Microbial Ecology

Vertical Distribution of Nitrogen-Fixing Phylotypes in a Meromictic, Hypersaline Lake

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Abstract

We investigated the diversity of nitrogenase genes in the alkaline, moderately hypersaline Mono Lake, California to determine (1) whether nitrogen-fixing (diazotrophic) populations were similar to those in other aquatic environments and (2) if there was a pattern of distribution of phylotypes that reflected redox conditions, as well as (3) to identify populations that could be important in N dynamics in this nitrogen-limited lake. Mono Lake has been meromictic for almost a decade and has steep gradients in oxygen and reduced compounds that provide a wide range of aerobic and anaerobic habitats. We amplified a fragment of the nitrogenase gene (*nifH*) from planktonic DNA samples collected at three depths representing oxygenated surface waters, the oxycline, and anoxic, ammonium-rich deep waters. Forty-three percent of the 90 sequences grouped in *nifH* Cluster I. The majority of clones (57%) grouped in Cluster III, which contains many known anaerobic bacteria. Cluster I and Cluster III sequences were retrieved at every depth indicating little vertical zonation in sequence types related to the prominent gradients in oxygen and ammonia. One group in Cluster I was found most often at every depth and accounted for 29% of all the clones. These sequences formed a subcluster that contained other environmental clones, but no cultivated representatives. No significant nitrogen fixation was detected by the $^{15}N_2$ method after 48 h of incubation of surface, oxycline, or deep waters, suggesting that pelagic diazotrophs were contributing little to nitrogen fluxes in the lake. The failure to measure any significant nitrogen fixation, despite the detection of diverse and novel nitrogenase genes throughout the water column, raises interesting questions about the ecological controls on diazotrophy in Mono Lake and the distribution of functional genes in the environment.

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Introduction

Mono Lake is an alkaline (pH 9.8), hypersaline (75–85 g/kg) lake at an elevation of 1945 m on the eastern slope of the Sierra Nevada (38°N, 119°W) in California. The Mono Basin was formed by tectonic faulting of the eastern Sierran escarpment and is the site of recent volcanic activity. The salinity of the lake doubled following diversions of inflowing streams out of the basin in 1941 and subsequent decreases in lake volume. Unusually high runoff resulting from El Niño climate conditions [19] and reduction in water diversions led to a rapid rise in lake level in 1995 and the onset of persistent chemical stratification, or meromixis. The absence of an annual period of holomixis has led to persistent anoxia and the accumulation of ammonium and sulfide in the bottom water (>25 m), whereas the upper layer undergoes seasonal thermal stratification accompanied by a pronounced oxycline developing in late spring.

In Mono Lake, high annual rates of primary productivity (270–1050 g C $m^{-2}y^{-1}$) are sustained by internal recycling of nutrients [15]. Soluble reactive phosphorus is superabundant (350–450 μM) in the lake, and dissolved inorganic nitrogen (DIN), which varies seasonally and interannually, often limits primary productivity [15, 17]. Stream inputs of DIN are low and the lake DIN pool is dominated by ammonia–ammonium as the nitrate plus nitrite pool is always low [18]. An earlier study [15] suggested ammonia supply from upward vertical fluxes and *Artemia* excretion could meet algal demand during much of the seasonal periods of thermal stratification, but more recent analysis of sestonic particulate ratios collected over the 11-year period from 1990 to 2000 are indicative of

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strongly nitrogen-limited algal growth during the summer and moderate nitrogen limitation during several winters following the onset of meromixis in 1995 [17]. Despite overall nitrogen limitation and very low ($\ll 1$) dissolved inorganic N:P ratios [17], an anecdotal report of failure to detect nitrogen fixation in the water column of Mono Lake [26] suggested that nitrogen fixation by planktonic prokaryotes may be relatively unimportant in this environment. Nitrogen fixation was measured, however, in epibenthic detritus and algal aggregates in the littoral zone [26], demonstrating that the unusual chemistry of the lake is not incompatible with diazotrophy. In this study, we revisited the issue of nitrogen fixation in Mono Lake from the molecular ecological perspective.

Microbial ecologists have learned much about the composition and dynamics of microbial populations in many freshwater, estuarine, and oceanic waters using molecular approaches based on rRNA sequences [6–8, 14, 24]. By focusing on the analysis of functional genes involved in specific metabolic pathways, the distribution and diversity of microbes involved in various steps of the nitrogen cycle, including nitrogen-fixing prokaryotes (or diazotrophs), have also been studied [45]. Molecular ecological investigations of diazotrophs have naturally focused on the genes encoding nitrogenase, which catalyzes the reduction of atmospheric nitrogen gas (N_2) to ammonium [45]. Nitrogenase is a multisubunit protein, encoded by the *nifH*, *nifD*, and *nifK* genes, that is widely distributed among prokaryotic phyla. Nitrogenase activity is sensitive to oxygen inactivation, and nitrogen fixation is energetically expensive, requiring 16 ATP and 8 reducing equivalents per molecule of N_2 fixed to NH_3. As a result, the nitrogenase genes are tightly regulated in response to oxygen and ammonium concentration in many species [12].

The pronounced chemical and physical gradients in Mono Lake make it an interesting habitat in which to investigate how environmental factors regulate the distribution and activity of N_2-fixing microorganisms. Ribosomal RNA studies have shown that the lake harbors unique, vertically stratified microbial assemblages [11, 14, 34]. The objectives of this study were to determine whether the genetic capacity for nitrogen fixation is present in the plankton of Mono Lake using the *nifH* gene as a diagnostic indicator, to compare the diversity and phylogenetic relationships of *nifH* sequences retrieved from Mono Lake with those from other environments, and to determine whether the pronounced chemical and physical gradients in Mono Lake affect the distribution and activity of N2-fixing microorganisms.

Materials and Methods

Sample Collection. Water samples for DNA extraction were collected on 18 May 2000 at station 3 (Fig. 1) from depths of 2, 17, and 26 m using a 5-L Niskin bottle deployed from a small boat. These depths were chosen as representative of the mixolimnion, mid-oxycline, and anoxic monimolimnion, respectively. Water was filtered through 120-μm nylon mesh to remove the invertebrate *Artemia* and collected in acid-washed, collapsible polyethylene containers, which were stored in coolers for transport to the lab. Water samples for measurements of $^{15}N_2$ fixation were collected on 6 Aug 2001 at station 6 (Fig. 1) from depths of 2 m (mixolimnion), 18.5 m (base of the oxycline), and 30 m (monimolimnion). Water samples were transferred to 500-mL polycarbonate bottles using techniques to minimize gas exchange. Silicone tubing attached to the nipple of the Niskin bottle was placed at the bottom of the bottle to reduce oxygenation of samples. The bottle was filled with minimal turbulence and allowed to overflow for three bottle volumes. The tubing was slowly withdrawn as the flow was gradually stopped by pinching the tubing. Bottles were immediately capped with polypropylene screw-caps fitted with silicone septa ensuring that there was no headspace. Samples were placed in a cooler for transport to the lab.

A *Ctenocladus circinnatus* aggregate was sampled from the littoral zone on the southwestern edge of the lake in approximately 10 cm of water. These aggregates are ball shaped tufts comprised of the chlorophyte *C. circinnatus*, a filamentous, nonheterocystous cyanobacterium, and various aerobic and anaerobic bacteria and have previously been shown to fix N_2 [26]. The aggregate was picked from the water with stainless steel forceps, deposited in a Whirl-Pak bag (Nasco), and stored chilled for ~10 h, then transferred to a −20°C freezer.

Hydrography. Conductivity, temperature, and pressure were measured with a SeaBird SeaCat profiler. Oxygen profiles were obtained with an oxygen meter (YSI) using a Clarke-type electrode. Water samples for ammonium analysis were collected at various depths in the water column using a Niskin bottle. Ammonium concentrations were determined by the phenol hypochlorite method [37]. The samples were filtered through 0.2-μm pore-size filters, stored on ice in the dark, and analyzed within 6 h. Replicates of each sample (5 mL) were allocated into separate analysis tubes. Lower water column samples were diluted by a factor of 10 with aerated Mono Lake surface water to conform to standard concentrations dictated by the spectrophotometric method and to eliminate interference from hydrogen sulfide. A reagent blank and standards ranging from 0.5 to 40 μM were prepared using 0.2-μm filtered, N_2-purged Mono Lake surface water and a 1.5 mM ammonium chloride stock solution. A 10-μM spike of ammonium was added to replicate samples from selected depths to evaluate percent recovery (internal standards).

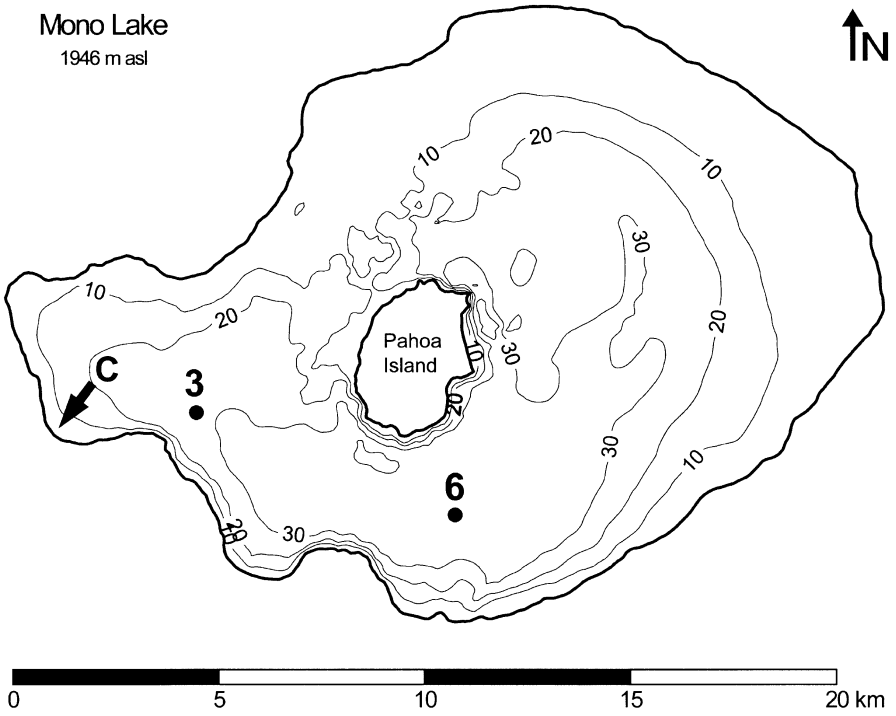

Figure 1. Map of sampling locations. Numbered spots indicate the pelagic stations. Arrow labeled C indicates the collection site for the *Ctenocladus* aggregate. Lake contour lines indicate depth in meters.

DNA Extraction. Water samples for DNA analysis were filtered onto 0.2-μm pore-size polyvinylidene fluoride membranes (Durapore, Millipore) using a peristaltic pump with acid-washed silicone tubing and 47 mm in-line filter holders. Occlusion of the pump was set to allow a maximum pressure of 15 psi. Pumping was continued until the filters clogged. The volumes filtered were 725 mL (2 m), 425 mL (17.5 m), and 400 mL (26 m). Filter holders were disconnected from the tubing and a 60-cc syringe was used to force residual water through the filters. Filters were stored in Whirl-Pak bags with 1 mL of SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris · Cl, pH 7.6) and stored frozen (−20°C, then −80°C). After thawing, lysozyme was added (5 mg/mL, final) and filters were incubated at 37°C for 1 h. Proteinase K (2 mg/mL final) and SDS (0.5% final) were added and the filters incubated at 60°C for 2 h. Liquid was removed from the Whirl-Pak bags, transferred to a 2-mL centrifuge tube, and extracted twice with phenol:chloroform:isoamyl alcohol (PCI; 25:24:1). After a final chloroform:isoamyl alcohol extraction, DNA was concentrated by ethanol precipitation [36].

A subsample of the *C. circinnatus* aggregate was transferred to a microcentrifuge tube with 500 μl 1× TE (10 mM Tris, 1 mM EDTA) and 25 mg of 0.1 mm glass beads which had been acid-washed and baked (350°C, 2 h). Lysozyme and proteinase K treatment were as described above. After proteinase K digestion, 500 μl of PCI was added to the tube and the tube was vigorously shaken in a FastPrep machine (Q-Biogene) for 30 s at 6 m s^{-1}. Tubes were centrifuged for 2 min at ≥16,000 *g*. The aqueous phase was transferred to a fresh tube and the DNA reextracted and precipitated as described above.

Amplification and Cloning of nifH Genes. An internal fragment of the nitrogenase (*nifH*) gene was amplified and cloned using nested PCR as previously described [43]. At a later date, the same DNA extracts were amplified a second time using a newly designed set of outer primers based on the CODEHOP strategy [35]. The primers are named nifH32F (TGAGACAGATAGCTAT YTAYGGHAA) and nifH623R (GATGTTCGCGCGGCA CGAADTRNATSA), where the numbers refer to the position of the 3′ end of each primer relative to the nucleotide numbering for the *nifH* coding sequence of *Azotobacter vinelandii* (GenBank accession no. M20568) and the terminal "F" or "R" letter designates them as forward and reverse primers, respectively. For this second amplification, first-round reactions were prepared in triplicate for each sample. After amplification, the triplicate reactions were pooled. One-microliter aliquots of pooled first-round product for each sample were added to triplicate second-round amplifications using the nifH1 and nifH2 primers [44]. After amplification, triplicate second-round products were pooled. Amplification products were purified in a 1% agarose gel, bands of the expected size were excised, and the DNA was purified using a QIAEX II kit (Qiagen). Gel-purified PCR products were stored at −20°C. The PCR products were cloned into the pGEM-T vector (Promega) using the manufacturer's recommended conditions. Blue-white

colony selection was ignored, since clones containing this *nifH* fragment inserted have been found to produce blue or white colonies depending on the orientation of the insert. Plasmids were purified using either a spin-column miniprep kit (Qiagen) or a 96-well format Montage miniprep kit (Millipore) following manufacturer's protocols.

Sequencing. All plasmid minipreps were screened for *nifH* inserts by single strand sequencing. Second-strand sequencing was performed only for representative sequences selected for deposition in GenBank. Cycle sequencing was performed using BigDye terminator sequencing kits (ABI) in either 1/4 reactions (ABI protocol) or 1/8 reactions (protocol supplied by Millipore) using SP6 or T7 primers. Unincorporated dye terminators were removed by isopropanol precipitation or by using 96-well format vacuum filtration plates (96_{seq}, Millipore). Precipitated reactions were resuspended in Template Suppression Reagent (ABI), heated to 90°C, then chilled on ice. Reactions purified by filtration were resuspended in sample loading buffer (Millipore). Resuspended products were transferred to sample tubes with septum caps and run on a capillary sequencers (ABI 310 and 3100). Sequence files were imported and vector sequence trimmed in SeqLab in the GCG Wisconsin Package software (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA). The resulting sequence lengths ranged from 324 to 330 bp.

Phylogenetic Analysis. The Mono Lake DNA sequences, along with 613 obtained from GenBank, were translated and the proteins aligned using a hidden Markov model built with HMMER 2.2 (http://hmmer.wustl.edu). The DNA sequences were then realigned according to the protein alignment and a phylogenetic tree was built using the neighbor-joining algorithm in ARB (http://www.arb-home.de/) considering only nucleotides in the region from 133 to 459 (*Azotobacter vinelandii* numbering, GenBank accession no. M20568). Sequences that did not completely span this region were not included in the tree. Mono Lake sequences sharing >95% identity were assigned a common sequence-type number, and at least one representative sequence of each type was submitted to GenBank. For clarity of presentation in the phylogenetic tree, any clusters of Mono Lake sequence types that were monophyletic (i.e., those that contained only Mono Lake sequences) were collapsed to a single branch. Branches were then assigned a sequence-group letter (A–Q), where a sequence group could then consist of just one or multiple sequence types. Percent identities between pairs of sequences were calculated using the program GAP in the GCG Wisconsin Package (Version 10.3, Accelrys Inc., San Diego, CA).

$^{15}N_2$ Incorporation. N_2 fixation was measured using the $^{15}N_2$ method [25]. $^{15}N_2$ was injected into inverted sample bottles through the silicone septa (1 μL $^{15}N_2$ per mL of sample). Surface water samples were incubated in a stream behind the field station under two layers of neutral-density black screen (approximately 25% surface light intensity). Temperature in the stream varied from 20°C during the day to 16°C at night. Bottles from the two deeper depths were incubated in the dark in a cold room at 4–6°C. At four time points (<1, 24, and 48 h) paniculate material was collected on baked (350°C, 2 h) glass-fiber filters (GF/F, Whatman) by gentle vacuum filtration of duplicate bottles for each depth. A single bottle from each depth was incubated for an additional 288 h (2 weeks total), either in the dark at ~22°C (2 m sample) or at 4°C (18.5 and 30 m samples), and then filtered. After filters were air-dried, they were folded in half (sample side in), wrapped in baked aluminum foil, and dried for 1–8 h at 60°C in an oven with desiccant. The $^{15}N/^{14}N$ ratios in particulate material on the filters was measured by continuous-flow isotope ratio mass spectrometry using a CE Elantech NA 2500 elemental analyzer interfaced to a Micromass Optima mass spectrometer by oxidation and mass spectrometry as previously described [25]. All isotope abundances are reported as $\delta^{15}N$ values in standard notation relative to atmospheric N_2.

Results

Hydrography. The water column in Mono Lake in May was characterized by an upper mixed layer within which temperature ranged from 9 to 10.5°C, salinity was 79 g kg^{-1}, and oxygen concentrations were between 4 and 5.5 ml L^{-1} (Fig. 2). The thermo- and oxyclines extended from approximately 10 down to 20 m depth. From 20 m to the bottom (~29 m) temperature was ca. 5°C and oxygen was below detection. The halo- and nutriclines were somewhat deeper than the oxycline beginning around 20–22 m. In this bottom layer, salinity increased with depth from 79 to ≥87 g kg^{-1}. Ammonium was consistently low in the surface waters (<0.27 μM), but increased with depth in the anoxic zone, reaching 450 μM at the bottom. This pattern of stratification persisted in August at Station 6 (Fig. 2B, but with some differences. In August, the surface layer was much warmer (ca. 20°C) and more saline (ca. 80 g kg^{-1}) with slightly lower oxygen concentration (3.7–4.3 ml l^{-1}). Thermo- and oxyclines were more defined, beginning at 11–12 m and extending to approximately 20 m. Below this depth the temperature was 3–5° C and oxygen was undetectable. As in May, ammonium was undetectable in the surface waters, but increased beginning at 18–20 m reaching 680 μM in the deepest sample at 35m. Salinity increased from

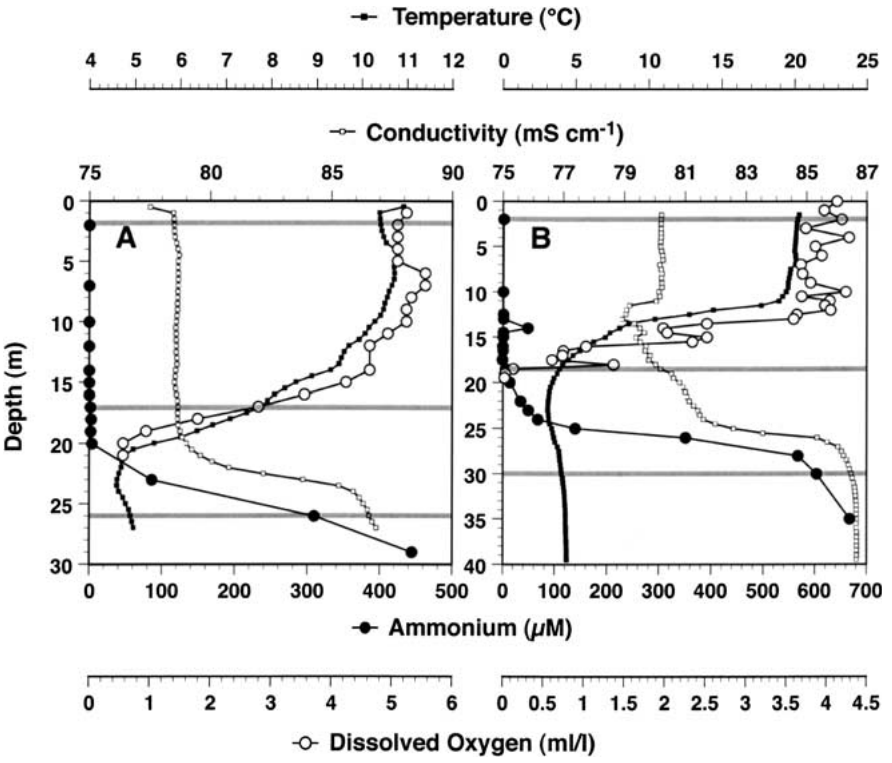

Figure 2. Depth profiles of temperature, conductivity normalized to 25°C, ammonium concentration, and dissolved oxygen concentration for (A) station 3 in May 2001 and (B) station 6 in August 2001. The depths sampled are indicated by shaded horizontal bars.

around 18 m and reached a maximum value of 86.5 g kg^{-1}, which extended from 30 m to the bottom.

Nitrogenase Gene Sequences. A total of 90 clones containing *nifH* inserts were sequenced and classified into 35 different sequence types (Table 1) which formed 17 distinct groups. Sequences were considered to be of the same type if they shared ≥95% DNA sequence identity. Sequence types were further placed into 17 sequence groups (lettered A–Q) where multiple sequence types were assigned to a common group if they formed a monophyletic cluster that contained only Mono Lake sequences. Only three of the *nifH* DNA sequences recovered as part of this study were closely related (>90% identity) to other sequences in GenBank with the remainder sharing ≤ 85% (Table 2).

All of the sequences grouped within two major clusters that are referred to as Cluster I and Cluster III [5] (Fig. 3). Mono Lake sequence groups A–E, comprising 43% of the sequences, fall within Cluster I along with many known aerobic and facultative microorganisms including some members of the *Proteobacteria* (alpha, beta, gamma, and epsilon subclasses), Gram-positive bacteria (*Paenibacillus* sp.), and cyanobacteria. Mono sequence groups F–Q, comprising 57% of the sequences, fall within Cluster III along with many known anaerobic organisms including delta-Proteobacteria, green sulfur bacteria, spirochaetes, and Gram-positive bacteria (*Clostridium* sp.). Sequences belonging to both Cluster I and Cluster III were found at all three depths at pelagic Station 3, but the relative proportion in each cluster varied with depth. The percentage of sequences in Cluster I at 2, 17, and 26 m was 42, 57, and 28%, respectively.

The sole member of Group A is a cyanobacteria-like *nifH* sequence that was obtained from a *Ctenocladus* aggregate collected in the littoral zone. This sequence is most closely related to *Phormidium*, a genus of filamentous, nonheterocystous cyanobacteria. No cyanobacteria-like sequences were found among the 89 clones from the pelagic samples.

All of the six clones comprising Groups B and C were retrieved from 17 m. Group B consists of two identical sequences that share 98% identity with a sequence retrieved from a plankton sample from the central Pacific Ocean. Other closely related sequences are from the beta subclass of *Proteobacteria* (*Burkholderia* sp. and *Herbaspirillum seropedicae*). Group C consists of four nearly identical sequences that cluster with those from gamma-Proteobacteria isolated from a marine mat and a clone retrieved from the Neuse River.

Group D and E sequences were retrieved from all three depths and cluster near those of gamma- and beta-Proteobacteria. Also nearby are a large number of sequences retrieved from a variety of environments including estuarine sediments, Lake George, NY, the Neuse River, NC, and from microbes associated with sea grass and rice plants. Sequences belonging to Group E were the most common at every depth and comprised 29% of all the clones analyzed.

Table 1. Names and accession numbers for the 52 representative *nifH* sequences deposited in GenBank out of the 90 total obtained in this study[a]

Clone name	*Accession no.*	*Sequence type*	*Total no. of type*	*Sequence group*
MO310A4	AY221804	1	1	A
M0170A06	AY221781	2	2	B
MO170A04	AY221779	3	4	C
MO170H13	AY221789			
MO163A16	AY221768	4	5	D
MO163H25	AY221774			
MO175H11	AY221805	5	1	
MO163H21	AY221772	6	11	E
MO170A02	AY221777	7	1	
MO175H25	AY221809	8	1	
MO175H32	AY221810	9	1	
MO170A07	AY221782	10	5	
MO163A04	AY221759	11	5	
MO163A07	AY221761			
MO163H19	AY221771			
MO170A03	AY221778			
MO170A09	AY221784			
MO163H11	AY221770	12	2	
MO170A10	AY221785	13	2	F
MO175A12	AY221803			
MO175A06	AY221797	14	1	
MO175A01	AY221792	15	1	G
MO163A02	AY221757	16	3	H
MO163A14	AY221767	17	3	
MO175A10	AY221801			
MO175A08	AY221799			
MO175A04	AY221795	18	1	I
MO163A05	AY221760	19	1	J
MO163A09	AY221763	20	3	K
MO175A07	AY221798			
MO163H23	AY221773	21	1	L
MO175A05	AY221796	22	2	M
MO170A08	AY221783	23	1	
MO170A12	AY221787	24	1	
MO163A01	AY221756	25	1	N
MO175A02	AY221793	26	1	
MO163A08	AY221762	27	2	O
MO163A13	AY221766			
MO170A05	AY221780	28	2	
MO175A03	AY221794			
MO175H13	AY221806	29	1	
MO163H31	AY221775	30	4	
MO175A09	AY221800			
MO163A03	AY221758	31	1	
MO170H17	AY221790	32	10	P
MO175A11	AY221802			
MO163H32	AY221776	33	1	
MO163A12	AY221765	34	7	Q
MO163H10	AY221769			
MO170H18	AY221791			
MO175H15	AY221807			
MO175H24	AY221808	35	1	
		Total	90	

[a]Also presented are the sequence type designations, the number of sequences of each type, and sequence group designations assigned as defined in the text.

They are part of a deeply branching subcluster in Cluster I that, at present, consists solely of sequences from uncultivated organisms from the aforementioned environments. The nearest sequence from a cultivated organism comes from a gamma-proteobacterium (75% identity).

Groups F and G are deeply branching in a subcluster that contains sequences from three species of Spirochaetes (*Treponema bryantii*, *Spirochaeta stenostrepa*, and *S. aurantia*), an Archaeon (*Methanosarcina barkeri*), a delta-Proteobacterium (*Desulfonema limicola*), and

Table 2. Distribution and affiliation of the major sequence types recovered from Mono Lake[a]

	# Clones from:			Nearest Neighbors to Sequence Group		
Sequence group	*2 m*	*17 m*	*26 m*	*Accession no. (name)*	*Identity (%)*	*Nearest cultivated*
lA[b]	0	0	0	U73132 (*Phormidium* sp.)	91	Cyano
B	0	2	0	AF299426 (Unidentified marine bacterial clone HT1901)	98	Beta
C	0	4	0	U43444 (Marine proteobacterium 'Tomales Bay wc2-3 sm wh')	80	Gamma
D	3	1	2	AJ297529 (*Pseudomonas stutzeri* A15)	80–82	Gamma
E	11	9	6	AY091867 (Uncultured nitrogen-fixing bacterium clone Sp3-3)	83–85	Gamma
F	0	1	2	U23636 (Unidentified marine eubacterium isolate Lower Mat 29)	71–73	Delta
G	0	0	1	U23636 (Unidentified marine eubacterium isolate Lower Mat 55)	79	Delta
H	4	0	2	U23644 (Unidentified marine eubacterium isolate Upper Mat 413)	79–81	Delta
I	0	0	1	U23644 (Unidentified marine eubacterium isolate Upper Mat 413)	79	Delta
J	1	0	0	U23644 (Unidentified marine eubacterium isolate Upper Mat 413)	73	Delta
K	2	0	1	M63689 (Unknown marine bacterium HRD2)	78	Delta
L	1	0	0	U23634 (Unidentified marine eubacterium isolate Lower Mat J7)	78	Delta
M	0	2	2	AY091909 (Uncultured nitrogen-fixing bacterium Sp16-3)	75–78	Delta
N	1	0	1	AY040518 (Uncultured bacterium clone BS0799 (2130) R06)	76–80	Delta
0	4	1	5	U23641 (Unidentified marine eubacterium Lower Mat 47)	78–82	Delta
P	3	5	3	AY040514 (*Desulfovibrio vulgaris*)	75–79	Delta
Q	3	3	2	AF329994 (Uncultured nitrogen-fixing bacterium clone MB57S4)	77–79	Green sulfur
Total	33	28	28			

[a]Sequence Group designations were assigned as described in the text and are cross-referenced to Table 1 and Figure 1. Nearest neighbors were determined from position in a neighbor-joining tree and pair-wise sequence comparisons. For groups with multiple sequence types, identities are given as the range for members within the group. Affiliation of the nearest cultivated neighbor is based on 16S rRNA sequence, where Cyano refers to cyanobacteria, Beta, Gamma, and Delta refer to subclasses of the Proteobacteria, and 'Green sulfur' refers to the green sulfur bacteria.
[b]A single clone of this group was obtained from a *Ctenocladus* aggregate collected in the littoral zone.

a Gram-positive bacterium (*Clostridium pasteurianum*). The uncultivated members of the group are from estuarine sediments, marine microbial mats, and microbes associated with copepods or rice plants.

Sequences comprising Groups H–K are distributed between two subclusters in which the only identified members are delta-Proteobacteria (*Desulfovibrio salexigens*, *Desulfobacter curvatus*, and *D. latus*). The environments from which the uncultivated representatives in these subclusters were obtained are similar to those mentioned above for Groups F and G.

Group O is within a small subcluster containing sequences from the delta-proteobacteria (*Desulfovibrio* and *Desulfomicrobium*), a spirochaete (*Spirochaeta aurantia*), and sequences of unknown affiliation from a marine mat and a rice plant–associated microorganism. Group P is nearby, but branches more deeply and has similar distance to the above sequences as well as to unidentified microorganisms associated with sea grass.

Group Q falls within a subcluster containing sequences from green sulfur bacteria (*Chlorobium tepidum* and *C. limicola*) and sequences of unknown affiliation from estuarine sediment and from sea grass. The next nearest subcluster contains sequences from spirochaetes (*Treponema* sp. *and Spirochaeta zuelzerae*) and others of unknown affiliation retrieved from termite guts and rice roots.

^{15}N Natural Abundance and N_2 Fixation. The natural (i.e., T_0) $\delta^{15}N$ values for particulate matter at 2, 18.5, and 30 m at station 6 were 7.45 ± 0.36, 7.74 ± 0.31, and 14.96 ± 0.62 (mean ± s.d.), respectively (Table 3). There was no significant difference between the natural $\delta^{15}N$ of particles collected at 2 and 18.5 m (*t*-test, $p = 0.47$), but both were significantly different from the $\delta^{15}N$ of particles collected at 30 m in the monimolimnion (*t*-test, $p = 0.0045$). During the time course incubations, there were no significant changes in $\delta^{15}N$ at any depth after 24

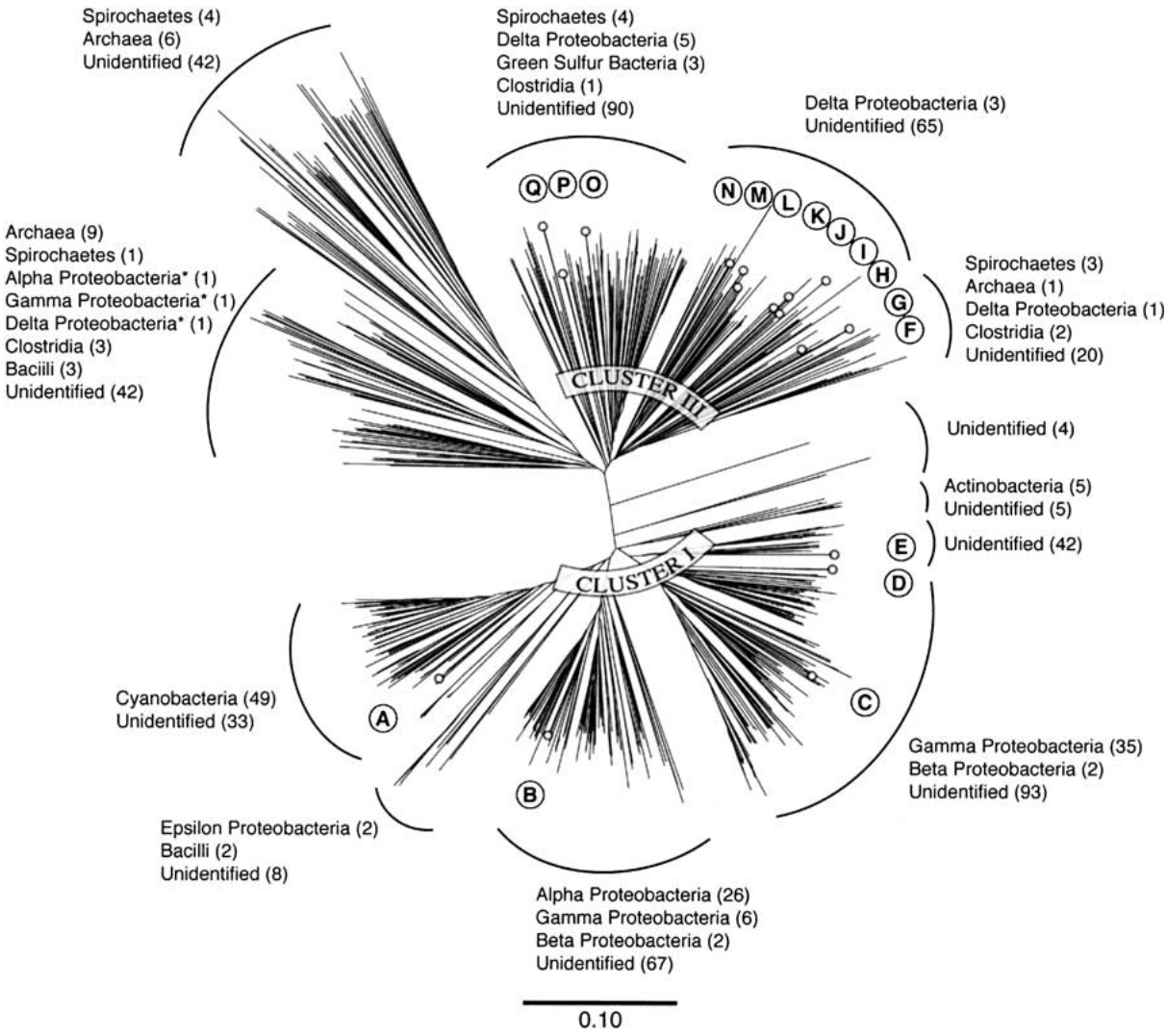

Figure 3. Unrooted radial tree showing the relationship among the *nifH* sequences retrieved from Mono Lake and 613 other *nifH* and *nifH*-like sequences retrieved from Genbank. Branches representing Mono Lake sequence groups are marked with a terminal dot and a Mono Lake sequence group letter. The letters, appearing distal to the Mono Lake branches, were assigned sequentially moving counterclockwise around the tree from the lower left. Within each cluster of sequences marked with the solid curved lines, the phylogenetic affiliations of cultivated microorganisms from which *nifH* genes were obtained are listed along with the number of each type in parentheses. Sequences obtained by PCR and cloning of environmental samples or uncharacterized isolates are listed as unidentified. Names marked with an asterisk indicate sequences of alternative (vanadium or nonmolybdenum, nonvanadium) nitrogenases. The scale bar indicates substitutions per 100 bases.

or 48 h (*t*-test, $p = 0.08$–0.82). After 2 weeks, values were higher in samples from all three depths compared to T_0 (by 0.7 to 1.1‰), but there were no replicates for this final time point and the statistical significance of this increase could not be determined.

Discussion

Our analysis of *nifH* gene fragments suggests that there are many diverse and novel diazotrophs present in Mono Lake, with all but two of the 35 Mono Lake *nifH* sequence types having very low similarity ($\leq$85% identity) to other known nitrogenase sequences in GenBank. One of the exceptions was Sequence Type 1, a *Phormidium*-like sequence (91% identity) recovered from a *Ctenocladus* aggregate. Recovery of this type of cyanobacterial sequence from the aggregate is consistent with previous evidence from microscopy and assays of nitrogenase activity [10, 26]. Experiments with metabolic inhibitors suggested that N_2 fixation in *Ctenocladus* aggregates is sometimes due solely to heterotrophic bacteria, but at other times is largely attributable to a nonheterocystous cyanobacterium [26].

The second exceptional sequence was Sequence Type 2, which is very closely related (98% identity) to a clone from the Pacific Ocean [43]. This sequence is also similar

Table 3. Measurements of $\delta^{15}N$ at time points during $^{15}N_2$-based nitrogen-fixation assays[a]

	Time			
Depth (m)	*0*	*24*	*48*	*336*
2	7.45 (0.36)	7.67 (0.11)	7.68 (0.11)	8.65
18.5	7.74 (0.31)	7.40 (0.38)	7.53 (0.11)	8.60
30	14.96 (0.62)	14.91 (0.03)	14.85 (0.04)	15.64

[a]Samples were collected at the indicated depths at station 6 in Mono Lake on 6 August 2001.

to amplicons that have sporadically appeared in negative controls [41]. Although it is possible these microorganisms exist in Mono Lake and other aquatic habitats, we cannot exclude the possibility that this, and closely related sequences deposited in GenBank by other researchers, come from low-level contamination of bacterial DNA in commercial reagents [38].

Despite the overall diversity and novelty among the Mono Lake *nifH* sequences, all were assignable to just two of four previously defined major clusters [5]. Cluster I sequences, comprising many from known aerobic or facultative bacteria, are commonly recovered from aquatic environments [1, 22, 43], but are also found in soils [31, 40] and marshes [21]. A significant departure from previous studies of aquatic environments is the very high proportion of Mono Lake sequences grouping in Cluster III, which comprises sequences from many known anaerobic bacteria.

The vast majority (72%) of *nifH* sequences from the 26 m sample grouped in Cluster III, which is consistent with observed anoxia in the monimolimnion. Somewhat surprising, however, was the high proportion of anaerobe-like sequences found in the oxygenated surface waters and the oxycline. Cluster III sequences have been retrieved from oxygenated waters in other studies, but not with such high frequency as we observed in Mono Lake. Only two of 21 clones from Lake Michigan were in Cluster III [22], as were only two of 101 sequences from the Neuse River, NC [1]. The source and significance of these Cluster III sequences in oxygenated waters is not clear.

It is possible, perhaps likely, that not all *nifH* sequences in Cluster III derive from strictly anaerobic bacteria. If they do represent anaerobes, those bacteria may be surviving in oxygenated waters by inhabiting anoxic microzones, perhaps within particles, or associated with zooplankton and their fecal pellets. The brine shrimp, *Artemia monica*, is the only mesozooplankton in Mono Lake and it is extremely abundant (few to hundreds of thousands m^{-2} [16]. Cluster III *nifH* sequences have been found in association with copepods in seawater [3], and a similar association between N_2-fixing bacteria and crustaceans may also occur in Mono Lake. The production of fecal pellets can be quite high during peaks of *Artemia* in the summer [18], and the interior of these or other organic particles may harbor microzones of reduced oxygen and N_2-fixation [28, 29].

Vertical zonation of bacterial communities in Mono Lake was observed in previous investigations of 16S rRNA gene diversity [11, 14]. Bottom-water samples had the highest sequence diversity (twice as many sequence types as surface or mid-water) and were dominated by Firmicute-type ribosomal RNA sequences including *Clostridium* sequences [14]. No *Clostridium*-like 16S rRNA sequences were retrieved from surface waters [14]. The dominance of anaerobe-like 16S rRNA gene sequences in the deep water is consistent with our results for *nifH*, but the absence of the anaerobe-like 16S rRNA sequence types in the surface waters suggests a greater degree of vertical zonation than implied by this study. The diversity of *nifH* phylotypes also appeared to be more evenly distributed than was observed for the 16S rRNA genes with ten, nine, and twelve of the 17 total sequence groups represented in surface, oxycline, and bottom waters, respectively. Direct correspondence between patterns of 16S rRNA and *nifH* sequences is not necessarily expected, however, since the diazotrophs probably comprise a small fraction of the entire bacterial community.

Previous attempts to measure N_2 fixation in the water column of Mono Lake or the chemically similar Big Soda Lake yielded negative results [26]. Given the ease with which we were able to amplify diverse *nifH* genes from three major strata in the water column, we considered that previous failures to detect water-column N_2 fixation might have been due to the limited sensitivity of the acetylene reduction assay. As well, we expected, a priori, that N_2 fixation in at least some bacteria would be inhibited in surface waters by oxygen, and in anoxic deep waters by the very high ammonia concentrations (300–600 μM). For these reasons we used the more sensitive $^{15}N_2$-incorporation method [25] and targeted one of our samples at a depth just under the oxycline and just above the chemocline in a narrow stratum having low levels of both NH_3 and O_2. Even with the more sensitive method, however, we found no detectable N_2-fixation at any depth after 48 h of incubation in the presence of $^{15}N_2$. Only a hint of N_2-fixation activity was detected after 2 weeks of incubation. Although this final time point was not replicated, the roughly 1‰ change we observed after 2 weeks in the samples from all three depths suggests a maximum specific N_2-fixation rate of about 10^{-7} h^{-1}, or a Mono Lake nitrogen turnover time on the order of a millennium. Clearly, N_2-fixation was not supporting the

production of new biomass in Mono Lake at the time of our experiments.

Salinity seems likely to play some role in limiting nitrogenase activity since increasing salinity was found to reduce N_2-fixation rates in Mono Lake sediments [9] and is reported to limit dissimilatory metabolism of microorganisms in general [27]. Salt concentrations of 200 mM have been shown to directly affect N_2 fixation through regulation at the promoters of nitrogenase and nitrogenase regulatory proteins [39]. Molybdenum availability in relation to sulfate concentration has also been suggested to control nitrogen fixation in saline, sulfate-dominated systems [13, 23]. Although this latter hypothesis has been somewhat controversial [30], Mono Lake does have high sulfate-to-molybdenum ratios. High ammonia concentrations also inhibit nitrogen fixation [32], making diazotrophy in the ammonia-rich monimolimnion unlikely.

Whatever the causes, the repeated failure to detect nitrogen fixation in the pelagic waters of Mono Lake raises important questions about what controls the activity and distribution of diazotrophs in the environment. It seems paradoxical that diverse and novel nitrogenase genes are present in the nitrogen-limited surface waters of Mono Lake, where no N_2 fixation appears to be occurring as well as in the deep, ammonia rich, anoxic waters where N_2 fixation was neither detected nor expected. A recent survey of nitrogenase sequence diversity [42] indicates that the microbial community of Mono Lake is probably not unique in displaying a discord between a genetic potential for N_2 fixation and the rates actually measured.

One potential explanation for the paradox is that nitrogen fixation does occur in such environments, but on temporal or spatial scales that have not been adequately sampled. The overall chemistry of Mono Lake, for example, changes over time, and nitrogen fixation may respond to changes in freshwater input, which can alter the chemistry of the lake seasonally or, more dramatically, as the longer period alternations between holo- and meromixis [19]. Pelagic nitrogen fixation may also be restricted to microenvironments such as in association with zooplankton or particulate material suspended in the water column as discussed above. The presence of anaerobic microenvironments within particles could explain the high proportion of anaerobes in surface waters. The sinking of such particles might also explain both the presence of nitrogenase genes in the ammonium-rich monimolimnion and the enrichment for Cluster III types there. Mechanical disruption of particles and the exclusion of *Artemia* by the prefiltration during sampling may be responsible for the absence of detectable fixation rates.

Another intriguing explanation for the presence of nitrogenases in ammonia-rich, anoxic waters such as the monimolimnion of Mono Lake is that nitrogenases have an alternate function in such environments. Nitrogenases can reduce a variety of substrates besides N_2 including acetylene, hydrogen cyanide, carbonyl sulfide, carbon disulfide, carbon dioxide, and thiocyanates [4, 33], and there has been speculation that the enzyme could have first evolved as a cyanide detoxification mechanism [32]. Gene expression studies will resolve whether or not these genes are actually being transcribed, even under conditions that would not seem appropriate for using nitrogen fixation as a source of N for growth.

It is also possible that the genes detected in Mono Lake and other environments without measurable nitrogen fixation are relics maintained by the introduction of microorganisms from other environments where nitrogen fixation is favorable. This would require a continual microbial input to the lake, since bacteria are believed to have high deletion rates that tends to eliminate genes in the absence of positive selection [2, 20]. There is at least some evidence suggestive of global microbial exchange among limnetic environments as observed in 16S rRNA sequences [24].

In summary, this report contributes to the characterization of the biogeographical diversity of nitrogenase genes by revealing novel sequences, but no detectable N_2 fixation, in an unusual environment. The results highlight significant gaps in our understanding of the function of this enzyme in nature. Making sense of the molecular ecology of nitrogenase, an ancient enzyme with many potential substrates, will require coordinated investigations on multiple fronts. These could include coupled measurements of gene expression and biogeochemical tracer studies, direct experimental manipulations, environmental genomic analysis, and concerted efforts to cultivate nitrogenase-containing microorganisms from diverse habitats for detailed molecular and biochemical characterization.

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