshwater-

preferring” strains from the clone library. A particularly interesting finding is that the two *Komvophoron* strains clearly show a preference for brackish water and an inability to grow above 5% NaCl (50 ppt TDS), yet the vast majority of clones at the consistently hypersaline south-site were close allies of the *Komvophoron* isolates. It is possible that these environmental clones instead represent halophilic phylotypes (as witnessed in Fig. 2), and the high number of replicate clones amplified from the south site supports this reasoning.

The discrepancy between halotolerance and environmental habitat was not reflected by the halointolerant heterocystous isolate (*Chlorogleopsis* sp. 606-1) and heterocystous-lineage clones, which were all found exclusively at the less saline north site. Whereas the north site can be hypersaline over dry periods, it experiences the highest frequency of freshwater inputs. This is consistent with previous reports [19, 35, 40] that did not find heterocystous cyanobacteria under high-salt conditions, yet attributed the occurrence of nitrogen fixation in these systems to nonheterocystous taxa such as *A. halophytica*, *Microcoleus chthonoplastes*, and Synechococcal-like cyanobacteria. The reason for the lack of heterocystous cyanobacteria in hypersaline environments remains unclear, but may be due, in part, to the metabolic trade-offs between ionic balance and heterocyst production/nitrogen fixation, which are all energy-intensive processes [7, 47]. However, heterocystous cyanobacteria are able to persist and maintain diversity, although their environment is suboptimal for growth. Like heterocystous cyanobacteria from arid environments [48], the heterocystous cyanobac-

teria at the GSP appear to be well-adapted to the desiccating conditions that frequently occur at the north site [27]. It is this adaptation (i.e., mucilage production and other physiological traits) that may also protect them from external salinity increases, which occurs concurrently with desiccation.

Not surprisingly, discrepancies exist between what we have taxonomically identified as *Geitlerinema* and *Phormidium* compared to the generic names assigned to related taxa from GenBank. *Geitlerinema* is a subgenus of *Phormidium*, and as such, both names are commonly used synonymously. It is interesting to note that the *Geitlerinema* lineage in our phylogenetic tree appears to be monophyletic, in contrast to *Phormidium*, which appears to be polyphyletic. These results emphasize the need to unify classical taxonomy with phylogenetic systematics in the microbiological and botanical nomenclature. The disparity between phylotype and halotolerance shown in this study also stresses the importance of including whole-organism physiological assays with PCR-based approaches when characterizing microbial functional diversity (i.e., it is important to know “who” does “what”). Moreover, we have shown that there is a higher degree of physiological diversity (i.e., variation in halotolerance) than taxonomic or phylogenetic diversity within the same sample pool. Thus, rather than there being a convergence of halotolerance traits at the GSP, there appears to be a greater incidence of divergence, resulting in a variety of ecotypes.

Nubel et al. [32] found that algal biodiversity increased with significant variations in environmental parameters such as salinity. It has also been shown that gut and pathogenic bacteria have higher rates of mutation, or hypermutation, when chronically exposed to changing environmental conditions [21, 30, 31]. Likewise, some GSP *Halomonas* isolates exhibited high rates of spontaneous mutation [46]. With increased rates of mutation, the probability that an organism will acquire a beneficial adaptation to their environment increases. A broad range of conditions at the GSP are dynamic, particularly diel temperature and occurrences of freshwater shock [27]. Thus, the poikilotrophic nature of this environment could promote the development of a diversity of ecotypes. As the average pore water salinity at the GSP is no less than 150 ppt TDS, we can infer that all of the taxa isolated, including those that match numerous environmental clones, are not always growing at the GSP. We know that these taxa remain viable under hypersaline conditions because the vast majority of isolates were collected from salt-saturated soil and pool samples (>300 ppt TDS). Therefore, the ability to survive periods of growth-inhibitory conditions, rather than evolve a wide halotolerance range, appears to be the most common survival strategy among cyanobacteria at the GSP.

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