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Preliminary studies of cyanobacteria, picoplankton, and virioplankton in the Salton Sea with special attention to phylogenetic diversity among eight strains of filamentous cyanobacteria

A. Michelle Wood[1], Scott R. Miller[1,3], W.K.W. Li[2] & Richard W. Castenholz[1]
[1]*Department of Biology, University of Oregon, Eugene, OR 97403, U.S.A.*
[2]*Biological Oceanography Section, Bedford Inst. of Oceanography, P.O. Box 1006, Dartmouth, Nova Scotia, Canada B2Y 4A2*
[3]*Present address: Department of Genetics, North Carolina State University, Raleigh, NC 27695, U.S.A.*

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Abstract

Cyanobacterial diversity in the Salton Sea, a high-salinity, eutrophic lake in Southern California, was investigated using a combination of molecular and morphological approaches. Representatives of a total of 10 described genera (*Oscillatoria*, *Spirulina*, *Arthrospira*, *Geitlerinema*, *Lyngbya*, *Leptolyngbya*, *Calothrix*, *Rivularia*, *Synechococcus*, *Synechocystis*) were identified in the samples; additionally, the morphology of two cultured strains do not conform to any genus recognized at present by the bacteriological system. Genetic analysis, based on partial 16S rRNA sequences suggested considerable cryptic genetic variability among filamentous strains of similar or identical morphology and showed members of the form-genus *Geitlerinema* to be distributed among three major phylogenetic clades of cyanobacteria. Cyanobacterial mats, previously described from the Sea were, in fact, composed of both filamentous cyanobacteria and a roughly equivalent biomass of the sulfur-oxidizing bacterium *Beggiatoa*, indicating their formation in sulfide rich regions of the lake. Flow cytometric analysis of the water samples showed three striking differences between samples from the Salton Sea and representative marine waters: (1) phycoerythrin-containing unicells, while abundant, were much less abundant in the Salton Sea than they were in typical continental shelf waters, (2) *Prochlorococcus* appears to be completely absent, and (3) small (3–5 μm) eukaryotic algae were more abundant in the Salton Sea than in typical neritic waters by one-to-two orders-of-magnitude. Based on flow cytometric analysis, heterotrophic bacteria were more than an order of magnitude more abundant in the Salton Sea than in seawater collected from continental shelf environments. Virus particles were more abundant in the Salton Sea than in typical neritic waters, but did not show increases proportionate with the increase in bacteria, picocyanobacteria, or eukaryotic algae.

Introduction

The Salton Sea is a large, eutrophic, saline lake located in the Southern California desert. The basin is more than 200 feet below sea level and the Sea was formed by an accidental and temporary diversion of the Colorado River to the Salton Sink in the early 1900s (Walker, 1961). While originally a freshwater lake, the Sea has changed dramatically in size, salinity, and biology during its 100-year history. Agricultural and municipal wastewaters now supply 88% of the inflow to the lake, the present salinity is greater than that of seawater (41–45 g l^{-1} increasing at 0.3–0.4 g l^{-1}), and summer conditions are characterized by periodic deoxygenation events during which surface sulfide levels can exceed 0.5 mg l^{-1} (Tostrud, 1997; Watts et al., 2001).

The Sea has a long history of use for sport fisheries and its fish stocks are also important to numerous migrating and breeding bird species. However, recent fish kills and avian deaths, combined with the pattern of increasing salinity, have increased concern over

the long-term future of the Sea. Salinity levels, which could reach 91 g l^{-1} by 2010 (Imperial Irrigation District, 1989), may already place stress on key elements of the food chain and could exceed the physiological tolerance of many organisms.

It is difficult to evaluate the ecological condition of the Sea because of the relative paucity of information about the biotic composition of the ecosystem. Only two studies provide information about primary producers in the Sea (Carpelan, 1961; USDI, 1970). Both of these studies were completed before widespread recognition that the 'microbial loop' community consisting of heterotrophic bacteria, protistan grazers, viruses, and ultraphytoplankton constitutes a dynamic food web that can be responsible for a large percentage of material and energy flow in aquatic ecosystems (Pomeroy, 1974; Fuhrman, 1997; Azam, 1998). The dominant primary producers in the microbial loop of the open ocean are two forms of picocyanobacteria, *Synechococous* and *Prochlorooccus*, both of which could have been introduced to the Sea multiple times as ocean water was added to the sea with fish stocks. Carpelan (1961) noted the presence of visible massed growths of 'bluegreen algae' on the bottom of shallow regions of the main body of the lake and on pilings and buoys, but his work was completed long before the role of picocyanobacteria in the microbial loop was recognized. Thus, it does not include any consideration of the possible role of picocyanobacteria in the plankton.

The need for further information regarding cyanobacterial diversity and dynamics in the Salton Sea extends beyond the importance of considering the role of cyanobacteria in the microbial loop of the Sea. As noted previously, Carpelan (1961) describes several forms of filamentous cyanobacteria in benthic mats; he also mentions that pieces of the mats are occasionally seen floating in the water column, a phenomenon that frequently occurs in the summer under current conditions (Hurlbert, pers. comm.). This is not surprising since cyanobacteria are widely recognized for their ability to grow and flourish in extreme conditions, particularly in the presence of sulfide, high temerpature, or high salinity. Blooms of cyanobacteria occur in many lacustrine environments and are often associated with anthropogenic pollution (Edmondson & Lehman, 1981; Thornton, 1982) and/or fatal poisonings in mammals, fish, and birds (Carmichael, 1992, 1994, 1997; Dow & Swoboda, 2000). Thus, sulfide levels and high nutrient concentrations in the Sea may promote the growth of cyanobacterial mats, and these mats may be sources of toxins that affect the many birds and fish that depend on the Sea.

In this study, we report the results of a preliminary survey of cyanobacteria and associated organisms in the Salton Sea. The study is based on material collected in 1999 and includes consideration of the overall composition of 'cyanobacterial mats' as well as the first flow cytometric characterization of the microbial loop community of the Salton Sea. Taxonomic identification of field and cultured material conforms to modern bacteriological standards as published in Bergey's Manual of Systematic Bacteriology (2nd edn., Boone et al., 2001). We also used molecular genetic approaches to reveal some of the morphologically cryptic phylogenetic diversity associated with forms of that were common in the Sea.

Methods

Field sampling and examination of field material

Water samples containing planktonic organisms and samples of benthic algae scraped from rocks or pilings and/or embedded in salt crusts were collected from the shore in January and June, 1999. Sites sampled in January were located along the southern and eastern shores of the lake (dikes along the shoreline between New and Alamo Rivers, Garst Road, Red Hill Marina, Bombay Beach, Varner Harbor and the open beach at the State Recreation Area Headquarters; see Fig. 1 in Lange & Tiffany, 2002). In June, samples were collected at these locations as well as six additional sites on the western and southern shores (Desert Shores, Salton Sea Beach, Riviera Keys, and Obsidian Butte; cf. Fig. 1, Lange & Tiffany, 2002). Samples for flow cytometry were immediately preserved with 1% (final concentration) paraformaldehyde and frozen in liquid nitrogen. The remaining material was stored, refrigerated, until it was examined microscopically and used for strain isolation.

Isolation of cultured strains

Pure cultures were obtained from samples collected in January. Nutrient composition of the culture medium corresponded to that of the standard cyanobacterial medium BG11 (Castenholz, 1988) prepared with 24 g l^{-1} 'Instant Ocean'^{TM}. In order to increase the chances of isolating a physiologically diverse range of strains, media were also prepared at two higher salinity levels (43.5 and 70 g l^{-1}) by adding NaCl.

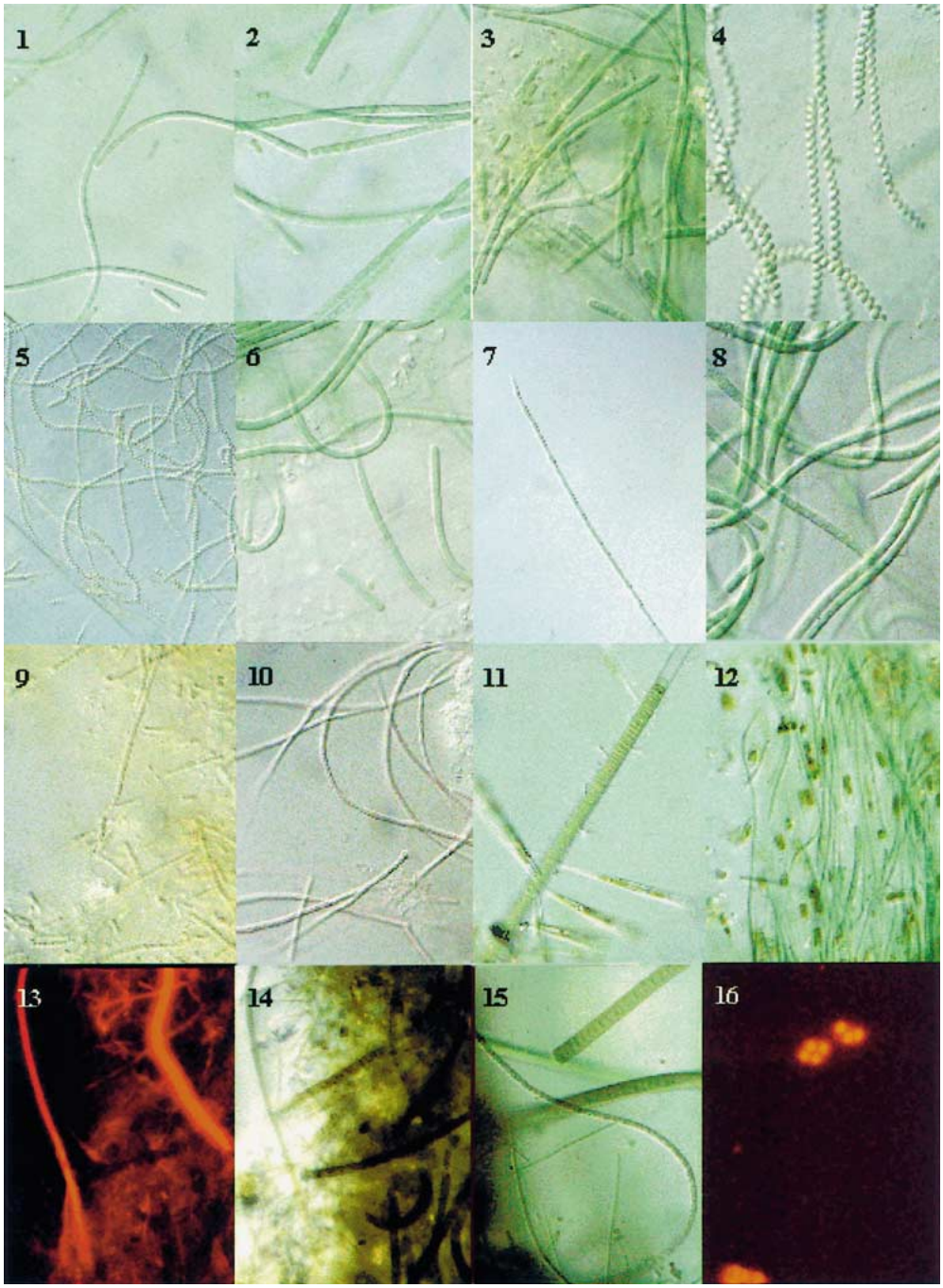

Figure 1. Field Samples and selected clonal isolates of cyanobacteria from the Salton Sea viewed by Nomarski Differential Interference Contrast (Panels 1–12, 14, 15) or epifluorescence microscopy (Panels 13, 15). Strain identifications are CCMEE 5439 (Panel 1); 5438 (Panel 2); 5433 (Panel 3); 5419 (Panels 4, 5); 5403 (Panel 6); 5446 (Panel 7, 8); 5407 (Panel 9); and 5435 (Panel 10). Panels 11 and 12 show the close association of pennate diatoms with epilithic *Lyngbya* (Panel 11) and multi-species mat material (12). Panels 13 and 14 show the same mat sample illuminated by transmitted light (14) and green light with epifluorescence illumination (13); the brightly red fluorescing cells and filaments are cyanobacteria with phycocyanin as the dominant light harvesting pigment; the filaments that are prominent in Panel 14, but which do not show any autofluorescence in Panel 13 are filaments of the sulfide oxidizing bacterium *Beggiatoa*; *Beggiatoa* was common in essentially all samples of mat material we examined by microscopy (see text). Panel 15 shows filaments of *Oscillatoria, Leptolyngbya*, and several thin filaments of *Geitlerinema* in a mat sample. Panel 16 shows autofluorescence from tetrads of planktonic unicells identified as *Synechocystis* sp; individual cells are $\sim$1.5 μm in diameter. All images were obtained using 1000$\times$ magnification except those in Panels 5 and 7 which were obtained using 400$\times$ magnification.

Thus, cells were isolated into media reflecting current conditions in the lake (~43.5 g l^{-1}), as well as conditions considerably less and considerably more saline than the current lake environment (24 and 70 g l^{-1}). At the beginning of the isolation process, media for enrichment cultures also contained cyclohexamide (final concentration 400 mg l^{-1}) to inhibit the growth of eukaryotic algae. Media was solidified for plating using 3% Difco^{TM} agar.

Several approaches were used to obtain isolates. Benthic samples consisting mainly of filamentous cyanobacteria were used as non-spread inoculum on agar plates, and motile gliding forms were allowed to separate from the mass. After filaments had radiated from the original point of inoculation, single filaments were picked off on small agar blocks with watchmaker's forceps and transferred to liquid medium. This enrichment was allowed to grow, examined by microscopy for uniformity of morphotypes in the culture, and replated on an agar plate (see Castenholz, 1988). A single filament from the population growing on the second plate was re-isolated into liquid media. The culture was considered a clonal isolate of the original morphotype after these two rounds of single filament isolation.

Dilution series were also used to isolate representatives of the most abundant cell types. In these cases, water samples or homogenates of benthic were inoculated into a dilution series of liquid medium, and then the community that grew up into the liquid medium used to inoculate an agar plate.

Water samples from the Sea were filtered onto polycarbonate filters (0.2 μm pore size) and agar plates were inoculated by dragging the filters across the agar surface. Colonies of unicells which grew on the plates were used to isolate clonal cultures using standard techniques of streak dilution. Additionally, unicellular forms isolated from benthic samples were inoculated onto plates using material that grew in the dilution series, general enrichments, or raw sample material and clonal cultures established from serial transfers of individual colonies that grew up on the plates.

Flow cytometry

Samples for flow cytometry were preserved and frozen in liquid nitrogen immediately after collection as described above; samples were stored at −80 °C until shipped to Bedford Institute of Oceanography on dry ice where they were again stored at −80 °C until analyzed. Samples were analyzed unstained and undiluted for the abundance of the major ultraphytoplankton groups (small phycoerythrin-containing cells, *Prochlorococcus*, and eukaryotic ultraphytoplankton) as described in Li (1995). For heterotrophic bacteria and viruses, the samples were diluted ten-fold in 10 mM Tris-EDTA buffer, stained with the nucleic acid-specific dye SYBR Green-I at 5×10^{-5} concentration of the supplier stock (Molecular Probes, Oregon), and the organisms enumerated according to Marie et al. (1999).

DNA isolation, amplification and sequencing

Genomic DNA of clones was isolated as described by Pitcher et al. (1989). Fragments of the 16S ribosomal RNA (rRNA) gene spanning *Escherichia coli* nucleotide positions 360–1326 were amplified in 50 μl reactions containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 200 μM of each dNTP, 1.12 mM $MgCl_2$, 1.25 U *Taq* polymerase (Perkin Elmer), ~10 ng genomic DNA, 0.2 μM each of primer CYA359F (Nübel et al., 1997) and primer PLG2.3 (5′CTTCA(C/T)G(C/T)AGGCGAGTTGCAGC3′), a modification of PLG2.1 (Urbach et al., 1992). Reaction conditions were 40 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. Amplification products were purified with the QIAquick-spin PCR purification kit (Qiagen), and cleaned amplification products were directly sequenced with an ABI Prism 377 at the University of Oregon's DNA Sequencing Facility.

Sequence alignment and phylogeny reconstruction

16S rRNA gene sequences were aligned as described in Miller & Castenholz (2000). Briefly, this entailed encoding secondary structure information for the mature 16S rRNA into a preliminary alignment prior to final pairwise alignment using Malign 2.7 (Wheeler & Gladstein, 1994) with pairwise addition of taxa and a gap cost to substitution cost of 3. Missing data were treated as unknown nucleotides.

Neighbor-joining, maximum parsimony and maximum likelihood phylogenies were inferred with PHYLIP 3.5c (Felsenstein, 1993). The neighbor-joining phylogeny was constructed with the NEIGHBOR program using a distance matrix calculated with DNADIST assuming Kimura's two parameter model (Kimura, 1980) with a transition to transversion ratio of 2. Maximum parsimony reconstructions were

Table 1. Cyanobcteria reported from the Salton Sea. (B-benthic samples; P-plankton samples) or among clonal isolates (C). Species reported by Carpelan (1962), which represents the only other survey of cyanobacteria from the Salton Sea, are included here for completeness

Taxon	This study		Carpelan[a]
	January	June	
Filamentous Forms			
Oscillatoria	B,C	B	B[g]
Spirulina	B,P,C	B	B
Arthrospira	B	B	
Geitlerinema	B,C	B	
Lyngbya	B,C	B	P
Leptolygnbya	B,C	B	
Calothrix	B	B	
c.f. Rivularia	B	B	
Phormidium tenue[a]			B
Plectonema calotrichoides[a]			B
Hydrocoleum sp.[a]			B
Undescribed	C		
Unicellular or Aggregates			
Synechococcus[b]	B,P,C	B,P	
c.f. *Synechocystis*[c]	P,C	P	
c.f. *Chroococcidiopsis*[d]	B,P		
c.f. '*Acaryochloris*'[e]	C		
Pleurococcus turgidus[a,f]			B
Pleurocapsa crepidinum[a]			B
Gomphosphaera lacustris[a]			P

[a] Used botanical classification system.
[b] Phycoerythrin and phycocyanin-dominant forms.
[c] 2 μm cells in tetrads.
[d] No baeocytes observed.
[e] See Miyashita et al. (1996); nomen nudem.
[f] Probable misspelling of *Chroococcus*; see text.
[g] Described as 'near *O. laetevirens*'.

obtained with DNAPARS, and a majority rule consensus diagram of equally parsimonious trees was found using CONSENSE. The maximum likelihood tree was inferred using DNAML with a transition to transversion ratio of 2 and sequential addition of sequences. The neighbor-joining and parsimony trees were bootstrap pseudoreplicated 1000 times, while the maximum likelihood phylogeny was bootstrapped 100 times. All trees were rooted with *Aquifex pyrophilus*, a hyperthermophile on an early branch of the Bacterial 16S RNA phylogenetic tree (see Madigan et al., 2000).

Results

Field samples

Microscopic examination of field samples revealed cell types that conformed to basic morphological features of eight genera of filamentous cyanobacteria and three genera of cyanobacteria that occur as unicells or aggregates (Table 1). Common genera referred to below are two genera of filamentous cyanobacteria which lack sheaths (*Oscillatoria* and *Geitlerinema*), both of which are motile, and which differ primarily in the cell dimensions. *Oscillatoria* (as currently defined) has disc-shaped cells that are wider than they are long and *Geitlerinema* has cells that are longer than they are wide, length being measured along the long axis of the filament (cf. Castenholz et al., 2001). *Lyngbya*, which was common in samples collected from rocks along the shore, has disc-shaped cells similar to those of *Oscillatoria*, but the trichome is encased in a distinct, persistent and firm sheath. Often the filaments appear brownish-colored because of the presence of an extracellular UV-protective pigment (scytonemin) bound in the sheath (see Garcia-Pichel & Castenholz, 1991).

Plankton samples, examined under epifluorescence microscopy, revealed a diversity of unicellular forms, the most common of which were representatives of the genera *Synechococcus* and *Synechocystis*. Individuals in both genera are short rods or coccoid cells ~1–2 μm in diameter; members of the two genera are distinguished by the number of planes of cell division: in *Synechococcus* cell division occurs along a single plane, producing pairs of dividing cells and, in *Synechocystis*, cell division occurs in two orthogonal division planes, occasionally producing characteristic tetrads of individual cells (e.g. Fig. 1.16). When excited with green light by epifluorescence microscopy, some of these small cells fluoresced orange, revealing the presence of phycoerythrin as the predominant light harvesting pigment and others fluoresced red under the same conditions. Since the red fluorescence disappeared when the cells were excited with blue light, it conformed to that expected from phycocyanin-dominant cells (see Wood et al., 1985). Single trichomes of filamentous cyanobacteria were observed in most water samples prepared for epifluorescence microscopy. Some of these trichomes were clearly members of the genus *Spirulina* based on their tightly coiled trichomes; others were not classified to genus but appeared to be members of Subsection III of the

Cyanobacteria (formerly Oscillatoriales, see Castenholz et al., 2001). Based on morphology, genera represented were *Oscillatoria*, *Geitlerinema*, *Leptolyngbya* and possibly others. Among the oscillatorian trichomes observed in the plankton, some had phycoerythrin and some had phycocyanin as the predominant photosynthetic pigment. Extremely small particles ($\ll$0.5 μm) that fluoresced orange when illuminated by green light were also common in the plankton samples; these particles, presumably containing phycoerythrin because of the golden-orange fluorescence, occurred in aggregates of many ($\sim$30–100) individually fluorescing cells or particles; we never observed any material like this in the enrichment cultures.

Great diversity of form was found among the filaments associated with floating mats and benthic samples collected from pilings and rocks. For example, a single subsample of mat material collected from a Varner Harbor piling in January revealed five different forms of oscillatorian cyanobacteria; these differed in the presence or absence and rate of motility, trichome width, presence or absence of phycoerythrin, presence or absence of a sheath, appearance of cross-walls, and presence or absence of constrictions at cross-walls. Mat samples collected at different locations were similar in color and consistency when examined macroscopically, but often contained different morphotypes of cyanobacteria that were easily distinguished from one another when samples were examined microscopically. Typical floating mat material was a mixture of one or two dominant morphotypes of filamentous cyanobacteria intertwined with filaments of the sulfide or sulfur-oxidizing chemolithotroph *Beggiatoa*. (Figs 1.13,1.14). For example, the dominant cyanobacterial morphotype in a *Beggiatoa*-rich sample of floating mat material collected from Salton Sea Beach in June was highly motile, with a trichome diameter of 4–5 μm and a curved, novel tip on terminal cells in the filaments; this morphotype was assigned to the genus *Geitlerinema* based on cell dimensions, lack of a sheath, and motility. This mat also included filaments of *Spirulina*, two morphotypes of *Beggiatoa* (4 and 8 μm wide), filaments of the green alga *Chaetomorpha*, and a large *Oscillatoria* (trichome width 8–10 μm). This motile filament was dark green, particularly at the cross-walls, and had a slight curve at the tip; while much less common than the *Geitlerinema* morphotype that dominated the mat sample from Salton Sea Beach, it was the dominant filamentous cyanobacterium in a sample collected the same day from Varner Harbor. Other morphotypes in the mat from Varner Harbor included a morphotype of *Geitlerinema*, a large *Spirulina*, a very large *Oscillatoria* (20 μm trichome width), and a smaller *Oscillatoria* (5μm trichome width) which was distinguished from another *Oscillatoria* of similar dimensions by the consistent granular appearance of the cross walls in one of the two morphotypes.

In most mat samples, the biomass of *Beggiatoa* was greater than, or comparable to, that of the filamentous cyanobacteria. Unicellular cyanobacteria, diatoms, and some other small photosynthetic eukaryotes were also apparent in the mat samples when they were examined with epifluorescence microscopy.

In January, 1999, we were interested in the possible winter refugia for organisms that formed benthic mats in the summertime. Samples of consolidated hard substrate, composed largely of barnacles, were collected in shallow water from the bottom of the lake off the beach near the State Recreation Area. Small ($\sim$10 mm^2) areas of bluegreen discoloration were observed in some places on the barnacle shells. Subsequent microscopic of a single sample scraped from one of these bluegreen areas revealed the presence of *Calothrix* (with phycoerythrin and heterocysts), *Spirulina*, a large morphotype (18 μm trichome width) of *Oscillatoria* containing phycoerythrin, two other smaller morphotypes of phycoerythrin-containing *Oscillatoria* that differed in trichome width, and at least one morphotype of phycocyanin-dominant *Oscillatoria*.

Lyngbya was especially common on rocks along the waterline at Desert Shore; most of these filaments were covered with pennate diatoms (Fig. 1.11). *Lyngbya* was found growing epilithically in both January and June, although diatom growth on the filaments was more luxuriant in the samples collected in June. The most common diatom found growing on Lyngbya was *Tabularia parva*, although *Achnanthes brevipes* and *Licomorpha* sp. are also found as occasional epiphytes on *Lyngbya* in the Sea (Mary Ann Tiffany, pers. comm.).

Flow cytometry

The results of flow cytometric analysis of water samples from three locations sampled in the Salton Sea in January, 1999 and two locations in the northern Gulf of Mexico sampled in October, 1998, are presented in Table 2. All samples were analyzed together, and the data from the Gulf of Mexico are presented as representative of ultra/picoplankton community structure in a marine community typical of neritic waters at

Table 2. Results of flow cytometric analysis of surface samples from the Salton Sea, with comparative data from samples collected at 2 m on the inner (I) and outer (II) West Florida continental shelf (Gulf of Mexico). Values $\times 10^3$ ml^{-1}; ND=Not detected

	Dike Road	Bombay Beach	Refuge Hdqtrs. Beach	Gulf Mexico-I	Gulf Mexico-II
Phycoerythrin-containing unicells	2.45	2.91	3.57	263.03	11.53
Prochlorococcus	ND	ND	ND	15.76	29.25
Small Eukaryotic Algae	128.89	91.41	111.55	4.02	0.79
Heterotrophic Bacteria	4366.02	4714.75	3972.41	886.93	219.68
Viruses-Type II	69 338.42	46 524.28	68 480.04	12 062.66	3901.02
Viruses-Type I	2698.34	4961.23	5353.38	1846.8	321.97
Total Viruses	72 036.76	51 485.51	73 833.78	13 909.46	4223.17

warm, temperate latitudes. Three dramatic differences between the Salton Sea picophytoplankton community and the neritic picophytoplankton community are apparent: (1) phycoerythrin-containing unicells, while abundant, were much less abundant in the Salton Sea than they were in typical continental shelf waters, (2) the very small divinyl-chlorophyll *a*-containing picocyanobacterial taxon *Prochlorococcus* appears to be completely absent from the samples we collected in the Salton Sea, and (3) small eukaryotic algae (typically cells ~3–5 μm effective spherical diameter; see Li & Wood, 1988) were more abundant in the Salton Sea than in typical neritic waters by one-to-two orders-of-magnitude. Not unexpectedly, because of the highly eutrophic conditions, heterotrophic bacteria were also more than an order of magnitude more abundant in the Salton Sea than in seawater collected from continental shelf environments. Interestingly, virus particles, while more abundant in the Salton Sea than in water samples from the continental shelf, did not show increases proportionate with the increase in bacteria or eukaryotic algae. Viruses were considerably more abundant, but roughly the same order of magnitude in the Sea as on the inner continental shelf. This trend was true for both Type I and Type II virus particles which, on the basis of nucleic acid staining intensity and apparent size, are thought to be possibly cyanophage and bacteriophage (Type II) or viruses of eukaryotic algae (Type I) (see Marie et al., 1999).

Cultured strains

Eighty-four clonal strains of cyanobacteria were isolated from the Salton Sea samples collected in January, 1999; all have been accessed into the NASA/University of Oregon Culture Collection of Microorganisms from Extreme Environments (UO-CCMEE; http://cultures.uoregon.edu). Because this project was of limited duration, isolates that grew quickly are disproportionately represented in the collection; most of these were isolated in medium of either low (24.5 g l^{-1}) or Salton Sea salinity (43.5 g l^{-1}). Only three clonal cultures were established in high salinity medium although the appearance of additional morphotypes in the enrichments during the project period indicates that many more could have been isolated if time had permitted; among the morphotypes that grew in high salinity enrichments, but which were not purified as clonal cultures, were several morphotypes of *Spirulina* and *Oscillatoria*, at least one morphotype of *Pseudanabaena*, and a number of unicellular forms (probably *Synechococcus* and/or *Synechocystis*). All three high-salinity isolates in the collection are filamentous forms (CCMEE Strains 5471, 5478, 5479).

The complete set of 84 strains isolated from the Salton Sea includes isolates from all sites sampled in January, and from samples of planktonic and benthic material. Filamentous and unicellular morphotypes were isolated from both water samples and benthic samples. The collection includes strains with phycoerythrin as the dominant light harvesting pigment, strains with phycocyanin as the dominant light harvesting pigment, and an unusual strain that appears to produce chlorophyll *d* as the predominant light harvesting pigment (A. M. Wood et al., in prep.).

While the collection does include several representatives of strains forming tightly coiled trichomes (*Spirulina* spp. sensu Castenholz, 2001) and several with thick persistent sheaths (*Lyngbya* spp. sensu Castenholz, 2001), the majority of the isolates are filamentous strains, most of which lack a sheath. Many of these are of morphotypes which were common in

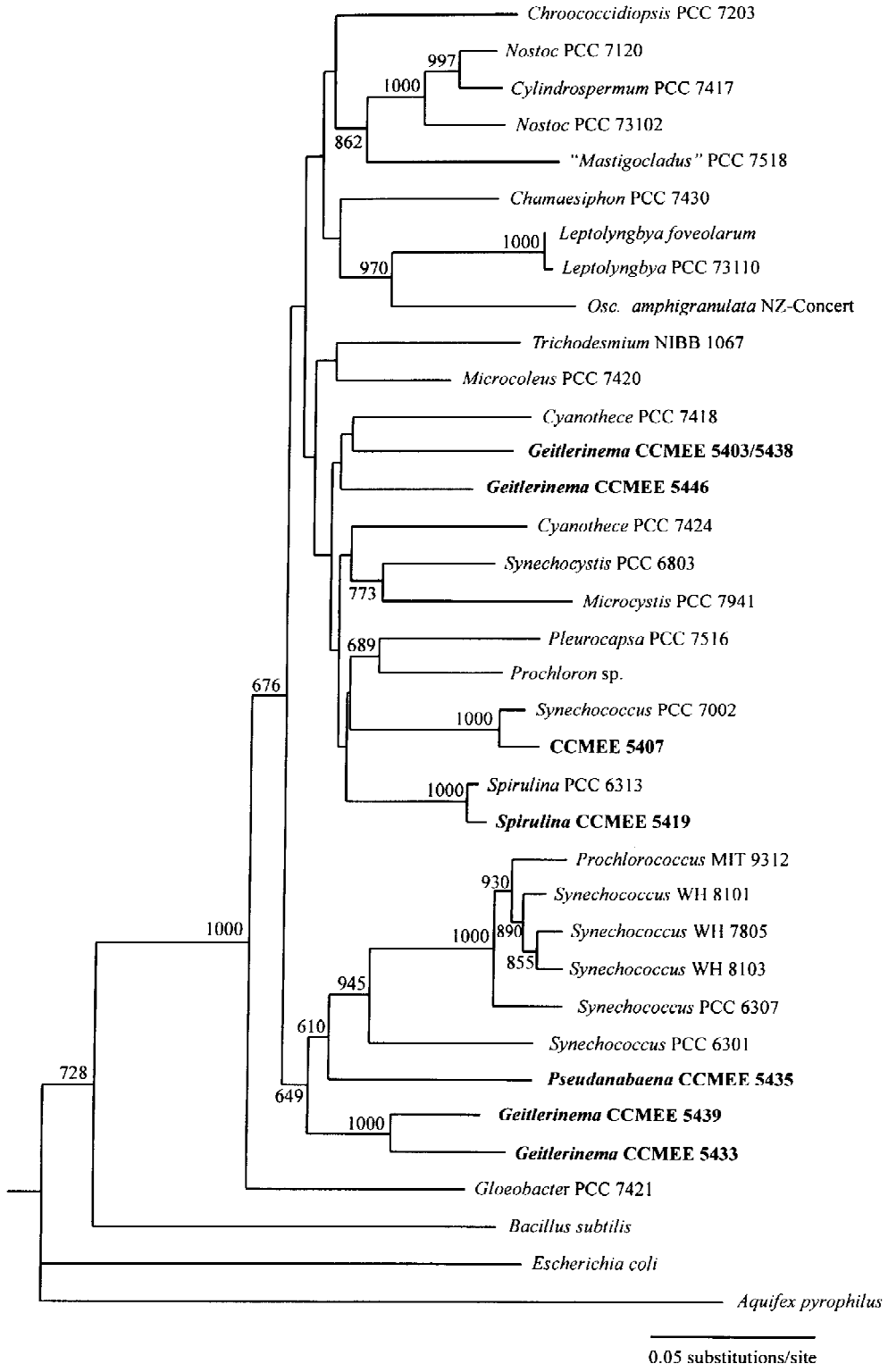

Figure 2. Cyanobacterial phylogeny inferred from ~960 bp of 16S rRNA sequence data using the Neighbor Joining method. A value at a node indicates the percentage of the time that the taxa to the right of the node formed a clade for 1000 bootstrap pseudoreplicates. Bootstrap values <50% are not shown.

Table 3. Cultured stains selected for phylogenetic analysis

Strain	Form-genus[a]	Width (μm)	Motility	Source
CCMEE5435	*Pseudanabaena*	1.2–3.0	Slow	Varner Harbor mat
CCMEE5439	*Geitlerinema*	1.0–2.0	Slow	Barnacle[b]
CCMEE5433	*Geitlerinema*	2.0	Very slow	Varner Harbor mat
CCMEE5438	*Geitlerinema*	2.0	Slow	Barnacle[b]
CCMEE5403	*Geitlerinema*	2.0	Rapid	Scraped off dike rip rap
CCMEE5419	*Spirulina*	1.2–1.3	Rapid	Sediment in boat harbor, Bombay Beach
CCMEE5446	*Geitlerinema*	3.0	Slow	Plankton sample collected from dikes
CCMEE5407	*Undescribed*	1.2	Nonmotile	Scraped off dike rip rap

[a] Taxonomic authority: Bergey's Manual of Systematic Bacteriology, 2nd Edn. (Boone & Castenholz, 2001).
[b] Barnacles collected in shallow water off the State Recreation Area headquarters.

mat material and which are very difficult to distinguish form one another based on morphology (cf. Fig. 1). Since several of the strains would key to the same species using the botanical system of classification commonly used in applied limnology, we were particularly interested in determining how much cryptic genetic diversity might exist among morphologically similar strains found in mats and attached to rocks and pilings. Thus, of the eight strains selected for further phylogenetic and morphological characterization, five would be placed in the same form-genus (*Geitlerinema*) and two (CCMEE 5403 and CCMEE 5419) were completely indistinguishable from one another morphologically (Table 3). Of the eight strains included in the phylogenetic analysis, all but strain CCMEE 5446 were isolated into low salinity medium (24 g l^{-1}); strain CCMEE 5446 was isolated into medium of intermediate salinity (43.5 g l^{-1}).

Phylogenetic analysis

Neighbor-joining (Fig. 2), maximum parsimony (Fig. 3), and maximum likelihood (Fig. 4) methods were employed to infer the position of eight clones isolated in this study within the cyanobacterial 16S rRNA gene phylogeny. All eight are classified taxonomically as members of Subgroup III of the cyanobacteria (formerly Oscillatoriales) under the current system (Castenholz et al., 2001). The maximum parsimony phylogeny shown (Fig. 3) is a consensus diagram of the three equally most parsimonious trees (2064 steps). The maximum likelihood phylogeny (Fig. 4) had a ln likelihood = −11389. These trees are similar to published cyanobacterial phylogenies (e.g. Wilmotte, 1994) in that they do not resolve many of the internal nodes. However, in most cases they provide consistent, robust information about the phylogenetic affinities of the Salton Sea clones included in the analysis. Each is discussed in detail below.

In trees produced by all three analyses, CCMEE 5435 (Fig. 1.10) was the basal lineage in a clade including marine *Synechococcus* and *Prochlorococcus* isolates, although this association was only robust for the neighbor-joining phylogeny (Fig. 2). The sequence is ∼98% similar to that for *Leptolyngbya* PCC7375 (not shown), a phycoerythrin-containing filamentous cyanobacterium isolated from a marine plankton sample (Rippka et al., 1979).

Strains CCMEE 5439 (Fig. 1.1) and CCMEE 5433 (Fig. 1.3) are sister taxa in all three phylogenies with extremely strong bootstrap support (Figs 2–4). They are also a sister group to a large clade including strain CCMEE 5435 (discussed above), although this association is only supported by bootstrap analysis for the neighbor-joining phylogeny. In addition to having slightly wider trichomes, cross-walls at the cell junctions are more distinct in CCMEE 5439 than CCMEE 5433.

With the exception of showing faster motility, Strains CCMEE 5438 (Fig. 1.2) CCMEE 5403 (Fig.

1.6) are essentially identical to CCMEE 5433 morphologically. However, they show little sequence homology with CCMEE 5433, as reflected by the lack of a close phylogenetic relationship between these two strains and CCMEE 5433 in any analysis (Figs 2–4). The two strains, one isolated from scrapings off the rip rap along the dikes between the New River and Alamo river (CCMEE 5403) and the other isolated from a mat sample collected at Varner Harbor (CCMEE 5438), share identical 16S rDNA sequences but their phylogenetic status was unclear. They were the sister taxa of *Cyanothece* PCC 7418 in the neighbor-joining and maximum likelihood phylogenies (Figs 2 and 4), but this association was not supported by bootstrap analysis in these trees, nor was it obtained in the maximum parsimony reconstruction (Fig. 3). However, subsequent analysis indicated that their sequence is >99% similar to that of *Geitlerinema* PCC7105, a marine isolate of Subgroup III (formerly Oscillatoriales; see Castenholz et al., 2001) (data not shown).

According to all three phylogenetic analyses, Strain CCMEE 5419 (Figs 1.4–1.5) groups with *Spirulina* PCC6313, an isolate from brackish water, with 100% bootstrap support (Figs 2–4). The position of this clade within the phylogeny as a whole was not resolved. Grouping of CCMEE 5419 with PCC6313 in our analysis was expected; cyanobacteria forming tightly coiled trichomes with regular helical coils appear to be a monophyletic group (Nübel et al., 2000).

The phylogenetic status of Strain CCMEE 5446 (Figs 1.8–9) is unclear, as its position is variable across phylogenetic reconstruction methods (Figs 2–4). A BLAST search of the GenBank database found it to be most closely matched with *Cyanothece* ATCC 51142, but their similarity was only ∼93% (data not shown). Microscopically, this strain looks very similar to the other strains of *Geitlerinema* described above (compare Fig. 1.8 with 1.2 and 1.6), but the trichomes are slightly wider (∼3 μm) and distinguished by a novel twisted and tapered pointed tip (Fig. 1.7).

Strain CCMEE 5407 (Fig. 1.9) consistently showed a robust phylogenetic association with *Synechococcus* PCC7002 (Figs 2–4). Overall the strain exhibits a combination of features that do not allow it to be placed in any genus under the current system (see Castenholz et al., 2001) indicating that no cultured representative of this taxon has been described before. The strain is brownish yellow in culture and the constrictions at the cross-walls of adjoining cells are pronounced. Cells are nearly isodiametric but slightly longer than wide; the strain appears to be nonmotile. In the botanical tradition of cyanobacterial taxonomy, the morphology of the strain corresponds to *Phormidium foveolarum* Gomont (Frémy, 1930).

Discussion

We found a variety of morphological cyanobacteria in all habitats and samples we examined. Differences between the list of cyanobacterial genera identified in this study and those identified by Carpelan (1961) largely arise from differences in the systematic standards utilized at the time of his study. At least two of the strains identified as *Geitlerinema* by us would have been described as *Phormidium* in his study; *Plectonema calotrichoides* is unknown in the polyphasic system as no cultured material conforms to the morphotype of this botanical species. We also believe that the organism he described as *Pleurococcus turgidus* was actually *Chroococcus turgidus* (Kütz) Näg. since we cannot find the genus *Pleurococcus* in any taxonomic treatment of the cyanobacteria or blue-green algae. Carpelan's omission of several genera of picoplanktonic cyanobacteria that we found to be common in the Sea almost certainly results from the fact that these organisms were largely overlooked in all aquatic environments before the introduction of epifluorescence microscopy and flow cytometry. We consider it likely that these organisms were present in the Sea at the time of Carpelan's study.

Our study, and Carpelan's before it, identified cyanobacterial mats as a common feature in the Sea. However, our epifluorescence analysis showed clearly that the mat material is not solely composed of cyanobacteria, but also includes *Beggiatoa*, a sulfur oxidizing bacterium as a major structural and biomass component. This means that most mats are formed in association with sulfide layers in the water column or sediment and raises the possibility that some of the cyanobacteria in the sea may be among those which are capable of utilizing H_2S as an electron donor for photosynthesis instead of water (Cohen et al., 1986; Castenholz et al., 1991). Additionally, mat material collected in different parts of the Sea, while superficially similar in color and consistency, varied considerably in composition. As noted previously, some mat samples were dominated by a single morphotype of filamentous cyanobacteria and others contained two or more dominant morphotypes of filamentous cyanobac-

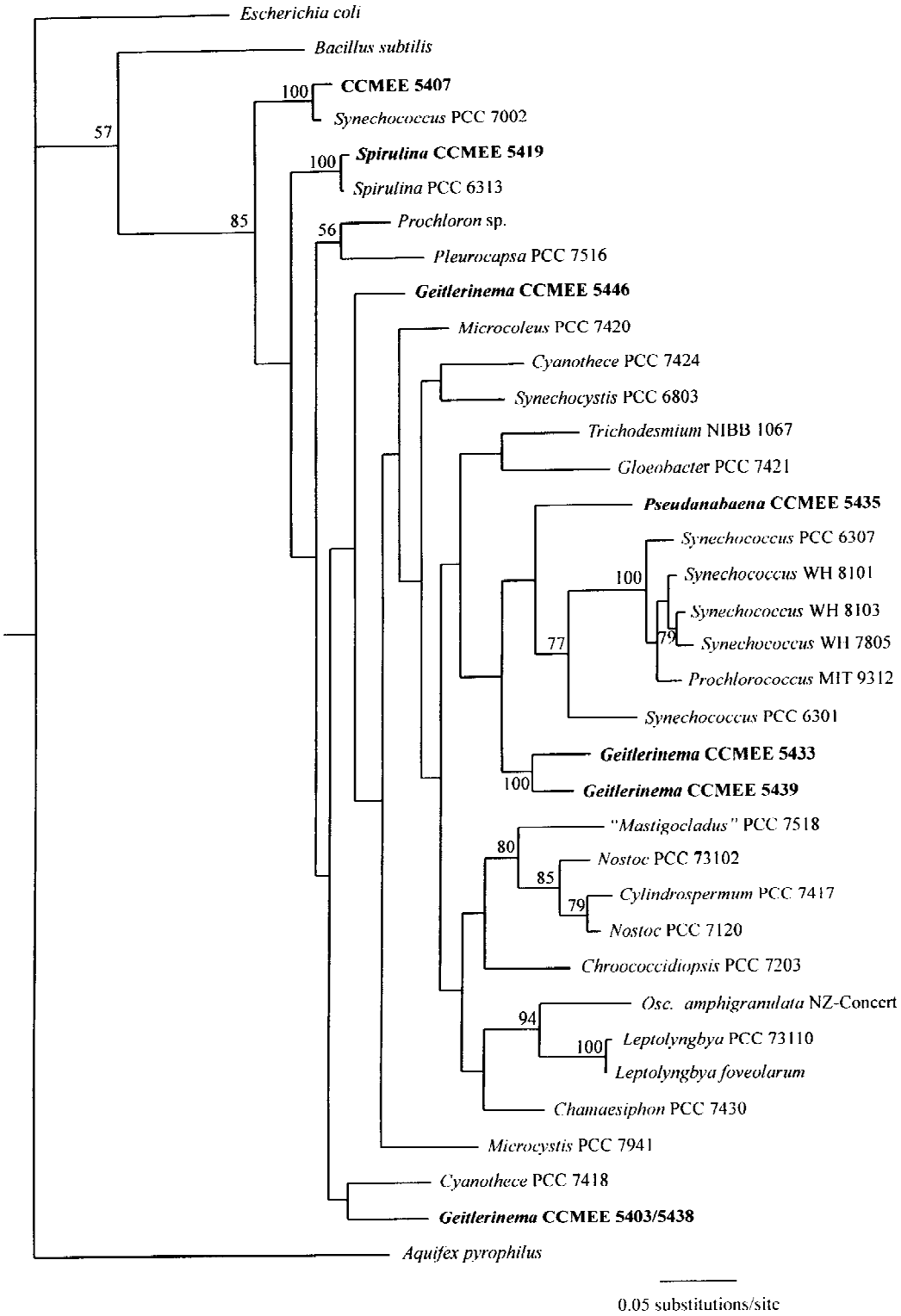

Figure 3. Cyanobacterial phylogeny inferred from ~950 bp of 16S rRNA sequence data using Maximum Likelihood method. A value at a node indicates the percentage of the time that the taxa to the right of the node formed a clade for 100 bootstrap pseudoreplicates. Bootstrap values <50% are not shown.

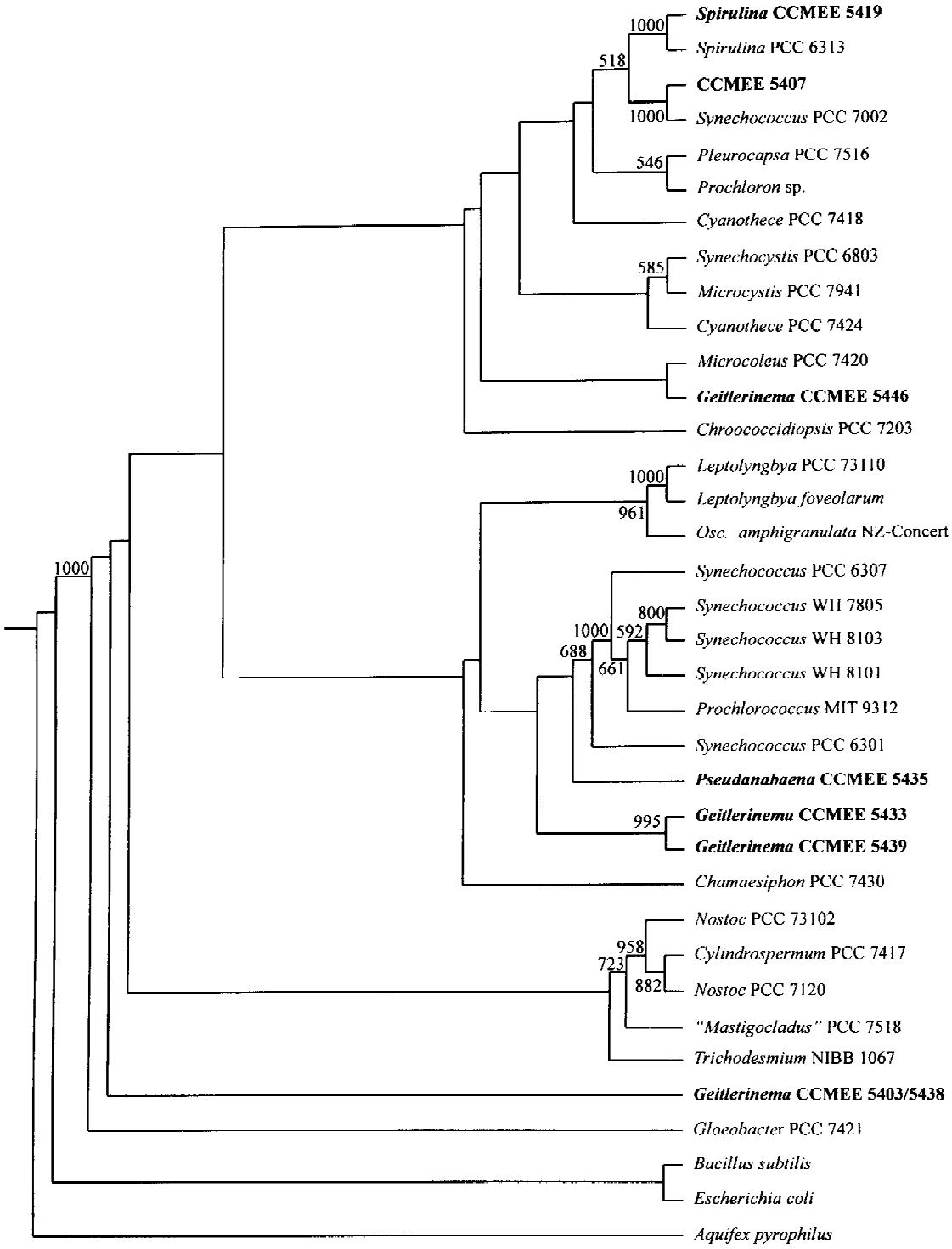

Figure 4. Cyanobacterial phylogeny inferred from ~950 bp of 16S rRNA sequence data using Maximum Parsimony. A value at a node indicates the percentage of the time that the taxa to the right of the node formed a clade for 1000 bootstrap pseudoreplicates. Bootstrap values <50% are not shown.

teria, as well as numerous less abundant morphotypes of filamentous cyanobacteria and many small diatoms and unicellular cyanobacteria. Even the small patch of blue-green material scraped off a barnacle colony revealed five morphotypes from four genera when examined by microscopy, and yielded two additional morphotypes from another genus when used as the inocculum for an enrichment culture. Thus, each mat community may have slightly different biogeochemical properties, possibly reflected in differences in productive capacity under different conditions of oxygen availability, level of irradiance, and/or sulfide concentration; these differences may, in turn, be reflected in differences in palatability to grazers.

In the plankton, the communities described by flow cytometry differ from those of neritic marine waters in ways that might be predicted by the difference in trophic status. For example, the high abundance of small eukaryotic algae is common in eutrophic estuaries and, while we do not know the taxonomic identity of these organisms, it is likely that the community includes a number of small flagellates that are difficult, sometimes impossible, to identify in preserved samples.

As noted before, *Prochlorococcus marinus* is widely regarded as an oceanic phytoplankter that succeeds primarily under conditions of extreme oligotrophy (Partensky et al., 1999). Thus, we interpret its absence from our samples as reflective of its general exclusion from the Sea due to its presumed intolerance of eutrophic conditions. The relatively low concentration of phycoerythrin-containing unicells was unexpected since these organisms reach very high abundance in warm, nutrient-rich water. The value of 10^5 cells ml^{-1} observed at one of our Gulf of Mexico stations is representative of values found in the Gulf of California in upwelling-influenced areas (Wood et al., unpublished data). Our culture collection and evaluation of the field samples by epifluorescence microscopy revealed the presence of small unicellular cyanobacteria which utilize phycocyanin as the primary light harvesting pigment as well as phycoerythrin-containing unicells in the Salton Sea. In the open ocean, these organisms are selected against because the red wavelengths of light they require for photosynthesis are rapidly absorbed by seawater (Wood, 1985). In the Salton Sea, these organisms are not under any particular disadvantage when competing with phycoerythrin-dominant strains. Because the euphotic depth is always very shallow, the spectral composition of available light should be relatively unaffected by the differential absorption of red and blue light by water. Thus, it is possible that the total abundance of picocyanobacteria in the plankton of the Salton Sea may be comparable to that of marine waters, but that it is composed of a greater range of pigment types. Ideally, methods for routine application of flow cytometry to the analysis of samples from the Salton Sea will be modified from those used for analysis of marine samples to take the presence of phycocyanin-dominant picocyanobacteria into account.

The high numbers of bacteria and viral particles observed in the flow cytometry study suggest that there is a very dynamic microbial loop operating in the Sea. The concentration of Type I viruses, thought to represent bacteriophage and cyanophage combined (Marie et al., 1999), exceeds the highest abundance of total viral particles observed during a spring bloom period in a Norwegian estuary by about an order of magnitude and exceed peak abundance of strain-specific cyanophage occurring in Woods Hole Harbor by several orders of magnitude (Bratbak et al., 1990; Waterbury & Valois, 1993). These high numbers may explain the fact that viral abundance, while higher in the Sea than in coastal marine waters, is not proportional to the even higher bacterial abundance. It is possible that the complex host-phage dynamics involved with infection and growth of viral particles has a maximum upper limit that is partially determined by the overall diversity of hosts (Cottrell & Suttle, 1991; Waterbury & Valois, 1993; Suttle & Chan, 1994; Paul & Kellogg, 2000). It is also possible that the lifetime of viral particles is shorter than that of the hosts (Noble & Fuhrman, 1999) and, thus, turnover accounts for the apparently 'low' relative increase in viruses relative to bacteria when we compare the Salton Sea values with other marine ecosystems. Regardless, viruses clearly play a role in termination of algal blooms in other marine environments (Bratbak et al., 1993; Paul & Kellog, 2000) and have a significant influence on particle flux (Proctor & Fuhrman, 1990; Fuhrman & Noble, 1995; Proctor, 1997). Given the values for viral particles and hosts in the Sea, it is clear that study of the details of the microbial loop will be a fruitful area for further research on the Salton Sea ecosystem.

Overall, our impression is that the Salton Sea contains a very diverse cyanobacterial flora. As is typical for this group of organisms, a considerable amount of morphological similarity masks a tremendous amount of genetic diversity; sequence data from only eight strains of generally similar morphology spans nearly the entire genetic distance encompassed by the cy-

anobacterial 16S rRNA tree (Figs 2–4). Our study is the first molecular characterization of genetic diversity within the form-genus *Geitlerinema*, but other investigators have obtained similar results in other large cyanobacterial genera (Wilmotte et al., 1997; Wilmotte & Herdman, 2001, Nadeau et al., 2001). These data highlight the difficulty any investigator encounters when trying to identify nominal species groups of cyanobacteria in field samples. Of the eight strains included in our phylogenetic analysis, five would have been classified as species of *Oscillatoria* using the botanical system or *Geitlerinema* under the current bacterial system, but they fall into at least two, and possibly three major clades of cyanobacteria based on their 16S rRNA sequences (Figs 2–4). Most notably, three strains included in our phylogenetic study (CCMEE 5433, CCMEE 5438, and CCMEE 5403) were nearly identifical morphologically and would all key to *Oscillatoria amphibia* Ag. (Frémy, 1929) in the botanical tradition of cyanobacterial systematics; these strains fell into two widely divergent clades based on 16S rRNA sequence (Figs 2–4). In culture, these strains differed slightly in their apparent rate of motility but this would not be apparent if one were examining preserved material. Overall, we conclude that the conservative approach of using form-genera and description of morphological types in field material without assigning species names is preferred for description of community structure. Without genetic characterization, assignment of the same species names to organisms of similar morphology suggests a level of common ancestry that cannot be assumed for many cyanobacteria of the forms that are common in the Salton Sea.

The close genetic association of Strain CCMEE 5435 with the marine unicellular strains *Prochlorococcus* MIT9312, *Synechococcus* WH8101, WH7805, and WH8103 suggests that this strain is of oceanic origin. Other authors in this volume (see Lange & Tiffany, Rogerson & Hauer) have discussed the many ways in which marine organisms have been introduced to the Sea during its short lifespan so that the presence of truly marine strains in the plankton or benthic community would be expected. Continuous introduction of freshwater forms of cyanobacteria via migratory birds and inputs from the New River and Alamo River can also be expected. Combined with our success at culturing strains of cyanobacteria from the Salton Sea at a wide range of salinities, this suggests that cyanobacteria will be a major component of the autotrophic community under nearly any management scenario.

Most proposed management scenarios for the Salton Sea project reduction of salinity, but only to conditions more typical of the open ocean environments. However, the Sea differs dramatically from marine environments by having very high nutrient concentrations, frequent occurrence of low oxygen and high sulfide levels in the water column, and high concentrations of dissolved organic carbon (Watts et al., this volume). Taken together, these conditions suggest that cyanobacteria, particularly the forms in mat communities with the sulfur-utilizing *Beggiatoa* may play an important role in carbon fixation and biogeochemical processes in the Sea because of their tolerance of H_2S and low dissolved oxygen levels.

Many cyanobacteria produce toxins that are particularly lethal to mammals, birds, and fish. Among taxa we have identified in our study, there is overlap at the generic level with five known toxin producing species (*Lynbgya, Oscillatoria, Pseudanabaena, Synechococcus, Synechocystis*). Since cyanobacteria do not have to occur as blooms to produce toxic effects in mammals and birds, especially when cyanobacteria can be concentrated in mat material that may be ingested by herbivores, the possibility that cyanobacteria are involved in bird and fish kills needs further evaluation.

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