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Potential N_2 fixation by sulfate-reducing bacteria in a marine intertidal microbial mat

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ABSTRACT: The first report that sulfate-reducing bacteria (SRB) can fix N_2 was published a half century ago. Over the last 50 yr, it has slowly emerged that N_2 fixation is widespread among Gram-negative, mesophilic SRB. However, the ecological role of SRB N_2 fixation is not well understood. In some marine systems, SRB may contribute significantly to observed N_2 fixation (acetylene reduction). To date, evidence of SRB N_2 fixation has relied on inferring the results from the use of metabolic inhibitors and most probable number counts. This study attempted to assess more directly the role of SRB N_2 fixation in a temperate marine microbial mat system within the Rachel Carson National Estuarine Research Reserve (RCNERR; Beaufort, North Carolina, USA). The SRB inhibitor sodium molybdate (a structural analog of sulfate) was employed to characterize potential SRB N_2 fixation at night. Sodium molybdate inhibited nighttime nitrogenase activity (NA) by as much as 64%. Sodium molybdate had no effect on daytime NA. Reverse transcription-polymerase chain reaction (RT-PCR) was employed to characterize organisms expressing the dinitrogenase reductase gene (*nifH*), an essential gene for N_2 fixation. Several *nifH* sequences obtained from RT-PCR were highly similar to the *nifH* sequences of anaerobic organisms, including several SRB. Estimates of ATP production, based on sulfate reduction rates, imply sulfate reduction is capable of supporting molybdate-inhibited NA. The evidence suggests that SRB may contribute to N_2 fixation in the RCNERR mats.

KEY WORDS: Microbial mats · N_2 fixation · Sulfate-reducing bacteria · *nifH* · PCR · RT-PCR · Sodium molybdate

INTRODUCTION

The first evidence suggesting sulfate-reducing bacteria (SRB) can fix N_2 was not presented until more than 50 yr after Beijernick reported the initial SRB isolate (Sisler & ZoBell 1953). Due to methodological concerns, though, the fact that any SRB could fix N_2 was not broadly accepted until Legall et al. (1959) demonstrated more convincingly that 2 *Desulfovibrio* spp. isolates were diazotrophic (Postgate et al. 1988). Since then, as more SRB strains have been tested (Riederer-Henderson & Wilson 1970, Postgate 1970, Widdel 1987, Widdel & Bak 1992, Braun et al. 1999), it has become more evident that diazotrophy is widespread among the mesophilic SRB.

A general understanding of the ecological niche SRB fill, with regard to N_2 fixation in the marine environment, also has progressed slowly. Sisler & ZoBell's original isolates were derived from deep-sea sediments. Recognizing the vast distribution of SRB in these sediments led them to speculate that SRB might have a role in supplying new nitrogen (N) via N_2 fixation to the oligotrophic oceans (Sisler & ZoBell 1953). SRB play an important role in terminal C mineralization in deep-sea sediments (Jørgensen 1982), but deep-sea sediment SRB have not been examined adequately as potentially important N_2 fixers. The relatively slow input of labile organic carbon to deep-sea sediments yields negligible N_2 fixation rates (Hartwig & Stanley 1977).

By contrast, highly productive coastal benthic systems such as sea-grass rhizospheres exhibit substantial rates of both N_2 fixation and sulfate reduction (Patri-

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quin & Knowles 1972, Carpenter et al. 1978, Hines et al. 1989, Visscher et al. 1992b). The input of labile organic carbon from sea-grass roots fuels high rates of rhizosphere-associated microbial activity (Herbert 1999). Rhizosphere-associated N_2 fixation by heterotrophic bacteria is estimated to supply 10 to 50% of the N requirement for photosynthetic growth in sea grasses (Patriquin & Knowles 1972, Capone 1988). For many systems, N_2 fixation by SRB may comprise a large percentage of total rhizome-associated N_2 fixation (acetylene reduction) (Herbert 1975, Nedwell & Abdul-Aziz 1980, Dicker & Smith 1980, Jones 1982, Gandy & Yoch 1988, Welsh et al. 1996a). There is also evidence suggesting SRB in the guts of marine zooplankton fix N_2 (Braun et al. 1999). Thus, in some marine environments, the input of new N via SRB N_2 fixation may be quite important.

Microbial mats are additional, highly productive coastal features that exhibit high rates of photosynthesis, N_2 fixation, and sulfate reduction (Carpenter et al. 1978, Stal et al. 1984, Fründ & Cohen 1992, Visscher et al. 1992, Bebout et al. 1993, Canfield & Des Marais 1993). Most of the major groups of bacteria found in mats include organisms that can fix N_2 (Young 1992). In earlier investigations, little evidence existed to suggest SRB or other heterotrophic bacteria play a significant part in the N_2 fixation of microbial mats, although their potential contribution has been recognized (Stal et al. 1984, Skyring et al. 1988, Bebout et al. 1993). The non-heterocystous cyanobacteria that dominate biomass and energy production of most phototrophic mats are suspected largely of conducting observed N_2 fixation (Carpenter et al. 1978, Bebout et al. 1993, Stal et al. 1984). Many cosmopolitan filamentous, non-heterocystous mat cyanobacteria, such as *Lyngbya* spp. and *Oscillatoria* spp., have been shown to fix N_2 (Stal & Krumbein 1985, Paerl et al. 1991, Bergman et al. 1997). Furthermore, the temporal patterns of N_2 fixation observed in several mats mimic the observed N_2 fixation pattern of diazotrophic mat cyanobacteria grown in culture (Stal et al. 1984, Stal & Krumbein 1987, Villbrandt et al. 1990, Bebout et al. 1993).

Recently, a partial genetic survey of the dinitrogenase reductase gene (*nifH*) revealed that a majority of the *nifH* sequences obtained from a mat located in the Rachel Carson National Estuarine Research Reserve (RCNERR) in Beaufort, North Caroline, USA were most similar to the *Desulfovibrio gigas nifH* sequence (Zehr et al. 1995). Additionally, several strains of mat-forming *Microcoleus* spp. were shown to be incapable of fixing N_2 (Steppe et al. 1996). *Microcoleus* spp. are a major cyanobacterial component of the RCNERR and other mats, and it has been postulated that they support N_2 fixation in several mats (Potts et al. 1978, Paling et al. 1989, Paerl 1990). These results led us to speculate that heterotrophic bacterial N_2 fixation in some mats may be greater than previously assumed (Steppe et al. 1996). Consequently, we decided to re-examine the question of whether SRB in the RCNERR mats fix N_2. In order to characterize SRB N_2 fixation potential and activity, experiments involving physiological rate measurements (N_2 fixation, photosynthesis, and sulfate reduction) and *nifH* gene expression (reverse transcription-polymerase chain reaction [RT-PCR]) were performed on microbial mats from the RCNERR. Results of these studies indicate SRB are significant contributors to RCNERR mat N_2 fixation.

MATERIALS AND METHODS

Study site. The RCNERR is a dredge spoil island near Beaufort, North Carolina, USA (34° 40′ N, 76° 42′ W). It is also referred to as Bird Shoal (BS). The mats are located on a sand flat in the high intertidal. They are exposed from 10 to 14 h per diel cycle, depending on tide levels and wind. In the phototrophic layer, the dominant cyanobacterial genera are *Microcoleus*, *Lyngbya*, and *Oscillatoria*. *Phormidium*, *Arthrospira*-like, and unicellular morphotypes are present to a lesser extent. Pennate diatoms are usually found in the upper surface of the mats, but their relative abundance in relation to the cyanobacteria varies greatly (Pinckney et al. 1995). During the summer, a pink to purple layer composed of anoxygenic phototrophs develops occasionally below the cyanobacterial layer. The sediments below the photic zone are black due to FeS precipitates and may be characterized as a sulfuretum.

Sampling. Four sections ($20 \times 35 \times 4$ cm) of microbial mat were collected in modified plastic trays and transported to an outdoor seawater system at the Institute of Marine Sciences (IMS; Morehead City, North Carolina, USA) during the afternoon on the day prior to an experiment. The trays with the mat samples were placed in a tank with running seawater (34 to 36‰) and left exposed to natural illumination. *In situ* tidal exposure was not simulated in the tanks. However, the water in the tanks was drained and the mats were exposed for 1 h before refilling the tanks at 18:30 and 06:30 h. Measurements were made over a 24 h cycle, starting at 06:00 h. Replicates were taken from each of the larger mat sections.

Rate measurements. Nitrogenase activity (NA) was determined by the acetylene reduction assay. Net O_2 production and dark O_2 consumption were determined by measuring changes in O_2 in water overlying mat pieces. CO_2 fixation was determined by measuring incorporation of $^{14}CO_2$. These methods are detailed elsewhere (Bebout et al. 1993, Pinckney et al. 1995, Paerl et al. 1996, Steppe et al. 2001).

Sulfate reduction rates (SRR) were determined by measuring the conversion of $^{35}SO_4^{2-}$ to $H_2{}^{35}S$. Over a diel cycle, SRR were assayed 3 times: at 06:30 h, sometime between 13:00 and 15:00 h, and sometime between 00:00 and 02:00 h. Thirty cm^3 syringes were used to house incubations. Labeled sulfate (carrier-free $Na_2{}^{35}SO_4$; 5 µCi, total) was vertically injected to a depth of 6.0 mm in 5 separate injections. The mat sample was overlaid with 5.0 cm of seawater and incubated under natural illumination. At the end of a 1 h incubation period, each replicate was quickly sliced into 2 sections, each 3.0 mm thick. Using 200 mM Na_2S, each section was washed into separate 60 ml serum vials containing 6.0 ml of 20 % zinc acetate. The vials were capped, sealed, and stored at –80°C. Serum vials were stored at –80°C until $H_2{}^{35}S$ determinations, usually no more than 3 d. Conversion of $^{35}SO_4^{2-}$ to reduced ^{35}S was determined using the 1-step reduced-chromium method (Fossing & Jørgensen 198). SRR were calculated for each slice based on the following equation (Jørgensen 1978):

$$\frac{\left[SO_4^{-2}\right] \times 1.04 \times \Phi \times H_2{}^{35}S}{(H_2{}^{35}S + {}^{35}SO_4^{2-}) \times h} = {}^{35}SO_4^{2-}\ \text{mmol cm}^{-3}\,\text{h}^{-1} \quad (1)$$

where $[SO_4^{2-}]$ is porewater sulfate concentration, Φ is sediment porosity, 1.04 is the isotopic fractionation correction factor, $H_2{}^{35}S$ is the recovered labeled sulfide, $^{35}SO_4^{2-}$ is the labeled sulfate in the porewater after incubation, and h is hours. Porewater sulfate concentrations were not determined. It was assumed there was no substantial sulfate depletion within the top 6.0 mm and that ambient sulfate concentrations were 28 mM. Depth-integrated values or SSRR were calculated by summing the average rates of the 2 sections. SRR measurements were made in 1998, but all measurements were made under artificially induced anaerobic conditions (dark conditions with N_2-sparged seawater). The data from those measurements are not included in this study.

Molybdate inhibition. Sodium molybdate has been shown to be a 'specific' metabolic inhibitor for SRB (Oremland & Capone 1989). When assessing the effect of sodium molybdate on daytime NA and primary production, small mat pieces (~6 cm × 6 cm) were removed from each of the larger sections sometime after sunset on the day prior to an experiment. They were placed in a 3 l plastic dish containing 2 l of seawater. Sodium molybdate (1.0 M in seawater) was added to the water in the plastic dish to a final concentration of 20 mM. The water in the container was lightly bubbled with air. The mat samples were incubated overnight and through the daylight hours in the presence of sodium molybdate. Samples for use in rate measurements were taken from the mat pieces incubated in sodium molybdate-amended seawater. The water in the plastic dish was exchanged with fresh seawater and sodium molybdate sometime between 06:00 and 07:00 h, and at least twice during daylight hours. When the effects of sodium molybdate on nighttime NA were assessed, mat pieces were cut from the larger mat sections and placed in the sodium molybdate-amended seawater at 18:00 h. When the effect of sodium molybdate on both daytime and nighttime was assessed, fresh mat pieces (i.e. not incubated in molybdate all day) were used for assaying sodium molybdate inhibition of nighttime NA. Appropriate incubation vials used in rate measurements contained sodium molybdate (20 mM final).

Enrichment cultures. Defined, N-free media intended to be selective for diazotrophic SRB growth was utilized for enrichment cultures. All basal media solutions, omitting N sources, were prepared under anaerobic conditions according to the recipes and methods outlined in Widdel & Bak (1992). Thirty ml of basal media was aliquoted into 60 ml serum vials. Lactate was added to serum vials (7 mM final concentration). Small mat cores (1.0 cm^2) were used to inoculate media. Initial inoculations were allowed to grow 3 mo in the dark, and then 0.5 ml was transferred to fresh media. After 6 wk, cells were harvested for DNA extractions. San Salvador lactate enrichment cultures were derived from a hypersaline pond, Salt Pond, on San Salvador Island, Bahamas, and were inoculated in March of 1996. BS enrichment cultures were inoculated in July of 1996.

DNA extractions and PCR. Small cores (~0.5 cm^2 × 0.6 cm) were collected from mat samples, placed in 1.0 ml of TE buffer pH 8.0 (10 mM Tris, 1 mM EDTA), and stored at –80°C until extraction. Purified DNA was obtained from mat pieces and SRB cultures according to the procedures outlined in Steppe et al. (2001). The *nifH* primers described in Zehr & McReynolds (1989) were used in all PCR experiments. The PCR conditions consisted of 30 cycles at 94°C for 60 s, 54°C for 60 s, and 72°C for 90 s.

RNA extractions and RT-PCR. At the halfway point of an incubation period, small mat cores (~0.5 cm^2) were placed in 2.0 ml screw cap tubes. Cores used for RNA extraction were collected close (<1 cm) to cores used in control NA measurements. RNA from only 1 core was used in subsequent RT-PCR reactions. The tubes were frozen quickly in a dry ice-ethanol bath. Alternatively, mat cores were suspended in 1.0 ml *RNALater* (Ambion). The tubes were stored at –80°C until extraction. Tubes were removed from the freezer and allowed to thaw. All cores were washed 4 times with diethylpyrocarbonate (DEPC)-treated TE buffer (pH 8.0). After the cores were washed, 0.5 ml of glass beads (150 to 200 µm) and 1.0 ml RNAwiz™ (Ambion) were added. Tubes were agitated using a 'bead

beater' for 3 min and then placed in a water bath (70°C) for no more than 1 h. At the end of the hour, bead beating was repeated, the tube was centrifuged at 14 000 × *g* for 1 min, and the supernatant was removed to a new tube. RNAwiz instructions were followed. After the first extraction, the crude pellets were suspended in 50 µl DEPC-treated dH_2O. RNA was purified twice more using the Totally RNA kit (Ambion) according to the manufacturer's instructions. After the third purification, the RNA solution was treated with DNase. The RNA solution was purified a final time using RNAwiz. In all, 4 purification steps were performed. The final pellet was suspended in 50 µl DEPC-treated dH_2O. To ensure there was no cross-contamination between different mat samples during extraction, tubes containing only reagent were processed (negative extraction control). The solution in these tubes was used as one of the negative controls in subsequent RT-PCR reactions. RT cocktail components were (final reaction concentrations) 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, 5.0 mM spermidine, 5.0 mM dithiothreitol (DTT), 100 nM of each deoxynucleotide triphosphate (dNTP), and 2.5 units AMV Reverse Transcriptase (Promega). One hundred ng each of *nifH* Aero (R) and Cyano (R) primers (Olson et al. 1998) was used in each reaction. After the RT cocktail was prepared, 47 µl aliquots were added to each reaction tube. RNA (50 to 150 ng) was added to the appropriate tubes. Negative controls were a reaction tube without reverse transcriptase and a reaction tube seeded with negative extraction control 'RNA'. To further assess whether RT-PCR reactions were contaminated with DNA, an additional negative control was added. This control consisted of purified RNA treated with RNase and then repurified to remove RNase. The RT reactions were incubated at 48°C for 30 min. Five µl of the RT reaction was used to seed the tubes used for PCR experiments. PCR experiments using RT reaction solution were performed using *nifH* primers described in Zehr & McReynolds (1989).

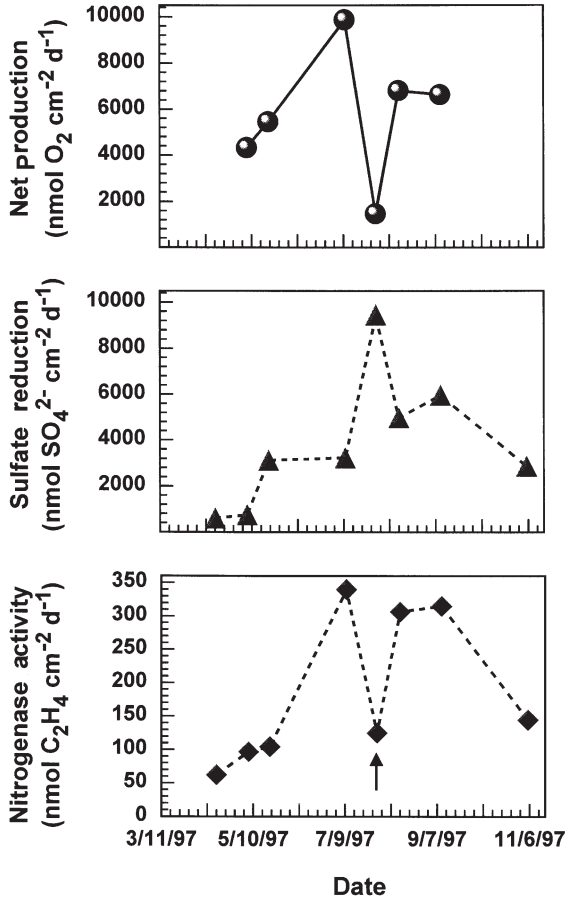

Fig. 1. Estimated total daily values of net O_2 production, sulfate reduction, and nitrogenase activity (acetylene reduction) for diel experiments conducted in 1997. All diel experiments were conducted on days without substantial cloud cover, except the diel experiment conducted on July 30, 1997. During this experiment, referenced by an arrow in the bottom panel, photosynthetically active radiation (PAR) never exceeded 300 µE m^{-2} s^{-1}. Dates given as mo/d/yr

Cloning, sequencing, and phylogenetic analysis. Amplification products were gel purified and cloned into the pCR 2.1 sequencing vector using the TA Cloning Kit (Invitrogen). The UNC-Chapel Hill Automated Sequencing Facility completed the sequencing on a Model 373A DNA Sequencer (PE-Applied Biosystems) using the *Taq* DyeDeoxyTM Terminator Cycle Sequencing Kit (PE-Applied Biosystems). Sequences were edited (i.e. primer sequences removed) and manually aligned with Seqlab in the GCG Genetics Suite (Genetics Computer Group). Phylogenetic analyses based on protein distances were done with Phylip 3.5 (Felsenstein 1995). Protein distances were obtained using the Dayhoff protein matrix. Phylogenetic trees were constructed using Phylip's Neighbor-Joining program.

Statistical analyses. Statistical analyses were performed using a software program, Statview™ (SAS Institute). One- or 2-way ANOVA was run on all results. *A posteriori* multiple comparison of means was achieved with the Bonferonni procedure ($p = 0.05$). Comparisons were made within treatments over a diel cycle, between treatments within individual incubation periods, between treatments during daytime hours (09:00 to 18:00 h), and between treatments during nighttime hours (21:00 to 06:00 h).

SRB isolates were kindly provided by R. Devereux (*Desulfosporosinus orientis* and *Desulfovibrio salexigens*) and D. Stahl (*Desulfovibrio africanus*, *Desulfomicrobium baculatum*, and *Desulfovibrio vulgaris*).

RESULTS

In 1997, total daily values of net O_2 production, NA, and SRR exhibited the seasonal patterns expected for a temperate microbial mat, such as the RCNERR mat (Paerl et al. 1996): the values were lowest in late winter to early spring, highest during the summer, and lower in the late fall (Fig. 1). The results show how light availability affects mat metabolism. On July 30, 1997, there was substantial cloud cover throughout the day. Photosynthetically active radiation (PAR) did not exceed 300 µE m^{-2} s^{-1}. While there was detectable NA on this day, it was greatly reduced compared to NA on July 10, 1997 or August 14, 1997 when light levels were higher. This supports Bebout et al.'s (1993) earlier studies that demonstrated NA to be tightly coupled to rates of photosynthesis in the RCNERR mat. In contrast, daytime (not shown) and nighttime SRR on July 30, 1997 were some of the highest measured in this study (Table 1). This suggests that high rates of oxygenic photosynthesis repressed SRR. In a separate experiment, the effect of eliminating oxygenic photosynthesis on SRR was determined by measuring SRR in cores in which photosynthesis was inhibited by darkness or by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) additions. Compared to the control, inhibiting photosynthesis significantly increased SRR ($p < 0.05$; Fig. 2). Visscher et al. (1992) also found that assaying SRR under reduced light conditions increased SRR.

Table 1. Average molybdate-inhibited nitrogenase activity (NA; C_2H_4 µM h^{-1}), Mo-NA as percentage nighttime (nt) NA, Mo-NA as percentage total NA (TNA), ratio of daytime (dt) to nighttime NA, and nighttime sulfate reduction rates (SRR; SO_4^{2-} µM h^{-1}) for diel experiments conducted in 1997, 1998, and 1999. SSRR and NA values represent activity in the top 6 mm of the mat. Molybdate-NA values were calculated by subtracting NA in molybdate-treated samples from control NA. SD: Standard deviation

Date	Mo-NA	% ntNA	% TNA	ntNA: dtNA	ΣSRR
16 Apr 97	0.6	50	14	0.7	5.4
07 May 97	0.3	11	2	0.4	5.4
21 May 97	4.4[a]	65	37	3.1	59
10 Jul 97	1.6	16	4	0.5	109
30 Jul 97	0.8	33	6	0.7	205
14 Aug 97	3.9[a]	31	9	0.7	217
10 Sep 97	5.2[a]	33	16	1.9	123
05 Nov 97	0	0	0	0.8	88
03 Jun 98	16.6[a]	45	18	1.2	–
30 Jun 98	4.3	20	8	6.4	–
29 Jul 98	5.1[a]	32	18	9.1	–
14 Oct 98	3.9[a]	48	26	5.1	–
09 Jul 99	5.4[a]	20	32	4.6	–
Average	4.1	31.1	14.6	2.7	101.5
SD	4.3	17.9	11.5	2.8	80.2

[a]Experiments where sodium molybdate significantly inhibited average NA from 21:00 to 06:00 h ($p < 0.05$)

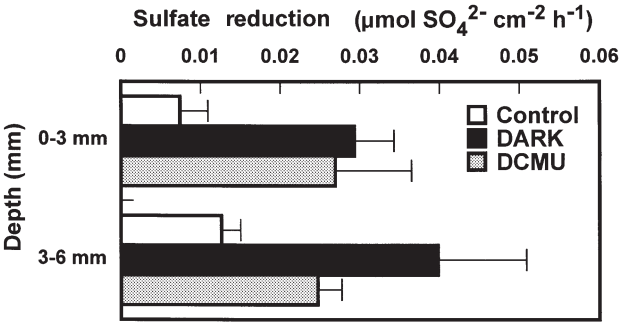

Fig. 2. Effect of oxygenic photosynthesis on daytime sulfate reduction. O_2 production was eliminated by covering samples with aluminum foil (dark) or by treating samples with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Data derive from experiment conducted on October 2, 1997 (13:00 to 14:00 h). Error bars represent standard deviation

In past studies, the inhibition of NA by sodium molybdate has been used as an indication of potential N_2 fixation by SRB (Gandy & Yoch 1988, Welsh et al. 1996a). Sodium molybdate effectively inhibited sulfate reduction in the RCNERR mats (Fig. 3). However, a 3 h pre-incubation in sodium molybdate-amended seawater was required to obtain >90% inhibition of sulfate reduction down to 6 mm depth (not shown). To determine whether sodium molybdate additions affected other metabolic processes, the impact of molybdate on photosynthesis (as measured by net O_2 production and $H^{14}CO_3^-$ uptake) and O_2 consumption was examined. Fig. 4 shows the effect of molybdate on $H^{14}CO_3^-$ uptake for 3 representative experiments. Molybdate

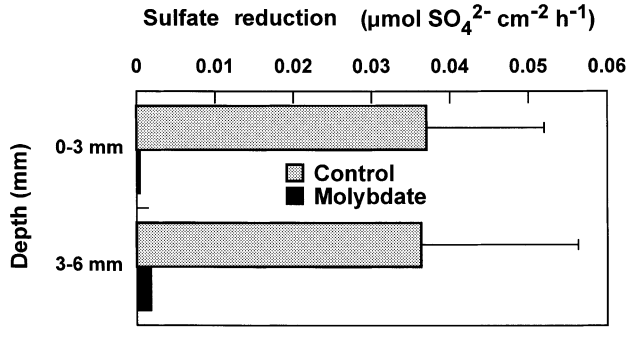

Fig. 3. Representative experiment demonstrating inhibition of sulfate reduction by sodium molybdate. Molybdate-treated samples were incubated in sodium molybdate-amended seawater (20 mM final concentration) for 3 h prior to addition of labeled sulfate. Data derive from the diel experiment conducted on May 21, 1997 (06:00 to 07:00 h). Error bars represent standard deviation. Deviation for molybdate-treated samples was less than width of error bars

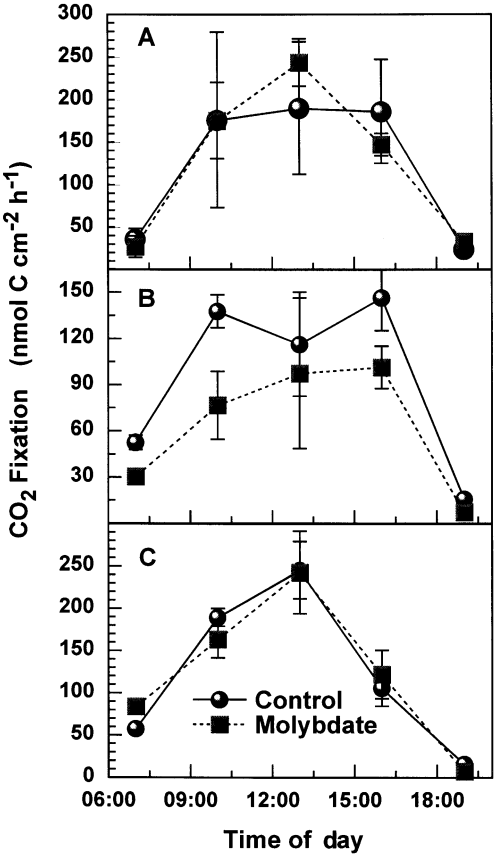

Fig. 4. Representative experiments demonstrating the effect of sodium molybdate on $H^{14}CO_3^-$ uptake. (A) July 10, 1997; (B) September 10, 1997; (C) November 5, 1997. Error bars represent standard deviation

appears to have had little effect on CO_2 uptake, except on July 10, 1997 (Fig. 4B). Treatment with molybdate decreased net O_2 production (Fig. 5A). Conversely, treatment with molybdate yielded more dark O_2 consumption during the day (Fig. 5B), and, although not significantly ($p > 0.05$), it consistently increased O_2 consumption at night.

Inhibition of NA by sodium molybdate was highly variable (Fig. 6). Historically, the RCNERR mats have exhibited a distinct diel separation of NA and photosynthesis during the spring and summer months (Paerl et al. 1996). This was not the general case for 1997, but the separation was observed more in 1998. Molybdate had little to no effect on NA during the daytime (09:00 to 18:00 h, cf. July 10, 1997 and November 5, 1997). Significant molybdate inhibition ($p < 0.05$) of nighttime NA (average NA value from 21:00 to 06:00 h) was observed generally when the ratio of nighttime NA (21:00 to 06:00 h) to daytime NA (09:00 to 18:00 h) exceeded 1 (Table 1). A few times, significant inhibition of NA in molybdate-treated samples was observed during the sunrise incubation period (06:00 to 09:00 h; e.g. July 10, 1997). The greatest percentage NA inhibition by molybdate was observed on May 21, 1997, when the average nighttime NA was inhibited 64%. The greatest molybdate-inhibited NA (control NA minus molybdate treatment) occurred on June 3, 1998, and was approximately 17 nmol C_2H_4 cm^{-2} h^{-1}. At no time did addition of Na_2S (200 µM final concentration) have a significant effect on molybdate-treated samples, even when there was significant inhibition of NA by molybdate (not shown). Nighttime SRR and molybdate-inhibited NA are also shown in Table 1.

In an effort to identify diazotrophs actively expressing the nitrogenase genes, RT-PCR was performed on RNA extracted from mat cores collected during 2 different diel experiments. Phylogenetic trees based on *nifH* sequences are characterized by several distinct groups of organisms (Ueda et al. 1995, Zehr & Capone 1996). The eubacterial clades are composed of cyanobacteria, alpha proteobacteria, beta/gamma proteobacteria, and anaerobic bacteria (SRB and *Clostridia* spp.). Fig. 7 depicts the *nifH* groups where the RT-PCR-derived sequences cluster, the time the cores

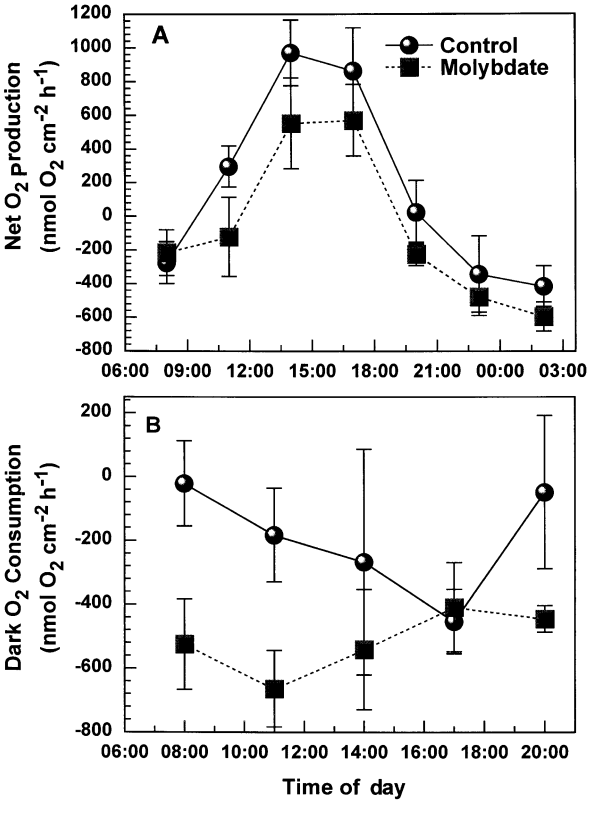

Fig. 5. Representative experiment (July 10, 1997) demonstrating (A) the effect of sodium molybdate on net O_2 production and (B) dark O_2 consumption. Error bars represent standard deviation

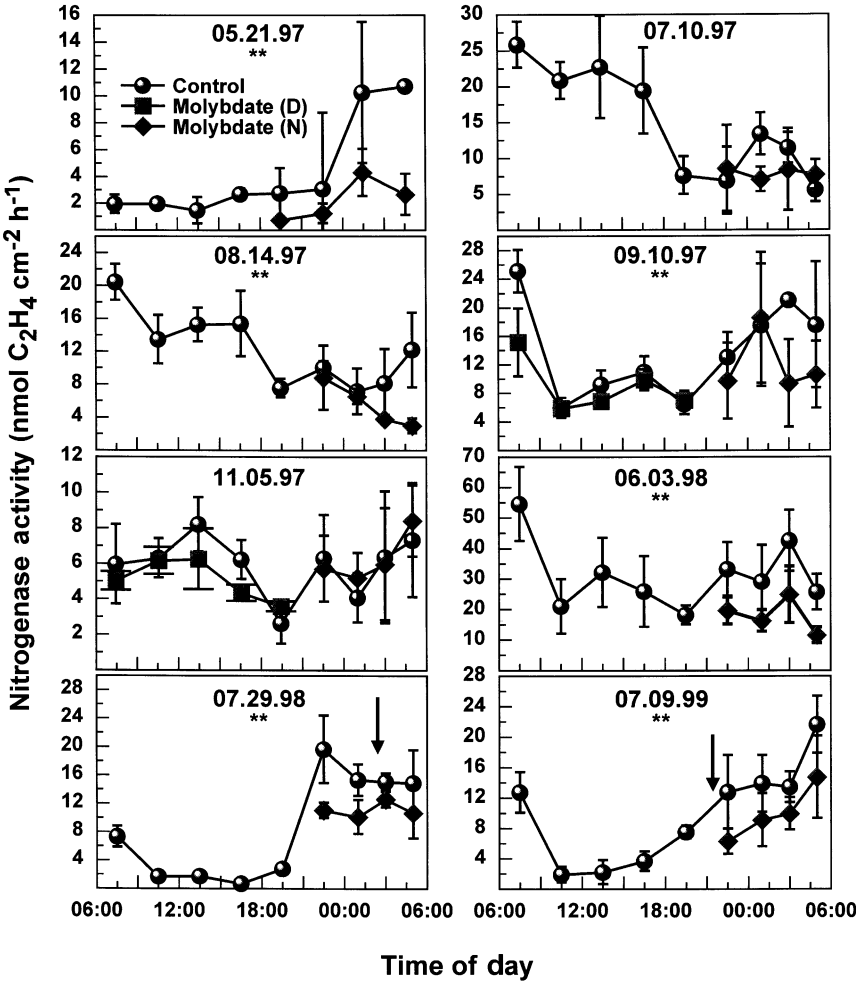

Fig. 6. Representative experiments showing diel variation in nitrogenase activity (NA) and effect of sodium molybdate on NA. **Experiments where sodium molybdate significantly inhibited average NA from 21:00 to 06:00 h ($p < 0.05$). Arrows show times when mat cores for RNA extraction were collected. Error bars represent standard deviation. D: daytime; N: nighttime

were collected, and the dates. On July 30, 1998, 3 unique *nifH* sequences were obtained from 10 clones; the other 7 sequences were repeats of 1 of these 3. One of these sequences clustered with the cyanobacteria and the other 2 within the anaerobe group. On July 9, 1999, 6 distinct *nifH* sequences were obtained from 12 clones. Five sequences clustered with the anaerobic group and 1 sequence clustered with the cyanobacterial group. NA in molybdate-treated mat samples was significantly reduced compared to control NA at the times control mat cores were collected for RNA extraction. Cores from molybdate-treated mat samples were not collected for RNA extractions.

To clarify the anaerobic *nifH* sequences, *nifH* sequence analysis was performed on SRB isolates (*Desulfovibrio africanus*, *Desulfomicrobium baculatum*, *Desulfovibrio salexigens*, *Desulfovibrio vulgaris*, and *Desulfotomaculum orientis*) and on lactate-based mat enrichment cultures designed to be selective for diazotrophic SRB (Fig. 8). Two unique *nifH* sequences were obtained from the San Salvador hypersaline mat culture, and 1 unique sequence was obtained from RCNERR enrichment culture. The enrichment culture sequences were consistently most similar to lactate-utilizing SRB, including *D. salexigens*, *D. vulgaris*, and *D. baculatum*. However, most RCNERR mat *nifH* sequences derived from mat genomic DNA (Zehr et al. 1995) and RT-PCR (this study) form new branches. Also included in Fig. 8 are sequences derived from RT-PCR.

DISCUSSION

Molybdate-inhibited NA

Studies conducted with estuarine sediments showed that sodium molybdate consistently inhibited NA by 75 to 90% (Nedwell & Abdul-Aziz 1980, Gandy & Yoch 1988, Welsh et al. 1996a). For the RCNERR mat, sodium molybdate inhibited NA by as much as 64%, but its effect varied greatly. Overall, if inhibition of NA by sodium molybdate is a reliable indicator of actual

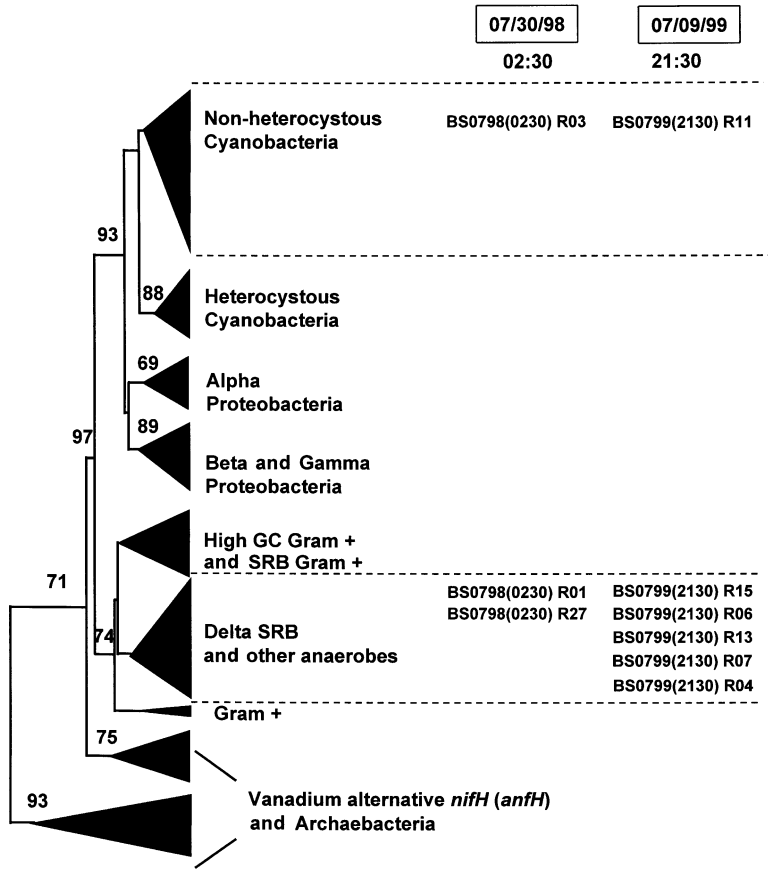

Fig. 7. *nifH* phylogenetic tree depicting the general bacterial groups where *nifH* sequences obtained by reverse transcription-polymerase chain reaction (RT-PCR) cluster. Tree topology was obtained using Dayhoff PAM matrix and Neighbor Joining in Phylip utilizing translations of an ~327 base pair (bp) section of the dinitrogenase reductase gene (*nifH*). Eighty nifH sequences were used in tree construction. The date and time when a mat core was collected for extraction are at the top of the figure. Sequence names are identified by location (BS for Bird Shoal), month and year, time core was collected, amplification method (R for RT-PCR), and clone number. Dates are given as mo/d/yr. SRB: sulfate-reducing bacteria

SRB NA, then nighttime SRB NA may be responsible for as much as 37% of total daily NA.

Inhibition of NA by sodium molybdate could have resulted from one or a combination of several direct and indirect consequences. Hence, there are alternative explanations for the decrease in NA attributable to sodium molybdate. Firstly, inhibition of sulfide production may have led to the inhibition of NA by bacteria dependent upon sulfide production by SRB, such as the sulfur-oxidizing *Beggiatoa* spp. (see also Bebout et al. 1993). Secondly, because microbial mats are metabolically coupled, highly diverse systems, the inhibition of sulfide production may have affected overall community respiration and metabolism, leading to non-specific inhibition of NA (Fig. 5). Thirdly, the inhibition of sulfide production may have altered pH, redox potentials, or both within the mat to levels inhibitory to NA (Tam et al. 1982, Villbrandt et al. 1990). Finally, the concentration of sodium molybdate (20 mM) at which mat samples were incubated may have specifically inhibited additional, thus far uncharacterized diazotrophic groups (Visscher et al. 1992a).

Sulfide additions did not stimulate NA in molybdate-treated samples during these experiments. Dissolved O_2 concentrations, pH, and H_2S concentrations were not concomitantly measured in the mat samples. Therefore, the method of sulfide addition or the quantity added may have not sufficiently compensated for potential NA inhibition due to unknown effects of sodium molybdate. For example, Villbrandt & Stal (1996) did not observe an increase in NA with the Na_2S concentration used in this study (200 µM) but did at a concentration of 5 mM Na_2S.

nifH characterization

Further evidence suggesting that the SRB within the RCNERR mat fix N_2 was obtained by sequencing *nifH* PCR products generated using RNA extracted from RCNERR mat samples. Nitrogenase may be subject to post-translational control. Therefore, sequences obtained by RT-PCR do not indicate actual NA. Furthermore, because the RT-PCR results were not quantitative, it is not possible to assign a quantitative significance to the *nifH* sequences obtained via RT-PCR. Nonetheless, it may be assumed that *in situ* conditions within the collected mat samples, sometime prior to the time of sampling, were favorable for transcription of the detected *nifH* sequences. The *nifH* sequence

analysis of RT-PCR-derived sequences revealed that organisms other than cyanobacteria expressed the *nifH* gene and that these sequences cluster within the anaerobic *nifH* group, which would be expected if SRB were transcribing nitrogenase genes.

Sequences obtained by RT-PCR have demonstrated the expression of alternative and anaerobic *nifH* sequences in termite guts (Noda et al. 1999). Additionally, Zani et al. (2000) detected a diversity of aerobic, non-cyanobacterial *nifH* transcripts in a freshwater lake. To our best knowledge, this is the first demonstration of *nifH* transcription by anaerobic diazotrophs in a marine system. *NifH* sequences from SRB, *Chlorobium tepidum* (green sulfur bacterium), and anaerobic Gram-positive organisms including *Clostridia* spp. and *Desulfotomaculum orientis* all cluster within the anaerobic *nifH* group. Our sequencing of *nifH* genes from cultures focused on incompletely oxidizing SRB such as *Desulfovibrio* spp. However, molecular genetic studies of SRB populations in mats have demonstrated that completely oxidizing SRB are dominant community components (Risatti et al. 1994, Minz et al. 1999). Hence, the lack of phylogenetic resolution within the anaerobic group, based on our analysis, prevents definitive identification of RT-PCR-derived sequences as diazotrophic SRB sequences. In the future, inclusion of more *nifH* sequences belonging to delta SRB (especially completely oxidizing SRB), purple and green sulfur bacteria, and other obligately anaerobic bacteria may provide greater resolution of the anaerobic *nifH* group, allowing reliable identification.

Energetic and ecological considerations

To date, investigations of microbial mats and marine sediments have consistently shown that the availability of photosynthetically produced organic carbon is a major factor regulating N_2 fixation (Dicker & Smith 1980, Nedwell & Abdul-Aziz 1980, Bautista & Paerl 1985, O'Neil & Capone 1989, Bebout et al. 1993, Paerl et al. 2000). Even those cyanobacteria that concomitantly conduct photosynthesis and N_2 fixation must support N_2 fixation by respiring the organic carbon

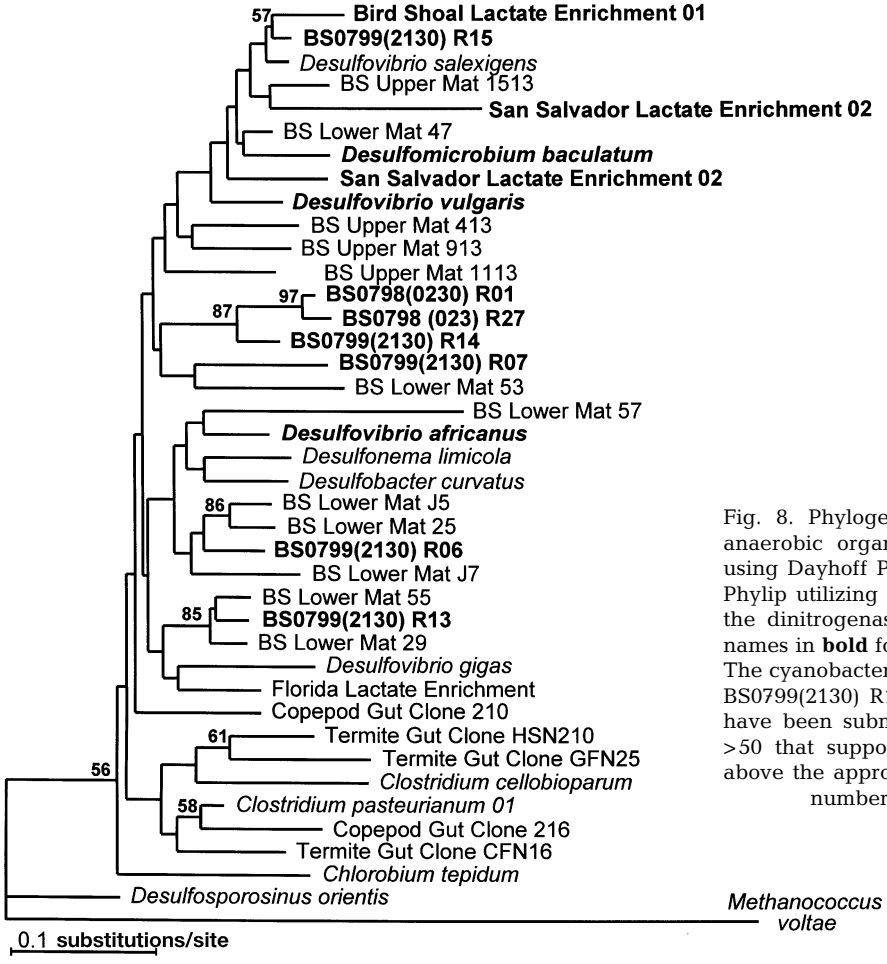

Fig. 8. Phylogenetic tree of *nifH* sequences from anaerobic organisms. Tree topology was obtained using Dayhoff PAM matrix and Neighbor Joining in Phylip utilizing translations of an ~327 bp section of the dinitrogenase reductase gene (*nifH*). Sequence names in **bold** font were generated during this study. The cyanobacterial sequences, BS0799(0230) R03 and BS0799(2130) R11, are not included in the tree, but have been submitted to GenBank. Bootstrap values >50 that support a particular branching are listed above the appropriate branches. GenBank accession numbers are AY040512 to AY040525

generated through photosynthesis. In marine rhizosphere sediments, where SRB are thought to be significant N_2 fixers, N_2 fixation is linked to the availability of the organic carbon exudates of sea grasses (Gandy & Yoch 1988, Welsh et al. 1996a). Likewise, in order for SRB or other heterotrophic bacteria to fix N_2 in the RCNERR mat, sufficient organic carbon must be available to meet the requisite ATP demand. SRB ATP production can be estimated using SRR. To assess whether the ATP available for SRB N_2 fixation was sufficient to cover the costs of molybdate-inhibited NA, ATP production by sulfate reduction and the ATP costs of molybdate-inhibited NA were calculated.

Theoretical values of ATP demand for N_2 fixation and conversion of acetylene reduction values into N_2 fixation values may be based upon the following formulas (Postgate 1982):

$$16ATP + 8e^- + 8H^+ + N_2 \rightarrow 16ADP + 16Pi + H_2 + 2NH_3 \quad (2)$$

$$C_2H_2 + 2e^- \rightarrow C_2H_4 \quad (3)$$

where Pi is inorganic phosphate. In marine systems, attempts to calibrate the ratio of acetylene reduction to $^{15}N_2$ rate measurements have yielded wide-ranging ratios of C_2H_2 reduced to N_2, but a ratio of 4:1, as suggested by the formulas, is not an unrealistic assumption (Capone 1988). Note that the 16 ATP per N_2 fixed or 4 ATP per $2e^-$ is a minimum theoretical ATP requirement. Actual estimates of ATP consumed per moles of N_2 fixed may be greater, e.g. 20 for *Clostridium pasteurianum* (Daesch & Mortensen 1967) or 29 for *Klebsiella pneumoniae* (Hill 1976). We assumed a requirement of 8 ATP per C_2H_2 reduced or 32 ATP per N_2 fixed. Porewater carbon substrate was not analyzed, but it was assumed that acetate was the dominant carbon source available to SRB (0.8 ATP produced per SO_4^{2-} reduced) (Widdel & Hansen 1991). However, the number of ATP produced per acetate consumed may be higher depending on the SRB species (Widdel & Hansen 1991). Additionally, carbon substrates that yield higher amounts of ATP, albeit to a lesser extent, such as glycollate or lactate, may have been available for use by SRB (Fründ & Cohen 1992). Considering these points, conversion of SRR to ATP in this study was set at 1.0 mol ATP per mol SO_4^{2-} reduced. Fig. 9 shows the percentage of nighttime sulfate reduction ATP yield (based on the 1 nighttime SRR value) that was required to sustain molybdate-inhibited NA for the 3 experiments in 1997, where molybdate significantly inhibited nighttime NA.

A similar examination of sulfate reduction and NA in *Zostera noltii* rhizome sediments found that molybdate-inhibited NA demand accounted for 17% of the ATP generated by sulfate reduction (Welsh et al. 1996b). Results of this study suggest that 14 to 59% of the sulfate reduction ATP yield was required to meet the molybdate-inhibited NA demand. A large percentage of the ATP generated by heterotrophic bacteria may be used to support N_2 fixation. Assuming no difference in maintenance costs between cultures, estimates based on the differences in Y_{ATP} (grams of dry cells per mole of ATP) between NH_3- and N_2-grown anaerobic cultures suggest that N_2 fixation may consume up to 60% of the total ATP generated by *Klebsiella pneumoniae* and 39% by *Clostridium pasteurianum* (Daesch & Mortensen 1967, Hill 1976). Because our estimates were based on total sulfate reduction values, they contain the assumption that all SRB reducing sulfate were fixing N_2. Most likely, not all SRB in the RCNERR mats are diazotrophic. If all of the active SRB were not concomitantly reducing C_2H_2, the actual amount of ATP available to support NA would be less than total sulfate reduction values indicate. As a result, the amount of ATP required by an individual or group of SRB to support N_2 fixation may be greater than our estimates suggest.

Neither the molybdate inhibition nor RT-PCR data, individually, are sufficient to establish the involvement of SRB in the N_2 fixation of the RCNERR mat. Together, however, these data suggest that SRB may be responsible for some portion of the observed NA within the RCNERR mat, either directly fixing N_2 or indirectly creating an anaerobic environment more conducive for N_2 fixation (Bebout et al. 1993, Villbrandt & Stal 1996). Due mostly to methodological concerns (i.e. potential effect of molybdate on photosynthesis) and the historical patterns of NA in the RCNERR mat (Paerl et al. 1996), this study focused on potential SRB N_2 fixation

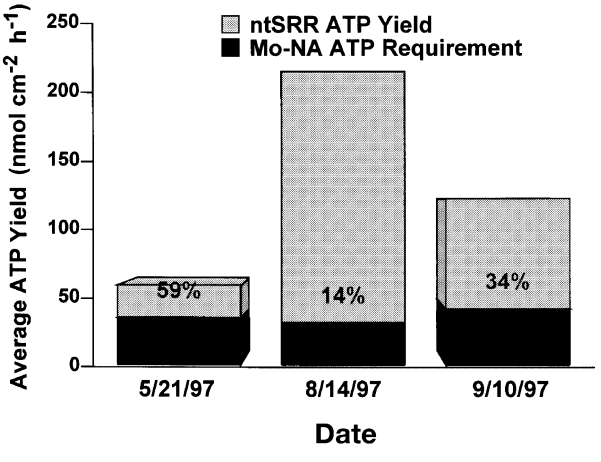

Fig. 9. Estimated amount of ATP generated by sulfate reduction required to sustain molybdate-inhibited NA. Estimates were calculated for the 3 experiments in 1997 where sodium molybdate significantly inhibited average nighttime (nt) NA. Calculations were based on values provided in Table 1. Dates are given as mo/d/yr. SRR: Sulfate reduction rates

at night. There is evidence suggesting that non-cyanobacteria, including SRB, may fix N_2 during the day in the RCNERR mats (Bebout 1992, Steppe et al. unpubl.), especially if low O_2 zones are maintained within the mat throughout the illuminated period. Therefore, the potential exists for SRB and other non-cyanobacteria to have an even greater N_2 fixation role in the RCNERR and other mats than this study suggests.

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