ECOPHYSIOLOGY OF A MONO LAKE CYANOBACTERIUM

by

CHARLES RYAN BUDINOFF

(Under the Direction of James T. Hollibaugh)

ABSTRACT

A cyanobacterium of the genus *Cyanobium* is found in alkaline hypersaline Mono Lake, California. I have studied its spatial and temporal distribution using epifluorescence microscopy and measured growth characteristics of an isolate (MLCB) to gain insight into the physiological ecology of the organism. The Mono Lake *Cyanobium* blooms in late summer (5.0×10^4 cells mL^{-1}). It has very low population densities in photic waters through spring and summer ($<10^2$ cells mL^{-1}), but maintains a significant population of cells (10^4 to 10^5 cells mL^{-1}) year round below 25 meters during meromictic periods. Complete turnover of the lake resulted in a large decrease (90%) in deep water cell concentrations. Bathymetric data was used to calculate the total number of cells at various depths throughout the lake to help determine whether sedimentation and/or littoral transport affected its distribution. Comparison of the salinity tolerance of MLCB to other members of the genus showed that strain MLCB was the most halotolerant, capable of growing at 10% salinity, compared to limits of 0 to 6% for the other strains.

INDEX WORDS: Cyanobacteria, *Synechococcus*, Meromixis, Phycoerythrin, Bacteria, Physiology, Halotolerance, Alkalitolerance, Mono Lake.

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B.S., Arizona State University, 2002

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIECNE

ATHENS, GEORGIA

2005

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December 2005

ACKNOWLEDGEMENTS

This work was made possible by a grant from the National Science Foundation (MCB 9977886). I wish to thank Ferran Garcia-Pichel for providing advice and cyanobacterial expertise, Anneliese Ernst for supplying cultures, Robert Jellison for his dedicated sampling of Mono Lake, and Mark Farmer and John Shields of the Center for Ultrastructure Research at UGA for help with TEM.

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CHAPTER 1

INTRODUCTION

Mono Lake lies on the eastern edge of the Sierra Nevada mountain range, at the western edge of the Great Basin. The lake lies in an area of active volcanism, being adjacent to one of the youngest volcanic ranges in North America, that stretches ten miles north to south, with elevations up to 9,000 ft. Mono Lake is endorheic, fed by melting snowfall and thermal springs via a series of alpine lakes and reservoirs. The basin's volcanic setting and endorheic characteristics contribute to its unique water chemistry (Table 1). Mono Lake is a sanctuary for millions of migratory birds that breed and molt while feeding on the trillions of brine shrimp in the lake, who in turn feed on unicellular alga production. Water diversions for municipal, industrial, and agricultural uses have been significant in the past 60 years, decreasing lake level by 1/4, doubling salinity, promoting monomixis, and threatening the habitat. Recent policy changes have led to a decrease in diversions. This, along with elevated snow pack, increased fresh water flow into the lake and triggered the onset of meromixis (persistent chemical stratification) that lasted from 1996 until 2003.

Eukaryotic phototrophs, mainly the green alga *Picocytis* and diatoms, are responsible for the majority of primary production in Mono Lake. Phytoplankton productivity is high (350 to >1000 g C m^{-2} yr^{-1}) with marked seasonal cycles of abundance (Jellison and Melack, 1993; Roesler *et al.*, 2002). Eukaryotic algae are abundant throughout winter and increase substantially in spring as the thermocline stabilizes. Grazing by the brine shrimp *Artemia monica* during summer rapidly reduces algal standing crop in the upper water column. The high levels of primary production and long residence times lead to a high concentration of dissolved organic carbon. During prolonged stratification, bottom waters become anoxic and accumulate

Location	California, east of the Sierra Nevada mountains, western edge of the Great Basin.
Watershed	Endorheic, volcanic, fed by melting snowfall & thermal springs.
Area (km^2)	180
Volume (km^3)	3.3
Max/Mean Depth (m)	47/18
Average extinction coefficients of PAR (m^{-1}) and average depth (m) to 0.1% I_0.	Summer 0.46 (23), winter 0.74 (12)
Ventilation	Meromictic, mixing 1988, '95, '03, '04, & '05
Salinity	8%
pH	9.8
Nitrogen (NH_4) (μM)	0-15 epilimnion, >500 beneath chemocline
Phosphorus (SRP) (μM)	500
Sulfate (mM)	130
Carbonate (mM)	400
Sulfide (μM)	>2000 beneath chemocline
Arsenic (μM)	200
Chlorophyll a (μg chl/L)	0.2-100
Primary Production (g $C/m^2/yr$)	350-1100

Table 1. Limnological properties of Mono Lake

high concentrations of reduced inorganic compounds such as sulfide and ammonia. In contrast to many other soda lakes, large-scale anoxygenic photosynthesis does not occur in Mono Lake, likely due to the depth of the mixed layer and low light levels at depths containing sulfide.

Oxygenic prokaryotic phototrophic phytoplankton are barely detectable in the photic zone of Mono Lake for most of the year and contribute little to primary production in Mono Lake. A single picoyanobacterium ecotype of the genus *Cyanobium* is found at high concentrations (10^5) in anoxic, sulfide-rich bottom waters. This is in contrast to the eukaryotic algae of Mono Lake who do not build up higher cell concentrations in the aphotic zone then the photic zone (Steward, G., unpublished data). *Cyanobium* species are considered halotolerate, but they have not been found in salinities above 4% (Rippka and Cohen-Bazire, 1983; Crosbie et al., 2003). Their cryptic presence in aphotic lake waters is not uncommon, especially in meromictic lakes, but the means by which they attain and maintain such concentrations is subject to speculation (Craig, 1987; Detmer et al., 1993; Malinsky-Rushansky et al., 1997). The puzzle posed by the presence of apparently healthy populations of a phototrophic *Cyanobium* in the aphotic, anoxic and sulfidic bottom waters of Mono Lake is my motivation for this research. The *Cyanobium*'s distribution raises interesting ecophysiological questions, particularly how it maintains its population of cells without light and how it tolerates anoxia and elevated salinity. I present data on the organism's seasonal abundances and examine the physiology of the isolated ecotype to elucidate its ecology.

Picocyanobacteria (Pcy) of the genera *Synechoccocus* and *Cyanobium* are ubiquitous in aquatic environments. Whether in oceanic waters, oligotrophic, eutrophic, ice-covered, or saline lakes, Pcy are important contributors to global carbon fixation and are considered a foundation for the microbial food web (Stockner et al., 2000). Their small size (0.2-2 μm) results in

increased surface to volume ratios, allowing Pcy to reduce the limitations of nutrient acquisition set by molecular diffusion, giving them an advantage over larger phytoplankton in low nutrient environments (Raven, 1998). However, they also compete well in eutrophic environments; some studies show that Pcy contribute 30-90% of total production in some eutrophic lakes (Maeda et al., 1992; Carrick and Schelske, 1997).

Phylogenetically, marine *Synechoccocus* and *Cyanobium* form a tight clade within the cyanobacteria (Wilmotte and Herdman, 2001) (Urbach et al., 1998). Currently there are >50 phylogenetically distinct isolates within the genus *Cyanobium*, representing a high species diversity (Crosbie et al., 2003; Ernst et al., 2003). In contrast to marine *Synechococcus* ecotypes (Rocap *et al*., 2002), *Cyanobium* show no significant correlations between pigment complement and phylogeny, suggesting that environmental factors other than light quality and quantity select for specific ecotypes. Determining these factors requires not only molecular-based approaches but also culture-based experiments using isolates. Factors such as salinity, sulfide concentration, UV light, hydrologic influences, and nutrient dynamics influence the genetic complement of the organism and define niches for individual Pcy species. Mono Lake provides an excellent habitat in which to examine physiological adaptations of the genus *Cyanobium* and to elucidate the factors influencing its speciation.

CHAPTER 2

METHODS

Water samples used in this study were collected from the central basin of Mono Lake at Station 6 (37°57.822' N, 119° 01.305' W; station S30 in previous reports) monthly to quarterly from September 2001 to April 2004 at various depths (2 to 40 m). The lake's bathymetry slopes steeply along the SW shore, adjacent to the deepest part of the lake where the bottom is esentially flat (Fig. 1). Samples were collected with a Niskin bottle, placed in airtight plastic bottles, and kept in the dark at 4°C until they were counted (within 10 days). Vertical profiles (Fig. 2) of temperature, pressure, photosynthetically active radiation (PAR, 400 to 700 nm; Licor 2π sensor), and fluorescence (WetLabs WetStar fluorometer) were obtained with a SeaBird SeaCat profiler. Oxygen profiles were obtained with a polargraphic oxygen sensor (YSI) equipped with a Clarke-type electrode. During meromixis, 0.1% of surface PAR is found at ~12 m in winter and ~23 m in summer.

Spatial and temporal distribution of the organism was quantified using a Leica epifluorescence microscope containing filter sets N2.1 (ex. 515-560 nm), A (ex. 340-380), and I3 (ex. 450-490) allowing for the differentiation of cells containing phycoerythrin (PE, $\lambda_{AbsMax} \cong 500$ nm) from those only containing phycocyanin (PC, $\lambda_{AbsMax} \cong 620$ nm). Bathymetric data (Pelagos, 1987) for the lake was used to construct a hypsographic curve that was used to calculate the total number of cells in the lake.

Deep, anoxic Mono Lake water containing the highest concentration of *Synechococcus*-like cells (10^5 mL^{-1}) was chosen for the enrichment of the organism. Placing Mono Lake water in the light encourages the growth of the eukaryote *Picocystis*, making it extremely difficult to

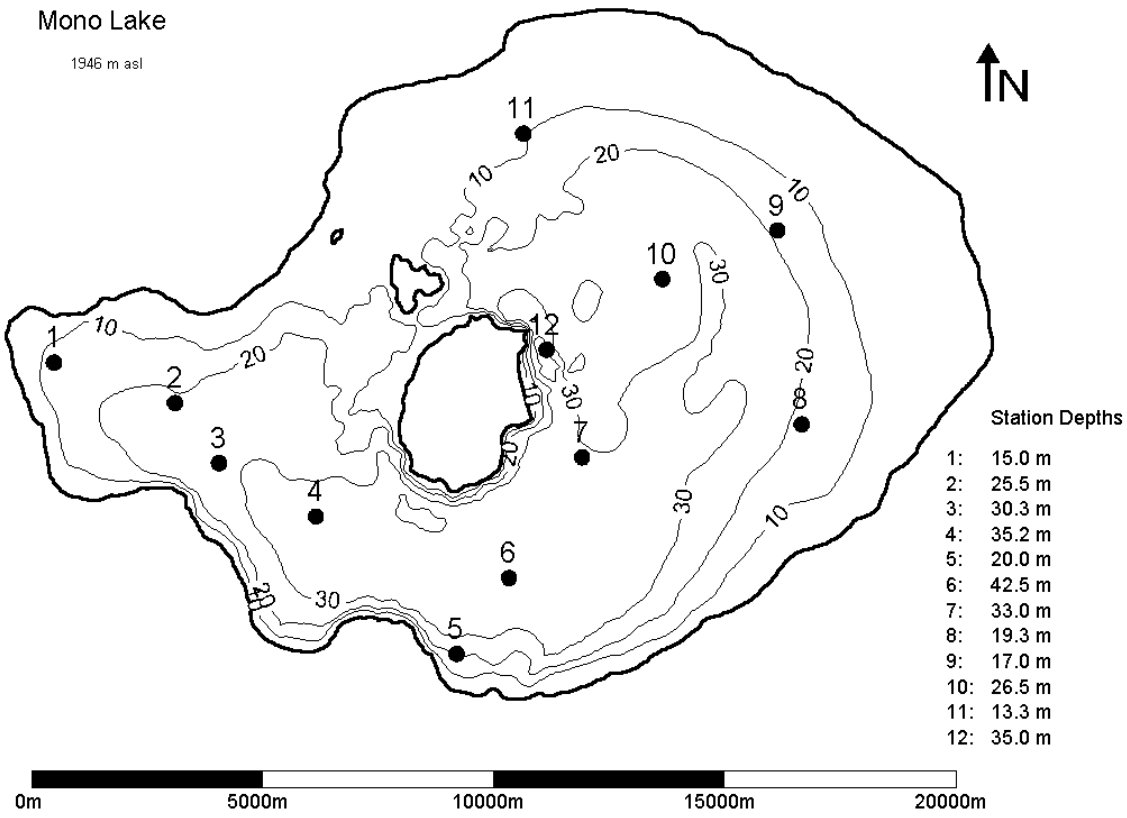

Fig. 1. Bathymetric map of Mono Lake showing sampling stations. Samples for this study were collected at station 6.

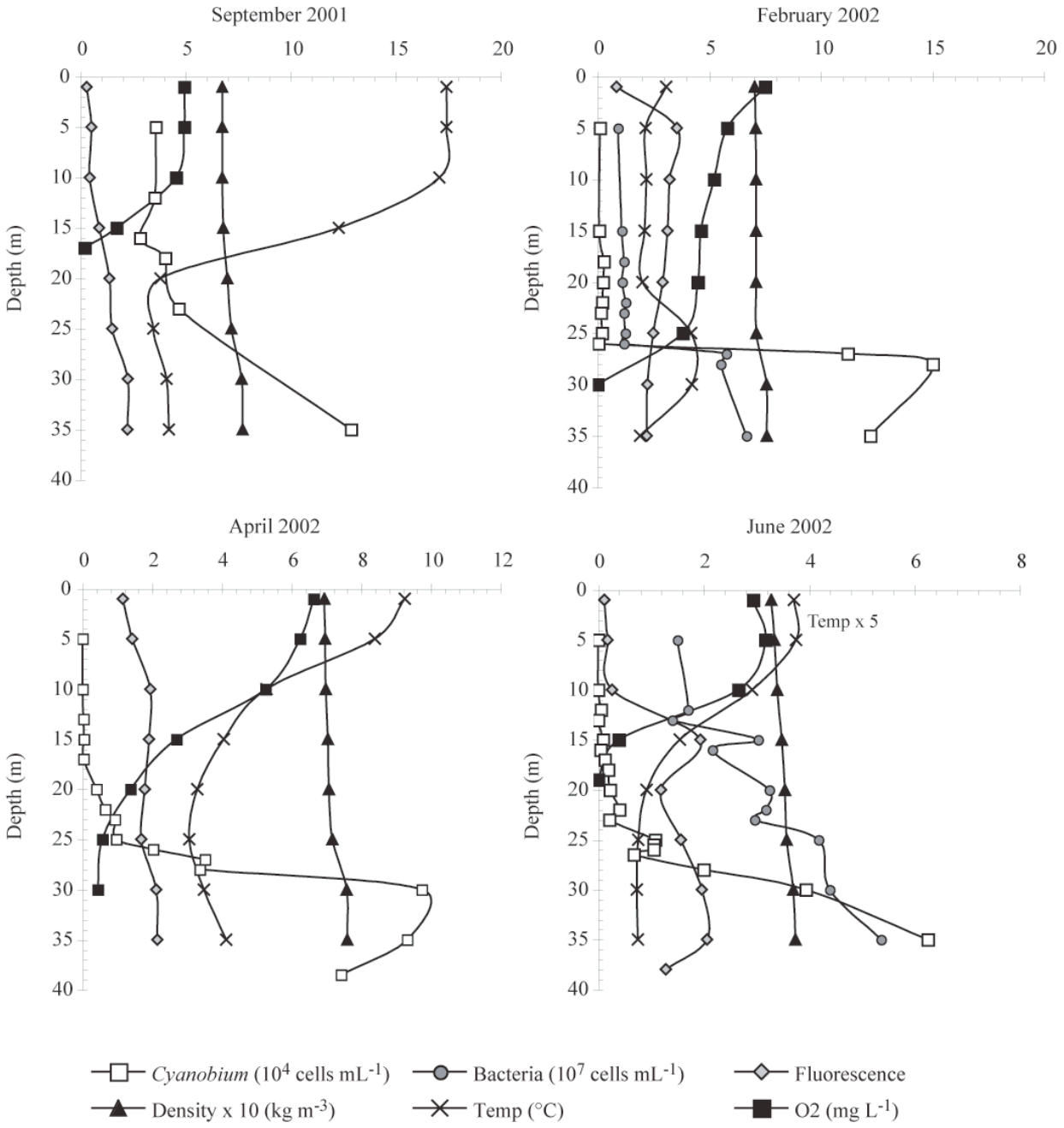

Fig. 2. Vertical profiles of temperature, density, photosynthetically active radiation (PAR, 400 to 700 nm), fluorescence, oxygen, *Cyanobium* abundance, and bacterial abundance for September 2001, February, April, and June 2002.

isolate other oxygenic phototrophs. Medium was amended with cycloheximide (5-10 mM) to discourage eukaryotic algal growth and allow for the successful isolation of the *Cyanobium* ecotype designated strain MLCB. In general, the organism did not grow well on agar plates, so that purification to axenic culture was achieved through serial dilution.

Growth media for all experiments contained the following per liter; 100 mg $MgSO_4$ x $7H_20$, 50 mg $CaCl_2$ x $2H_2O$, 25 mg K_2HPO_4, 420 mg $NaNO_3$. Vitamin, trace metal and iron stock solutions were added to this basal media (0.5 mL of each per 1 liter). The vitamin stock solution contained the following per liter: 5 mg folic acid (B_9), 5 mg cyanobalamin (B_{12}), 5 mg Biotin, 40 mg Ca-pantothenate (B_5), 70 mg thiamin (B_1), 40 mg nicotinic acid (niacin), 40 mg *p*-aminobenzoic acid, 20 mg pyridoxolium hydrochloride, and the trace metal stock solution contained (per liter): 1000 mg H_3BO_3, 450 mg $MnCl_2$ x 4 H_2O, 50 mg $ZnSO_4$ x 7 H_2O, 20 mg Na_2MoO_4 x $2H_2O$, 20 mg $Co(NO_3)_2$ x $6H_2O$, 5 mg Na_2SeO_4. The iron stock solution contained (per 200 mL): 300 mg $FeCl_3$, 500 mg citrate, 100 mg EDTA. NaCl was used to increase salinity, depending on strain and experiment. Nutrients (nitrogen, vitamins, trace metals, sugars, sulfide, etc.) were added to autoclaved salts as filter-sterilized solutions. Agar plates were prepared with purified agar at 0.8% w/v. Anoxic media were prepared in an anaerobic chamber and dispensed into serum bottles equipped with butyl rubber stoppers. Cultures were maintained and growth rate experiments were performed in a temperature-controlled incubator (20°C) with constant cool white light from fluorescent tubes (40 μE^{-1} m^{2-1} s^{-1}).

Instantaneous growth rates (μ) were calculated from the logarithmic change of *in vivo* fluorescence measured once per day with a Turner Designs fluorometer. Typically, growth was followed for 1-4 weeks, but under certain conditions (high salinity, low light), growth was slow

and was monitored for up to 8 weeks. All cultures were acclimated to their conditions for at least 3 transfers before measurements were made.

Adsorption spectra were obtained *in vivo* using a Shimadzu UV160U spectrophotometer by filtering homogenized suspensions onto Millipore type HA filters, which were then placed as close as possible to the light-sensor window of the spectrophotometer, as previously described (Garcia-Pichel and Castenholz, 1991). *In vivo* fluorescence spectra of phycoerythrobilin (PEB, λ_{AbsMax} ≅540-570 nm) and phycourobilin (PUB, λ_{AbsMax} ≅495-500 nm) were obtained using a Shimadzu RF-5000 spectrofluorophotometer as follows: exitation illumination ranged from 450 nm to 580 nm, with the excitation monochromator set at 560.4 nm, and the emmission monochromator set at 588 nm, and a bandpass of 3 nm. Complimentary chromatic adaptation potential was determined as previously described (Tandeau de Marsac and Houmard, 1988).

Morphology and ultra-structure of the isolate were determined by transmission electron microscopy (TEM) performed on a JEOL 100 CX TEM as previously described (Stanier, 1988).

Nucleotide sequences for the 16S rRNA gene, the 16S-23S rRNA internal transcribed spacer (ITS) region, and form I ribulose-1,5-bisphosphate carboxylase (*cbbL*) genes were obtained as previously described (Rocap *et al.*, 2002; Giri *et al.*, 2004). Neighbor-joining phylogenetic trees were constructed using the program ARB (www.arb-home.de). Seven major clades were identified and labeled based on the occurrence of three or more sequences grouping together with bootstrap values higher than 50%.

CHAPTER 3

RESULTS

Interrogation of Mono Lake samples using general bacterial primers (Hoeft *et al.*, 2002; Humayoun *et al.*, 2003) and form I ribulose-1,5-bisphosphate carboxylase (*cbbL*) (Giri *et al.*, 2004) indicated the presence of only one *Synechococcus*-like ecotype in the anoxic waters of Mono Lake. The sequences obtained were identical (>99%) to sequences from strain MLCB. Additionally, based on their fluorescence characteristics, all cell counts are of a PE-containing Pcy. Thus, I am confident that the epifluorescence microscopy counts are of the same organism as was isolated into pure culture.

Although sampling occurred over a period of 3 years, the majority of data presented are from 2001 to 2002. Monthly sampling during this period gave a more thorough description of seasonal changes in abundance. When we sampled in 2003 and 2004 we saw the same overall patterns of abundance.

Seasonal epifluorescence microscopy counts of strain MLCB are shown in figure 3. Cell concentration data were integrated to estimate the total population of strain MLCB in the photic zone and compared to the estimated population in the aphotic zone. This calculation indicated that abundance in the photic zone was never greater than in the aphotic zone and that abundance decreased in the aphotic zone over winter and spring until late summer (Fig 4). I used bathymetric data to calculate the volume of the photic and aphotic zones to compare the lake

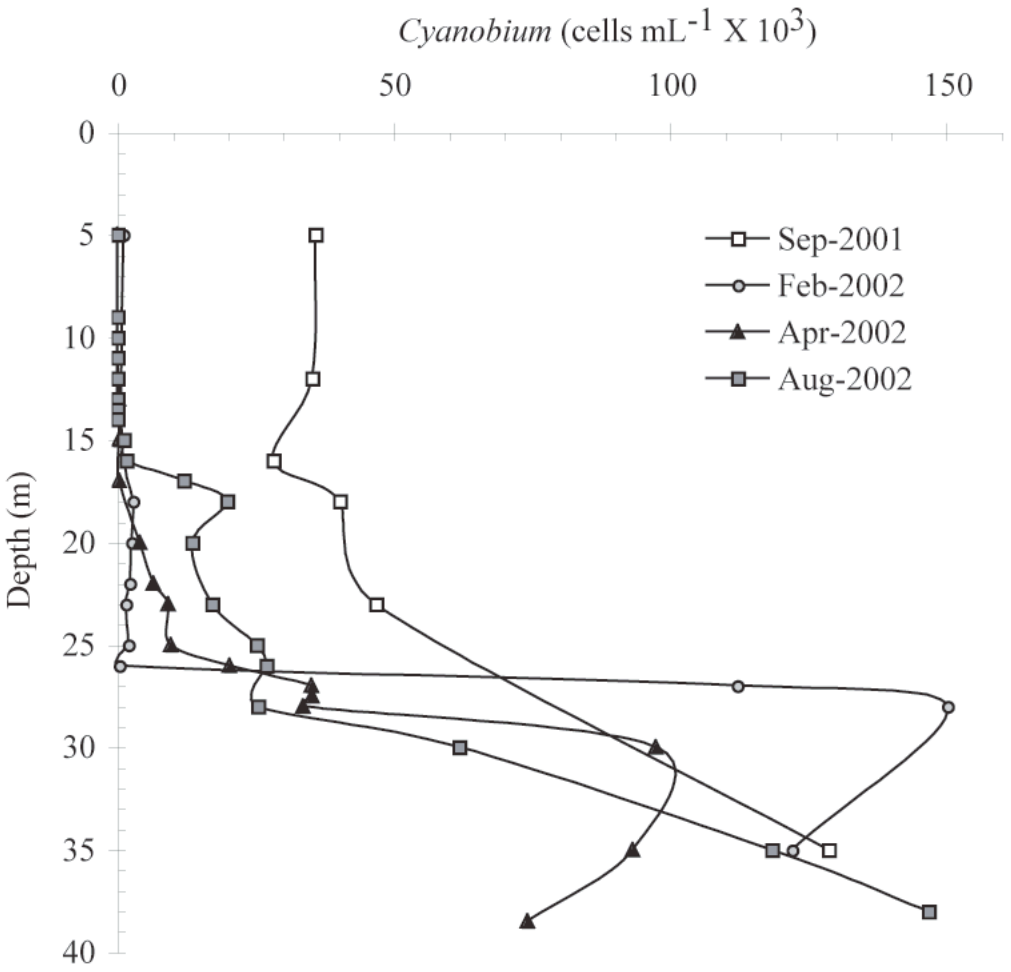

Fig. 3. Seasonal abundance of *Cyanobium*. Note the high abundance of cells in the aphotic region throughout the year, the low number of cells in the surface layer winter/spring/summer, and the autumn bloom.

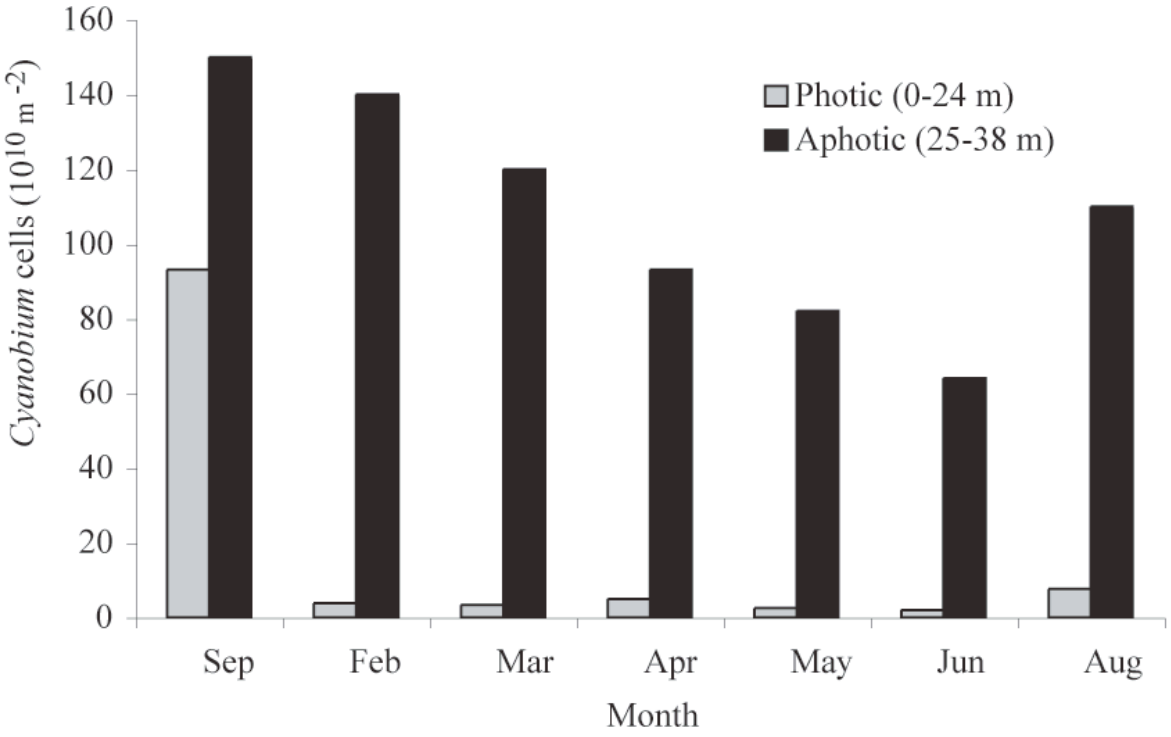

Fig. 4. Total abundance of strain MLCB cells in the photic (24 m^3) and aphotic zones of the water column (14 m^3). Note that the population of cells in the photic zone is never greater than in the aphotic zone and that the aphotic population slowly decreases until late summer, early autumn.

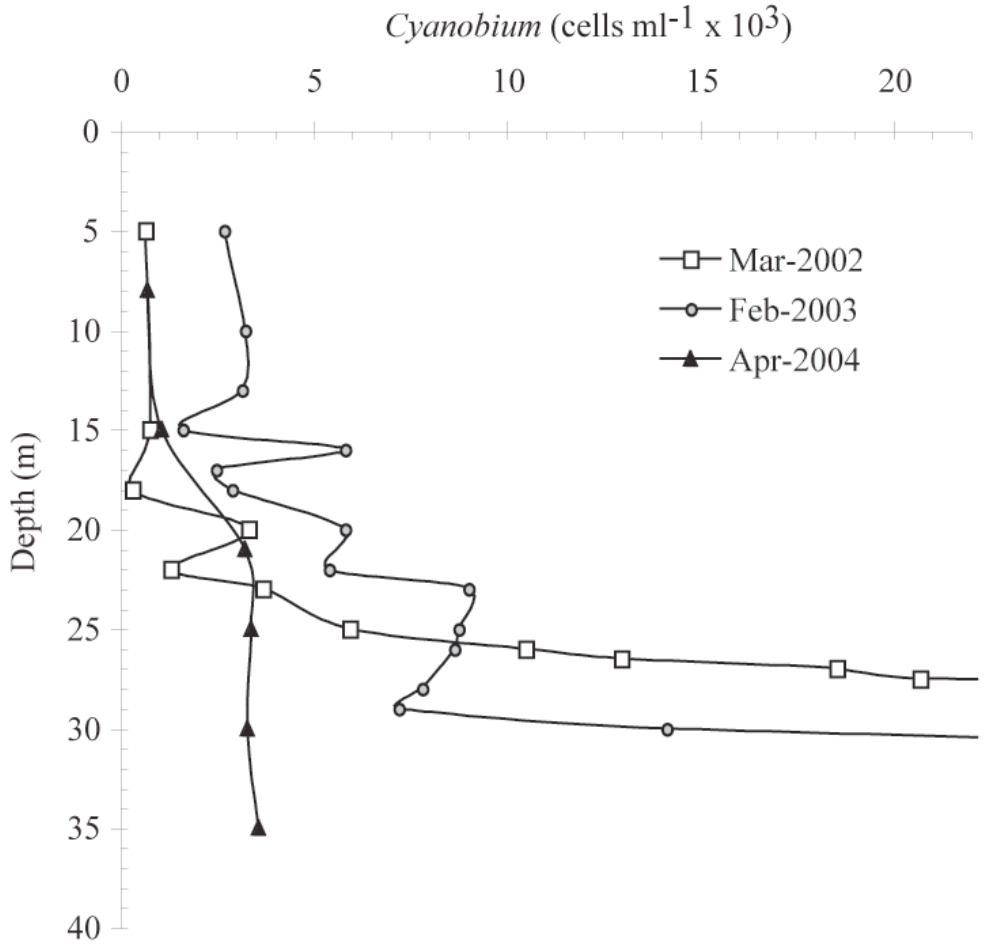

Fig. 5. Total number of strain MLCB cells in the entire lake using bathymetric data to correct for volumes associated with photic and aphotic zones. Note that the total number of cells in the photic zone is greater (Sept.) then that of the aphotic zone.

wide population of strain MLCB in the two layers. This calculation showed that abundance was only greater in the photic zone than in the aphotic zone in autumn (Fig. 5). The lake, which had been meromictic for 8 years, mixed completely in November of 2003. Comparing strain MLCB abundance from the following April (2004) with that of the previous two springs showed a large decrease (90%) in total abundance, mostly due to loss from the aphotic zone (Fig. 6 and 7). Strain MLCB was also enumerated in samples from a sediment core, where it was found from 0 to 4 cm at a concentration of 1.2×10^6 cells mg dw^{-1}.

Phylogenetically, strain MLCB grouped with the picophytoplankton clade (*sensu* Urbach *et al.*, 1998), thus being related to *Prochlorococcus* and marine *Synechococcus*-type strains. Analysis of 16S rDNA (Fig. 8) and the 16S-23S ITS sequences (data not shown) indicates the isolate to be a member of the genus *Cyanobium*, grouping most strongly with MBIC 10613, isolated in Kagawa, Japan (AB183569). The less conserved 16S-23S ITS region offers higher sequence divergence between strains, which would be valuable for phylogenetic comparisons of the closely related *Cyanobium* strains, but because of the small size of the ITS database, 16S rDNA provided a more informative phylogeny. Tree topology generally agreed with previous studies (Crosbie *et al.*, 2003; Ernst *et al.*, 2003) but with a few minor differences. Such as the Antarctic strains of clade V grouping within the *Cyanobium* instead of being a separate clade while strains LBP1 and MW28B3 did not group with clade III as shown previously. Because of the high similarity of the 16S rDNA gene among the *Cyanobium*, tree construction is very sensitive to number of species included, the treeing algorithm used, and software chosen. Salinity tolerance and habitat characteristics of isolates were not strongly correlated with genotypic distributions, but some inferences can be made (see Discussion).

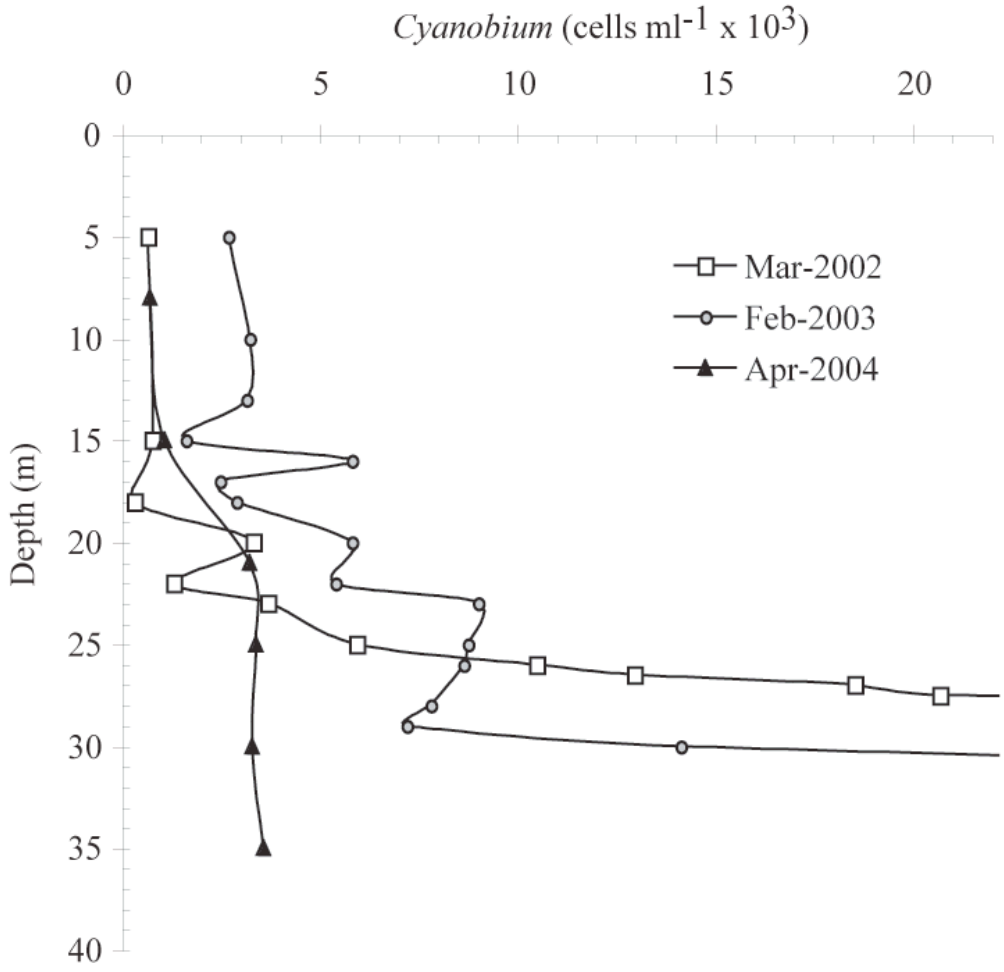

Fig. 6. Vertical distribution of strain MLCB over three consecutive winters.

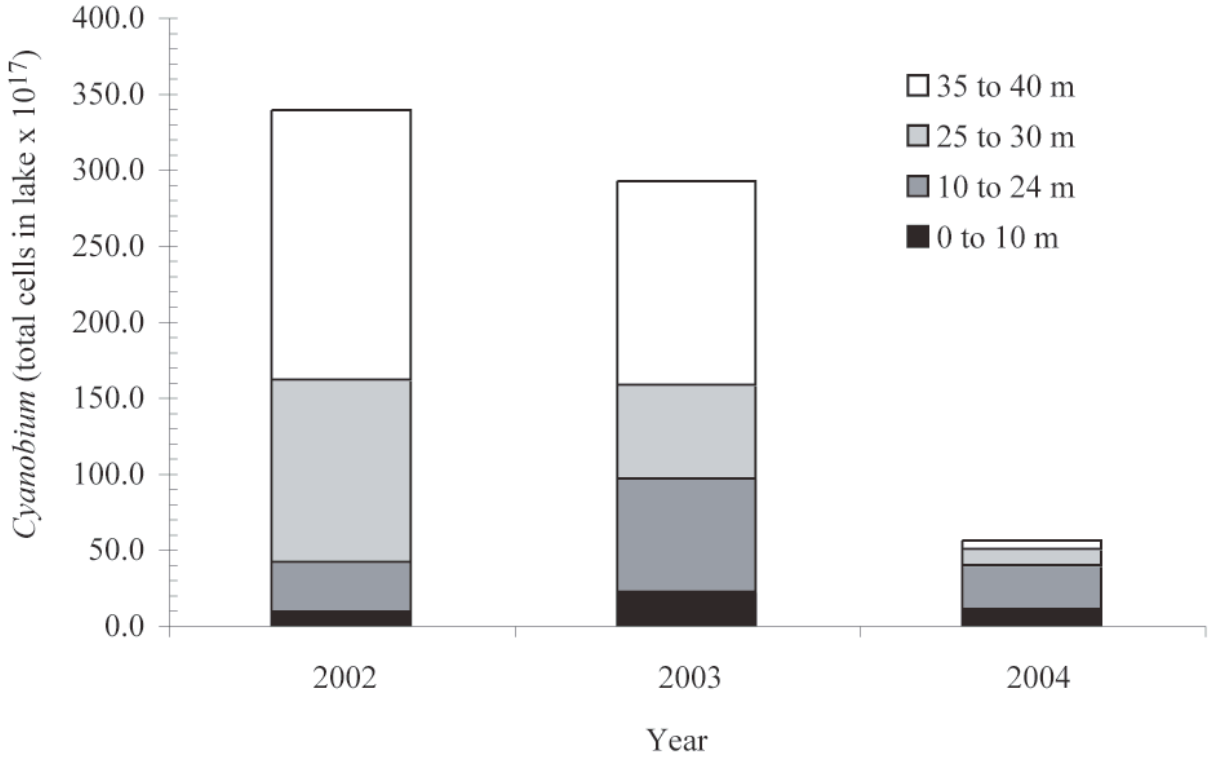

Fig. 7. Total number of strain *Cyanobium* cells in Mono Lake over three consecutive winters.

Fig. 8


PS838, Russian Coast, marine, AF448068
Cyanobium 'Bright', AY172837
HOS, Iriomote Is., Japan, brackish (marshland), AF448064
TAGS, Nagasaki, Japan, brackish (marshland), AF448067
T7cc1, Tokunoshima Is., Japan, brackish (estuary), AF448061
PCC 7001, Long Island Sound, brackish (mud), PE, AB015058 *
MBIC 10456, Okinawa, Japan, brackish (mud), AB058264
CCY 9504 (BS 4), Bornholm Sea, saline, PC, AF330252 *
CCY 9505 (BS 5), Bornholm Sea, saline, PE, AF330253 *
MH301, L. Mondsee, Austria, fresh, PE, AY224199
MBIC 10089, marine, AB058226
UBR, Iriomote Is., Japan, brackish, AF448063
LBG2, L. Biwa, Japan, fresh, PE, AF330249
LBB3, L. Biwa, Japan, fresh, PE, AF330250
uncultured bacterium, L. Kasumigaura, Japan, fresh, AB154318
PS717, L. Biwa, Japan, fresh, PE, AF216953
PCC 9005, pond, Thailand, fresh, PC, AF216950
BO 9404, L. Constance, central Europe, fresh, PE, AF317075
SAG 3.81, L. Zurich, Switzerland, fresh, PE, AF317076
MW74D6, L. Hallstattersee, Austria, fresh, PE, AY151242
MW73B4, L. Hallstattersee, Austria, fresh, PE, AY151250
BO 8807, L. Constance, central Europe, fresh, PE, AF317074 *
uncultured bacterium, L. Kasumigaura, Japan, fresh, AB154316
LB03, L. Biwa, Japan, fresh, AY183115
PS680, L. Biwa, fresh, AF216951
MH305, L. Mondsee, Austria, fresh, PE, AY224198
TAG, AF448066
PCC 6307 (C. gracile), lake, Wisconsin, fresh, PC, AF001477 *
BO 981502, L. Constance, central Europe, fresh, PC, AF317077
PS715, L. Akan, Japan, fresh, PE, AF216952
PCC 8966, Mediterranean, France, brackish (marshland), AF448072
NEIS 42, L. Kasumigaura, fresh, AB001724
PCC 7009, brine pool, California, saline, PC, AF216945
GL 150636, L. GrandLieu, France, AJ133176
PCC 7920, pond, Tolsa, Finland, PC, AF216948
CCY 0011, Schiermonnikoog, Netherlands, marine, PC, AY183114 *
MBIC 10007, Japan coast, marine, AB058205
ACE, Ace Lake, Antarctica, saline, PE, AF098370
PENDANT, Pendant Lake, Antarctica, saline, PE, AF098371
ABRAXAS, Lake Abraxas, Antarctica, saline, PE, AF098372
PS845, Russian Coast, marine, AF448070
MW97C4, L. Mondsee, Austria, fresh, PC, AY151251
P211, Bylot Island tundra pond, Artic, PC, AF098373
BO 8805, L. Constance, central Europe, fresh, PC, AF317073
WH 5701, Long Island Sound, marine, PC, AY172832
BO 0014, L. Constance, fresh, PE, AF330251
PS840, Russian Coast, marine, PC, AF448069
MBIC 10459, Okinawa, Japan, marine, AB058265
LBP1, L. Biwa, Japan, fresh, PE, AF330247 *
MW28B3, L. Mondsee, Austria, fresh, PE, AY151235
Minos01, Mediterranean Sea, marine, PE, AY172806
Minos11, Mediterranean Sea, marine, PE, AY172807
ML Cyanobium, Mono Lake, California, saline, PE *
uncultured bacterium MLS1228cl3, Mono Lake, California, saline, AF400152
MBIC 10613, Kagawa, Japan, brackish (mud), AB183569
Marine Synechococcus / Prochlorococcus
PCC 6301, PCC 7942, M. holsatica
65%
67%
70%
75%
55%
66%
98%
65%
99%
72%
I
II
III
IV
V
VI
VII
0.01

Fig. 8. Phylogenetic inference of the picophytoplankton clade (*sensu* Urbach *et al.*, 1998) based on 16S rDNA (>1200 bp). Trees were constructed used neighbor-joining, bootstrap (1000 replicates) analysis within the ARB program. Numbers at nodes indicate percent bootstrap support for that branching pattern. Terminal branches display the strain information; location, water type, dominant phycobilin, and GenBank accession number. Asterisk (*) indicates strains used for salinity tolerance experiment. Bracketed clades represent strains that had high bootstrap support (>50%). Strain information came from the following sources; Crosbie et al. (Crosbie *et al.*, 2003), Ernst et al. (Ernst *et al.*, 2003), Pasteur Culture Collection (France), and Marine Biotechnology Institute Culture collection (Japan).

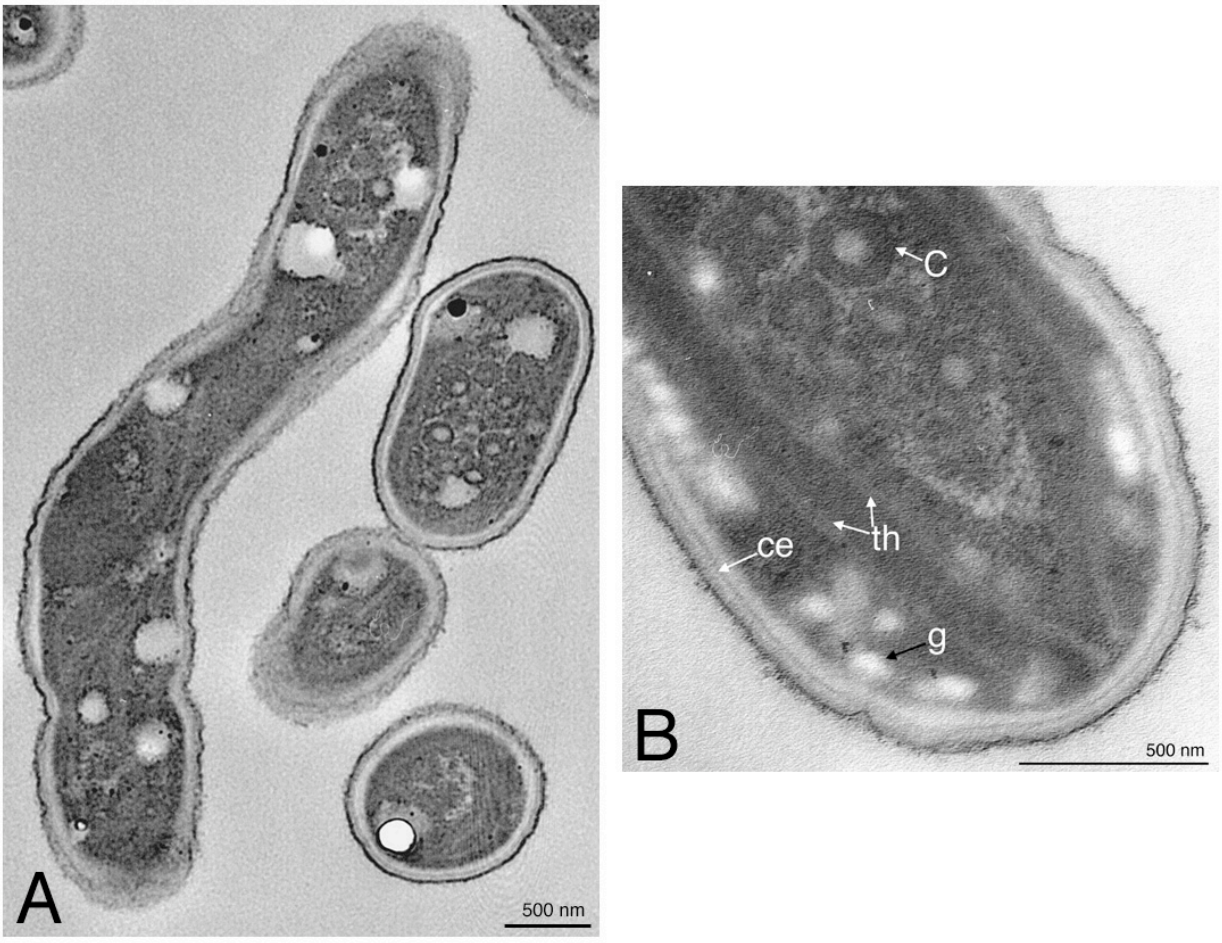

Fig. 9. Transmission electron micrographs of strain MLCB. (A) Dividing and elongated cell from a stationary phase culture, (B) Internal structures of the isolate. Carboxysomes (c), cell envelope (ce), thylakoids (th), and glycogen deposits (g).

Examination of field (not shown) and cultured cells by epifluorescence microscopy and TEM (Fig. 9) show strain MLCB to be 2 x 1 μm and to divide by binary fission. In culture, the cells are capable of elongated growth, where a long (10 μm) tube-like cell is present (Fig. 9 A). These cell morphologies occur at all salinities and temperatures, but are only found in stationary phase.

In vivo absorption spectra of exponentially growing cells in freshwater medium indicated PE as the dominant chromophore (Fig. 10). At low light intensities (<75 μE^{-1} m^{2-1} s^{-1}) and low salinities (<5%) cells retained a dark red color. At high light intensities (>100 μE^{-1} m^{2-1} s^{-1}) and high salinities (>8%) cells became yellow or light brown typical of cell stress. In comparison to other described PE-rich *Cyanobium* isolates (Ernst, 1991) strain MLCB showed a much higher absorbance at 500 nm in relation to the phycoerythrin peak at 575 nm. This could indicate a higher concentration of carotenoids than the other isolates. The isolate did not contain PUB and was not capable of complementary chromatic adaptation (CCA); the organism grew well in green light and slightly slower in red light while maintaining its PE to PC ratio. Currently, all described *Cyanobium* isolates are incapable of CCA. Instead, it appears that permanently down-regulating or deleting the PE operon is more favorable for these cyanobacteria. This is emphasized by the genetic and habitat similarity between PE-rich and PC-rich isolates (see Fig. 8, Bornholm sea strains).

Strain MLCB is euryhaline, growing from 0% to 10% salinity with a maximum growth rate of 0.45 d^{-1} at 3% and a minimum (measurable) growth rate of 0.15 d^{-1} at 8% (Fig. 11). Growth occurs at all light intensities tested (<2 to 200 μE^{-1} m^{2-1} s^{-1}) with a maximum of 0.45 d^{-1} at 40 μE^{-1} m^{2-1} s^{-1}, decreasing to 0.15 d^{-1} at 200 μE^{-1} m^{2-1} s^{-1} (Fig. 12). Maximal growth

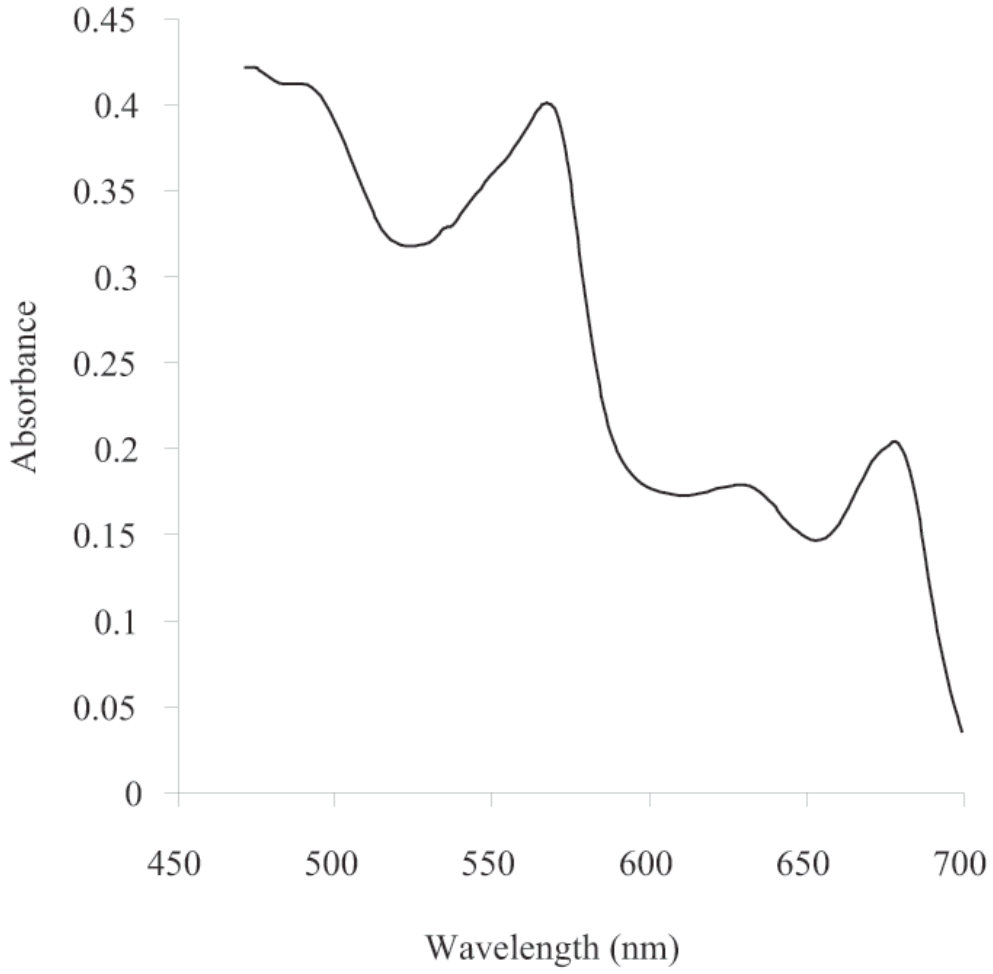

Fig. 10. *In vivo* adsorption spectra of strain MLCB. Absorbance peaks (nm) correspond to: chlorophyll *a* (679), phycocyanin (620), phycoerythrobilin (575), and cartinoids (~500).

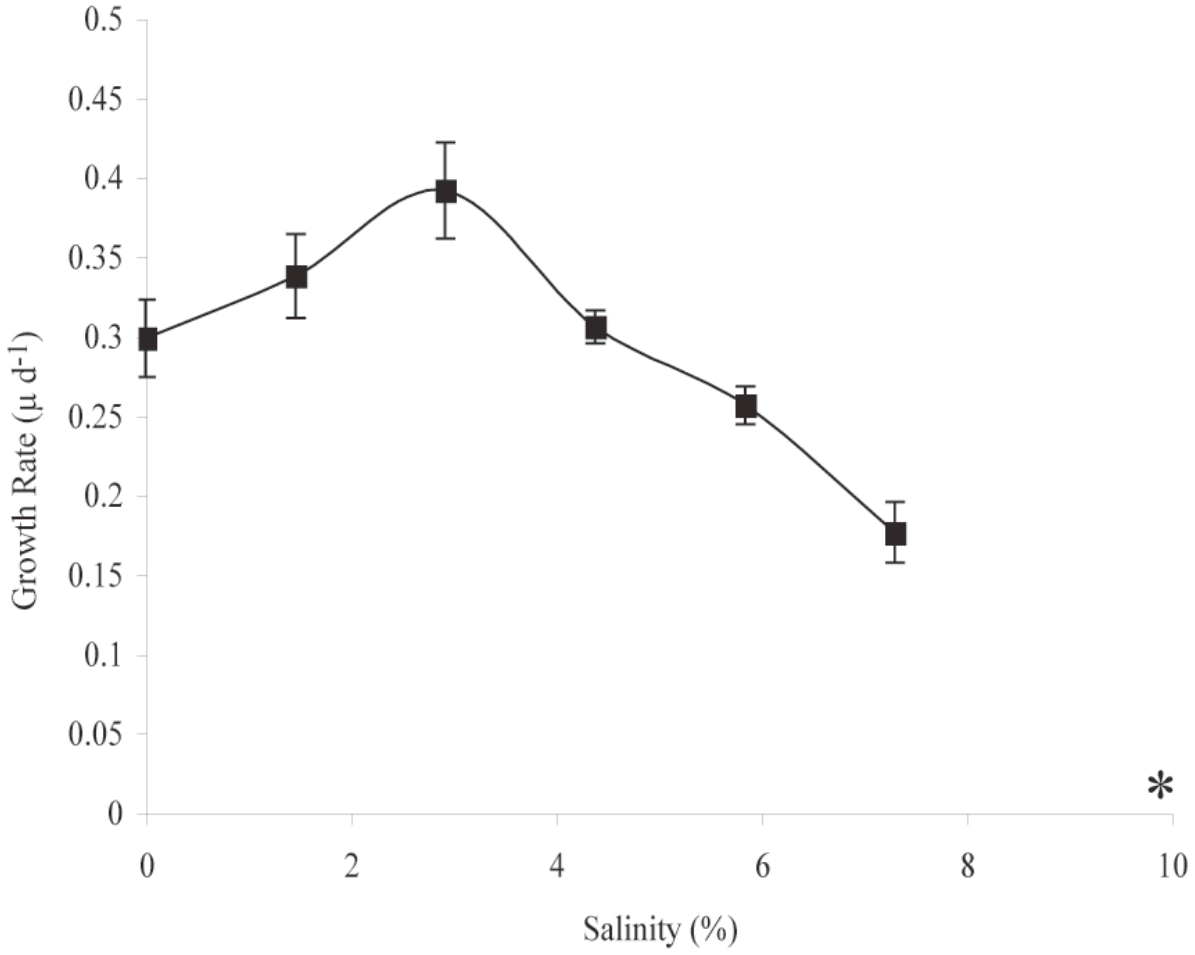

Fig. 11. Effect of salinity on the specific growth rate (μ d^{-1}) of strain MLCB. Data are means +/- SD (*n* = 3). Asterisk (*) indicates visible growth that was not measurable.

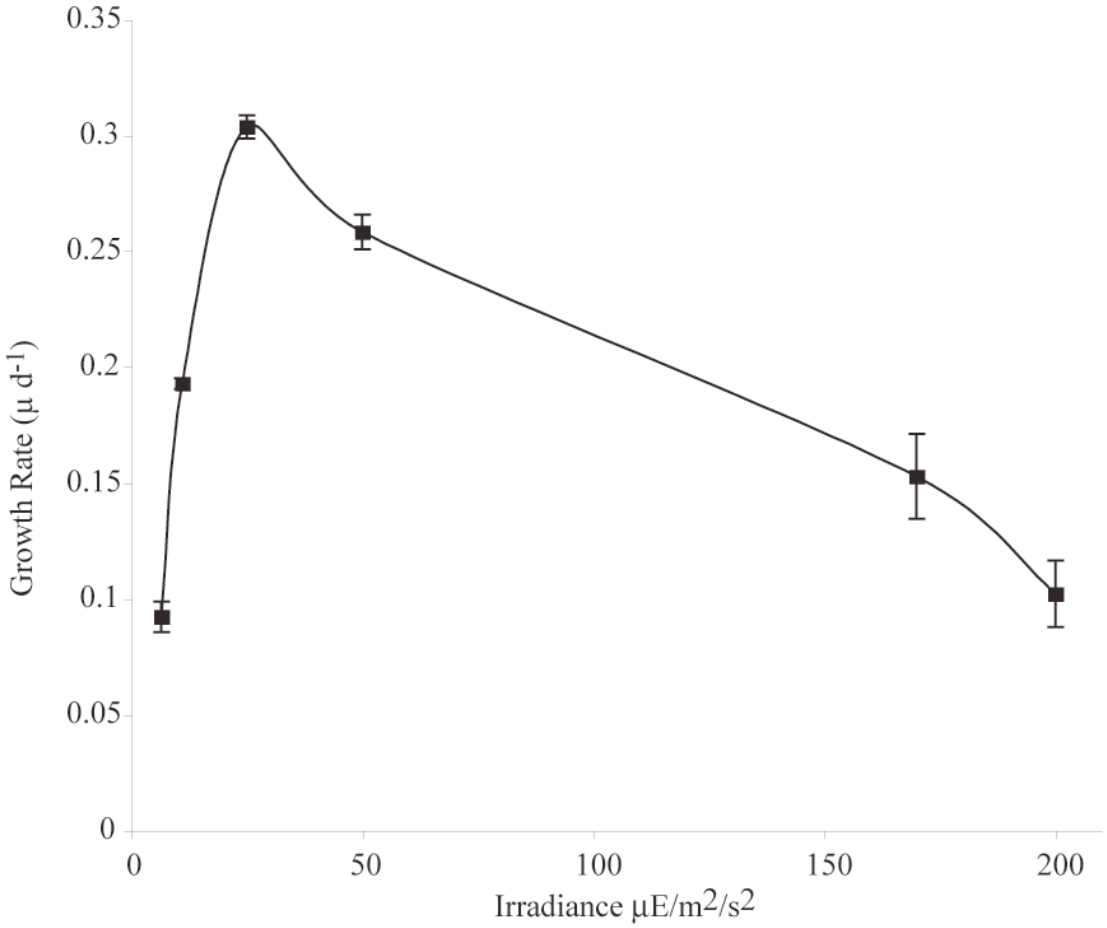

Fig. 12. Effect of light intensity on the specific growth rate (μ d^{-1}) of strain MLCB. Data are means +/-SD (*n* = 3).

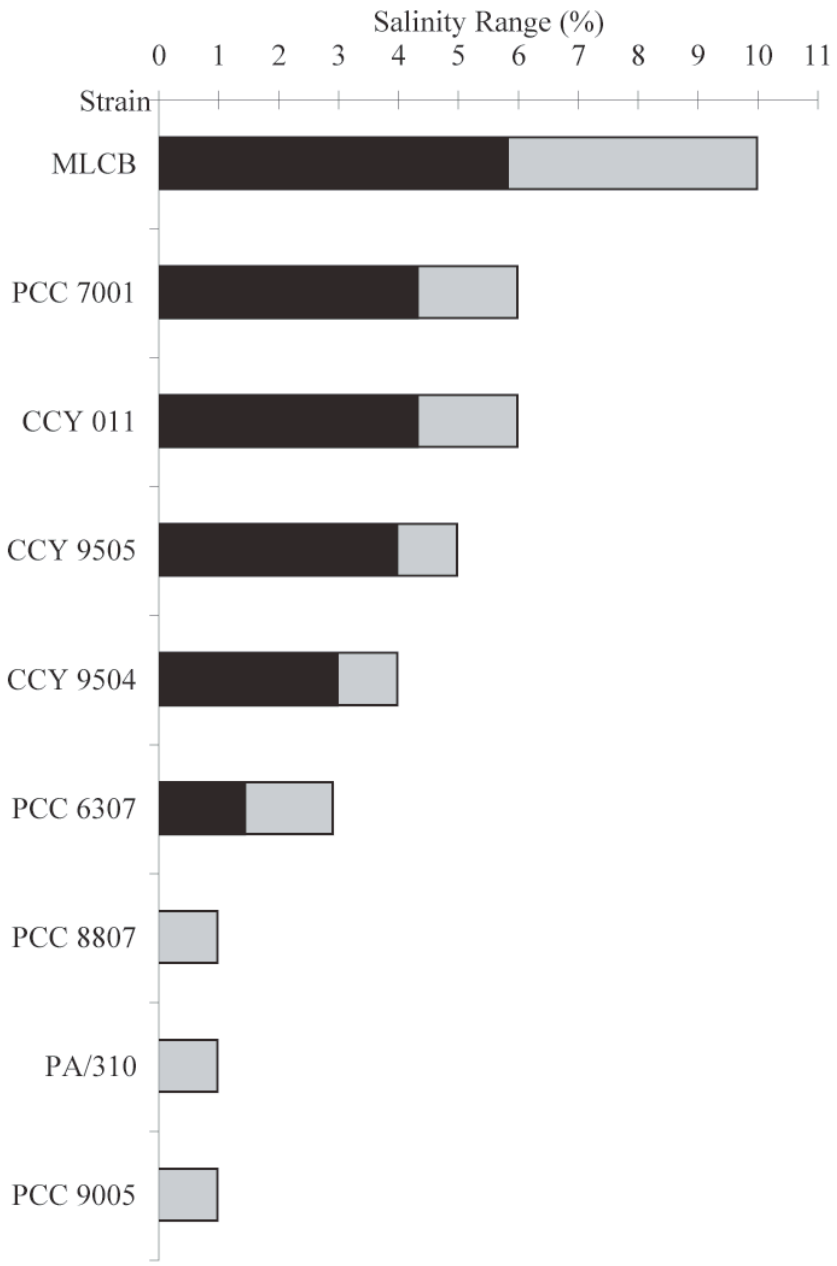

Fig. 13. Optimal growth (black bar), defined as salinities where growth rates were above 0.2, suboptimal growth (gray bar), where growth is positive, but obviously (<50%) less than optimal.

rates occur at ~20°C (4°C, 20°C, and 27°C were tested) and at temperatures above 27°C growth rates decrease rapidly along with the onset of noticeable stress (pigment loss) to the culture. The organism can perform oxygenic photosynthesis in up to ~400 μM total sulfide at a pH of 8.0, which is the typical concentration at which photosystem II is inhibited in cyanobacteria (Padan and Cohen, 1982). For comparison, salinity tolerances of 7 other *Cyanobium* strains were determined (Fig. 13). Strains isolated from salt waters showed the highest growth rates at 1.5 to 3% salinity. Strains from fresh water grew best when no salt was present.

Strain MLCB was not capable of dark chemoorganotrophic growth either aerobically or anaerobically using glucose, fructose, sucrose, yeast, or peptone as substrates. However, cells placed in the dark without a carbon source turned from dark red to pale green/yellow indicating the degradation of phycobilins, a common cyanobacterial reaction to stress, while the cells given sucrose remained dark red.

CHAPTER 4

DISCUSSION

Seasonal counts of strain MLCB show it reaches its highest concentrations in the aphotic/anaerobic zone of the lake (Fig. 3). Studies of other lakes have shown the presence of Pcy below 0.1% surface irradiance, but have not demonstrated year round concentrations 2 to 10 times that of concentrations in the photic region (Craig, 1987; Detmer *et al.*, 1993; Malinsky-Rushansky *et al.*, 1997; Padisak *et al.*, 1997). Three years of epifluorescence counts during meromixis show the organism to bloom in surface waters in late summer and early autumn, while remaining practically undetectable in photic zone waters from late winter through the summer. Such seasonal growth patterns are not uncommon for Pcy (Stockner *et al.*, 2000). As solar radiation increases in spring and nutrients are plentiful from winter mixing, eukaryotic algae out-compete strain MLCB and bloom. But as nutrients become exhausted by summers end and *Picocystis* concentrations are reduced by grazing, strain MLCB is able to out-compete eukaryotes and bloom in the upper 10 meters. As mixing continues through the autumn and winter, nutrients increase, eukaryotic algae resume their dominance, and strain MLCB concentrations decrease substantially in the photic zone while concentrations in the aphotic remain high.

Drought and water diversions can trigger the complete overturn (holomixis) of Mono Lake. Holomixis occurred in late October to early December 2003, for the first time since 1995. In November of 2003, concentrations of strain MLCB in the photic zone were high, consistent with the autumn bloom of previous years. But, when comparing cell numbers of April 2004 with those of spring 2002 and 2003, an obvious decrease in strain MLCB populations is evident (Figure 6 and 7). The population decrease was most pronounced in the aphotic zone, while

abundance in the photic zone was comparable to previous years. Lake turnover appears to eliminate the pool of deep-water MLCB cells, perhaps by mixing them into the surface layer where they are unable to compete successfully for nutrients and are subject to increased predation. Mono lake also mixed in 2004, and although I do not have strain MLCB abundance data from that autumn or following spring, I was able to obtain counts for September 2005, showing the typical autumn bloom profile, with 6.0×10^4 cells mL^{-1} in the photic region and 1.5×10^5 cells mL^{-1} cells in aphotic waters. It appears that even though winter overturn eliminates the bottom water population, come the following autumn bloom the cells are replenished.

A definite explanation for high deep water MLCB concentrations is not known and will most likely be the result of more then one contributing force. I can only offer the following 4 possible scenarios that might contribute to the build up of cells.

(1) Sinking of cells is an obvious explanation for the elevated concentrations of strain MLCB found in the aphotic zone. Although extremely small, with a negligible sinking velocity, Pcy are known to sink, possibly by attaching to detritus or fecal pellets thus increasing their sedimentation rate (Silver and Alldredge, 1981; Lochte and Turley, 1988; Simon *et al.*, 2002). For strain MLCB to achieve such concentrations in aphotic water by sinking at a constant rate until reaching the sediment requires the photic water above to have a higher number of cells at one point in the year. Comparing the total number of cells in the photic zone with those in the aphotic zone (cells/m^3) over the year failed to indicate a time when the photic region population was greater then the aphotic (Figure 4). But the data did reveal the slow loss of cells in the dark waters and their sudden replenishment come late summer, suggesting that a least part of the deep water population is dependent on growth in the photic zone above.

(2) Littoral transport and sediment re-suspension are other possible mechanisms for the build up of cells in the deep water. Applying a correction for lake morphology using bathymetric data allows the total number of cells in the photic region to out-number the total cells in the aphotic (Figure 5). This implies that it is possible for sedimentation alone to supply enough cells to the aphotic region but it requires horizontal transport from edge waters. Mono Lake has strong (6-8 m s^{-1}) diurnal winds for much of the year, setting up cyclonic flows and boundary mixing which could support horizontal transport into the study site (MacIntyre *et al.*, 1999).

(3) Besides horizontal transport, continuous production in the photic zone combined with a decreased sedimentation rate below the chemocline might also account for the concentrations found in the deep water. A slight density increase is seen below 25 meters corresponding with the increase in abundance of strain MLCB. Sedimentation rate differences throughout the water column are not known. Interestingly, eukaryotic algae would also be predisposed to sinking, littoral transport, and sediment re-suspension, but they do not show a higher concentration in the deep water.

(4) Chemoorganotrophic growth is another possible explanation for the high concentrations of strain MLCB in the aphotic zone. Some cyanobacteria are capable of dark chemoorganotrophic growth on exogenous carbon sources using the oxidative pentose-phosphate cycle, particularly filamentous and benthic cyanobacteria (Pelroy and Bassham, 1972). Strain MLCB was not capable of dark chemoorganotrophic growth. But, the isolate appears to be capable of using sugars for cell maintenance, particularly of pigment composition. It is of course possible that an unknown growth factor is required to stimulate chemoorganotrophic growth.

Part of the reason for higher deep-water cell concentrations of strain MLCB and not of the eukaryotic algae could be due to a difference in cell structure and physiology. For example,

its small size might allow for not only increased nutrient uptake rates but also a reduced sedimentation rate. Additionally, strain MLCB might have the ability to significantly reduce its mortality rate in sulfidic aphotic waters, possibly by entering a dormant state and maintaining cellular integrity via the uptake of organic nutrients. Lastly, selective predation pressures could also be responsible for contrasting distributions of strain MLCB and eukaryotic algae. Grazing studies at Mono Lake have been limited to the surface waters and have mainly focused on *A. monica* (Conte *et al.*, 1988). Protozoan grazing has not been examined, nor has grazing of any sort in the anoxic waters.

Many biological, chemical, and physical processes can influence cryptic Pcy populations. Determining the factors contributing to their presence will require multiple angles of research. For example, detailed physiological comparisons between prokaryotic and eukaryotic algae under dark/anoxic conditions including selective predation could reveal what, if any, adaptive advantage Pcy have. Also, an exhaustive quantification of Pcy abundance is needed that will provide a clear picture of horizontal and vertical distribution seasonally and during contrasting mixing regimes. Physical processes such as boundary mixing, internal waves, gyres, and sediment-water interface transport should also be addressed.

Salinity tolerance of strain MLCB is a central physiological trait that separates it from the eukaryotic algae of Mono Lake and to other members of the genus *Cyanobium*. Strain MLCB is able to grow in salinities up to 10%. *Picocystis* grows in salinities up to 26% (Roesler *et al.*, 2002) and experimental mesocosm studies of benthic diatoms from Mono Lake showed activity at 15% (Herbst and Blinn, 1998). The mesocosm studies also suggested a decrease in algal species diversity as salinity levels go over 5%. Strain MLCB's lower tolerance to salinity could hamper its ability to compete with the other algae. The increase in salinity of Mono Lake over

the past 50 years could also be responsible for the fact that only one dominant Pcy ecotype is appears to be present.

Cyanobium salinity tolerances can be separated into 4 phenotypes based on my data. Those not growing over 1%, those tolerating > 1% but <3%, those tolerating up to 6%, and those growing above 6%. There does not appear to be an obvious connection between habitat and 16S rDNA phylogeny, as freshwater strains group with marine or brackish strains. Although, as shown with PCC 6307, isolation of a strain from a freshwater environment does not preclude it from having the ability to adapt to elevated salinity and, unlike the trait of being either PC-rich or PE-rich, there is a range of salinity tolerances. Examining the phylogeny of the currently available sequences, one can make inferences about the salinity tolerances of certain clades. Clade I contains PC-rich strains from marine or brackish habitats while Clade III contains PE-rich strains from freshwater lakes. BO 8807 (Clade III) was found to be inhibited by the addition of salt (Fig. 13), probably making this clade truly 'freshwater'. Clades IV and V emphasize the close genetic relationship between freshwater and halotolerant strains. Whether all these freshwater strains are 'truly' freshwater phenotypes or whether they still maintain some capability for osmoadaptation like PCC 6307 is not known. Clades II and VI demonstrate a clear division between freshwater and salinity tolerate species, including LBP1 which is 'truly' freshwater, further emphasizing rapid diversification of the Pcy and the need for studies to resolve the phenotypic adaptations of these closely related strains. As mentioned earlier, the outcome of a phylogenetic analysis is somewhat dependent on the data set used. Isolation of *Cyanobium* strains will continue, altering the structure and composition of clades, leading us to modify our assumptions about the forces that drive ecosystem-dependent radiations.

Based on the range of salt tolerances and the fact that there is no definite phylogenetic correlation with salinity tolerance, it can be assumed that the ability to withstand elevated salinity has been lost or modified repeatedly in the evolution of the *Cyanobium* and perhaps involves multiple operons. Osmoadaptation can be separated into low or high salinity (>5%) responses (Galinski, 1995). Low salinity responses involve the increase in cytoplasmic levels of potassium, glutamate, trehalose, glycine betaine, and other sugars, as well as an increase in specific periplasmic porins. High-level responses involve not only a change to the cytoplasmic and interior of the cell and the periplasmic space, but also to the outer membrane by substituting anionic phospholipids for neutral phospholipids. Such membrane adjustments could separate halotolerate from halophilic type organisms. Given the high similarity of *Cyanobium* strains (>97% 16S rDNA) and their relatively simple genomes, it would be beneficial for the study of bacterial osmoadaption to see what genetic modifications are responsible for the differences in salinity tolerance among members of this genus. It is not known whether genes have been completely lost in euryhaline strains, or whether they have been modified, or a combination of both. For example, it is possible that strain MLCB has retained certain genetic elements and/or subsequently altered these to handle higher salt tolerances and that organisms like PCC 6307 have lost certain genetic elements and/or modified them to be more competitive in freshwater. Understanding the evolution of salinity tolerance mechanisms in the genus *Cyanobium* requires applying molecular techniques (whole genome sequencing, 2-D protein analysis) along with the cultivation and comparisons of isolates from diverse habitats. Connecting physiological attributes of related species with their corresponding genetic signature could allow us to determine the environmental factors that influence speciation within a genus and also the evolution of ecologically significant biochemical pathways.

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