APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2005, p. 3978–3986 Vol. 71, No. 7
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.7.3978–3986.2005

Diel Variations in Carbon Metabolism by Green Nonsulfur-Like Bacteria in Alkaline Siliceous Hot Spring Microbial Mats from Yellowstone National Park

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Received 9 July 2004/Accepted 11 January 2005

Green nonsulfur-like bacteria (GNSLB) in hot spring microbial mats are thought to be mainly photoheterotrophic, using cyanobacterial metabolites as carbon sources. However, the stable carbon isotopic composition of typical *Chloroflexus* and *Roseiflexus* lipids suggests photoautotrophic metabolism of GNSLB. One possible explanation for this apparent discrepancy might be that GNSLB fix inorganic carbon only during certain times of the day. In order to study temporal variability in carbon metabolism by GNSLB, labeling experiments with [^{13}C]bicarbonate, [^{14}C]bicarbonate, and [^{13}C]acetate were performed during different times of the day. [^{14}C]bicarbonate labeling indicated that during the morning, incorporation of label was light dependent and that both cyanobacteria and GNSLB were involved in bicarbonate uptake. ^{13}C-labeling experiments indicated that during the morning, GNSLB incorporated labeled bicarbonate at least to the same degree as cyanobacteria. The incorporation of [^{13}C]bicarbonate into specific lipids could be stimulated by the addition of sulfide or hydrogen, which both were present in the morning photic zone. The results suggest that GNSLB have the potential for photoautotrophic metabolism during low-light periods. In high-light periods, inorganic carbon was incorporated primarily into *Cyanobacteria*-specific lipids. The results of a pulse-labeling experiment were consistent with overnight transfer of label to GNSLB, which could be interrupted by the addition of unlabeled acetate and glycolate. In addition, we observed direct incorporation of [^{13}C]acetate into GNSLB lipids in the morning. This suggests that GNSLB also have a potential for photoheterotrophy in situ.

Chloroflexus aurantiacus and its phylogenetic relatives (i.e., *Roseiflexus*) are found within a deeply branching kingdom-level lineage in the domain *Bacteria* (30, 31). These phototrophs are major components of photosynthetic microbial mats in sulfidic and nonsulfidic hot springs in Yellowstone National Park, Wyo. (4, 5, 7, 13, 30, 50). On the basis of genetic and lipid biomarker evidence (30, 46, 48, 51), *Chloroflexus* appears to be dominant in sulfidic springs, whereas *Roseiflexus* appears to predominate in nonsulfidic hot springs. *C. aurantiacus*, the best-studied representative of the green nonsulfur bacteria available in pure cultures, can grow heterotrophically by aerobic respiration, photoheterotrophically (using light to incorporate prereduced organic compounds), and photoautotrophically (using light to fix inorganic carbon) (34). However, *Chloroflexus* and *Roseiflexus* are mainly regarded as photoheterotrophs in hot spring microbial mats. This is based on their preferred photoheterotrophic growth in culture (4, 13, 17, 26, 33) and in situ ^{14}C-labeling experiments (1, 41).

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The potential for photoheterotrophy in alkaline siliceous hot spring microbial mats was demonstrated by microautoradiography showing light-stimulated uptake of [^{14}C]glycolate (3) and ^{14}C-labeled fermentation products (1, 41) by filaments thought to represent green nonsulfur-like bacteria (GNSLB). Glycolate can be produced by cyanobacteria via photorespiration in superoxic, alkaline mats (14, 22, 29) and might be an important carbon source together with other excreted photosynthates during daytime. [^{14}C]bicarbonate was taken up during the midday by *Synechococcus* cells and, to a lesser extent, by filamentous bacteria (3). However, uptake by filaments could be reduced by the addition of unlabeled glycolate, suggesting photoautotrophy by *Synechococcus* and cross-feeding to the filamentous bacteria via glycolate (3). Nold and Ward (28) demonstrated that [^{14}C]bicarbonate was fixed mainly into polysaccharides, which appeared to be fermented predominantly to acetate in the dark. Like other fermentation products (1, 41), acetate was also photoincorporated into filaments. Some of these filaments were shown to cross-react with anti-*Chloroflexus* antiserum (52), suggesting that they were related to *Chloroflexus*. Nold and Ward (28) thus proposed that *Synechococcus* was the main photoautotroph and that most of its photosynthate becomes available to GNSLB via glycolate pro-

duction during the day and glycogen fermentation primarily to acetate at night.

Although *Chloroflexus* and *Roseiflexus* seem to prefer photoheterotrophic growth in culture, *Chloroflexus* spp. can also grow autotrophically with both sulfide (24, 25) and hydrogen (19, 27) as electron donors. There are no indications that *Roseiflexus* can grow photoautotrophically (17). Photoautotrophy by obligately phototrophic GNSLB in sulfidic hot springs has been described previously (13), and the potential for autotrophy in nonsulfidic mats has been suggested by 3-(3,4-dichlorophenyl)-1,1-dimethylurea inhibition of oxygenic photosynthesis and sulfide stimulation experiments (4). The natural abundance of stable carbon isotopic compositions of typical *Chloroflexus* lipids, such as wax esters and long-chain alkenes (45, 49), suggests that GNSLB are autotrophic in sulfidic mats (46, 48), as typical *Chloroflexus* lipids had stable carbon isotope values that were in agreement with inorganic carbon fixation via the 3-hydroxypropionate carbon fixation pathway (19, 49). In sulfidic mats, where the sulfide concentration is low enough for cyanobacteria to be present, the stable carbon isotopic composition of *Chloroflexus*-specific lipids suggests mixotrophic metabolism of the GNSLB population (48). The predominant GNSLB in nonsulfidic, alkaline, siliceous hot spring microbial mats are closely related to *Roseiflexus castenholzii* (30) and might therefore have the potential for heterotrophic metabolism (17). The stable carbon isotopic composition of wax esters typical for *Roseiflexus* (M. T. J. van der Meer, S. Schouten, J. S. Sinninghe Damsté, M. T. Madigan, and D. M. Ward, unpublished data) and *Chloroflexus* (48) found in such mats seems to suggest autotrophy as the dominant carbon acquisition mechanism.

In this study, we investigated the carbon metabolism of GNSLB in greater detail, focusing in particular on the possibility that photoautotrophy by GNSLB might play an important role only during certain periods of the day. In long-term anaerobic incubations of alkaline siliceous mats, sulfide production in the upper mat layers was reported (55). Revsbech and Ward (37) detected sulfide in dimly lit photic zones of alkaline siliceous mats and suggested the possibility of GNSLB photoautotrophy in the morning and evening. In this study, we measured the presence of both sulfide and hydrogen at these times in such mats using microsensors and investigated GNSLB carbon uptake at different times of the day using ^{13}C- and ^{14}C-labeled substrates. The ^{13}C label incorporation was measured in specific lipids of both cyanobacteria (heptadecane [44]) and GNSLB (wax esters [45, 47, 49]), enabling the study of both the carbon flow in hot spring microbial mats and the effects of sulfide and hydrogen addition on the inorganic carbon uptake by both types of phototrophic community members.

MATERIALS AND METHODS

Sampling sites. Core samples for labeling experiments were taken on 25 August 1997, 25 August 1999, and 10 October 2000 from Mushroom Spring, an alkaline hot spring close to Octopus Spring in the Lower Geyser Basin, Yellowstone National Park, Wyo. Over numerous years of studying both springs, we have found them to be nearly identical chemically (unpublished results) and biologically (9, 11, 12, 32, 35). On 25 August 1997, the temperature and pH at the sampling site were 64°C and 8.52, respectively, at the time of sampling. In 1999, the temperature and pH at the sampling site at the time of sampling were 61 to 62°C and 8.13, respectively. In October 2000, the temperature at the sampling site ranged from 54°C predawn to 60°C during the day. Small amounts of sulfide could be measured in the spring water only in the early morning and evening, when there was no direct sunlight on the mat.

Microsensor measurements. We used microsensors for O_2 (36), H_2 (8), and H_2S (10, 23) to monitor the chemical microenvironment in situ on 24 to 25 August 1999. The microsensors were connected to pA meters (PA2000; Unisense A/S, Denmark), and data were recorded on a stripchart recorder. All instruments were battery operated during the field measurements. The measuring tips of the microsensors were aligned, and the microsensors were fixed in a micromanipulator (Märtzhäuser, Germany), which was used to profile the microbial mat in steps of 0.1 to 0.2 mm several times over a diel cycle.

Oxygen microsensors were linearly calibrated from readings in the overlying water, the oxygen content of which was determined by Winkler titrations (15) and from readings in the anoxic part of the microbial mat. The H_2S microsensors were calibrated in the laboratory at in situ temperature using anoxic phosphate buffer solution (0.2 M, pH 7.8) with increasing amounts of sulfide added from a sulfide stock solution with a known concentration (53). We attempted to calibrate the H_2 microsensor in the field by flushing hot spring water with a 1% H_2–99% N_2 gas mixture. However, due to inefficient flushing as well as sensor drift at high temperatures, we did not succeed in obtaining stable calibrations in situ, and H_2 measurements are thus given in units of pA, which are linearly correlated to the dissolved H_2 concentration. In a recent in situ study, gas chromatography (GC) analysis of Mushroom Spring water showed 9 to 97 nmol H_2 liter^{-1} in the spring water during the late afternoon and early evening (M. Kühl and G. Ackerman, unpublished data).

^{14}C-labeling experiments. (i) In situ incubations. Core samples for radiolabeling experiments were taken on 24 August 1999 and 31 May 2000 from the Mushroom Spring microbial mat found at 61 to 62°C. Cores were transferred to 7-ml screw-cap glass vials containing 4 ml of water from the collection site. Control samples were killed by adding formaldehyde (4% final concentration). For incubations in the dark, some vials were wrapped with aluminum foil prior to addition of radiolabel. Each mat core received either 4 μCi of $NaH^{14}CO_3$ (50 mCi mmol^{-1}) or 2 μCi of [2-^{14}C]sodium acetate (54 mCi mmol^{-1}; NEN Life Science Products; Boston, MA). Vials were incubated at ambient-light intensity (from 300 to 2,000 μmol photons m^{-2} s^{-1}, during the incubation period) and hot spring temperature by placing them in the effluent channel at the site of sample collection for 3.5 h (7:30 a.m. to 11:00 a.m. and 8:00 a.m. to 11:30 a.m. with a 1-h preincubation in the dark in 1999 and 2000, respectively). For removal of visible or infrared light, vials were incubated under a cold or hot mirror, respectively (NT42-414 and NT43-453; Edmund, Barrington, NJ). To test for hydrogen and sulfide stimulation, hydrogen was added by injecting 4 ml of pure hydrogen gas into the vials, and sulfide was added to a final concentration of 30 μM. To stop biological activity, samples were fixed in 4% formaldehyde for 4 h. Subsequently, samples were washed in phosphate-buffered saline (130 mM NaCl, 10 mM sodium phosphate [pH 7.2]) and stored in a 1:1 mixture of phosphate-buffered saline and 96% ethanol at −20°C. Mat samples were radioassayed for ^{14}C content with a Tri-Carb liquid scintillation analyzer (model 1900 TR; Packard Instruments Co., Meriden, CT).

(ii) Cryosectioning and autoradiography. For cryosectioning, mat cores were embedded in Optimal Cutting Temperature embedding medium (Tissue-Tek, Miles, IN) at room temperature overnight. Cores were then frozen and sectioned in a cryomicrotome (2800 Frigcut; Reichert Scientific Instruments) at −17°C. Vertical sections from the center of the core (to avoid edge artifacts due to suspension of cores in liquid) were placed on gelatin-coated microscope slides. After air-drying, the Optimal Cutting Temperature Compound was removed by dissolving it in a drop of distilled water and carefully dipping the slide in distilled water. Slides were allowed to air dry again. Microautoradiography was performed as described previously by Nübel et al. (30).

^{13}C-labeling experiments. Labeling experiments were performed with ^{13}C-labeled sodium bicarbonate (99% ^{13}C, CLM-441; Cambridge Isotope Laboratories Inc.) and 1,2-^{13}C-labeled sodium acetate (99% ^{13}C, CLM-444; Cambridge Isotope Laboratories Inc.). Unless stated otherwise, labeling experiments were performed on small cores (no. 4 cork borer) taken just before incubation. The core samples were placed in 4 ml spring water in 7-ml glass screw-cap vials, which were capped with Teflon/silica septa and sealed with tape to avoid contamination of the Mushroom Spring microbial mat with ^{13}C-labeled material. The vials were incubated horizontally with the cores in the upright position at approximately the same temperature as that at the sampling site.

(i) 1997 ^{13}C-labeling experiments. In order to investigate the diel variability in inorganic carbon uptake by GNSLB, labeling experiments were performed at different times of the day. Octopus Spring water was acidified and purged to remove all inorganic carbon. After adjusting the pH to neutral, labeled sodium bicarbonate was added to approximately 15 mM (i.e., inorganic carbon concen-

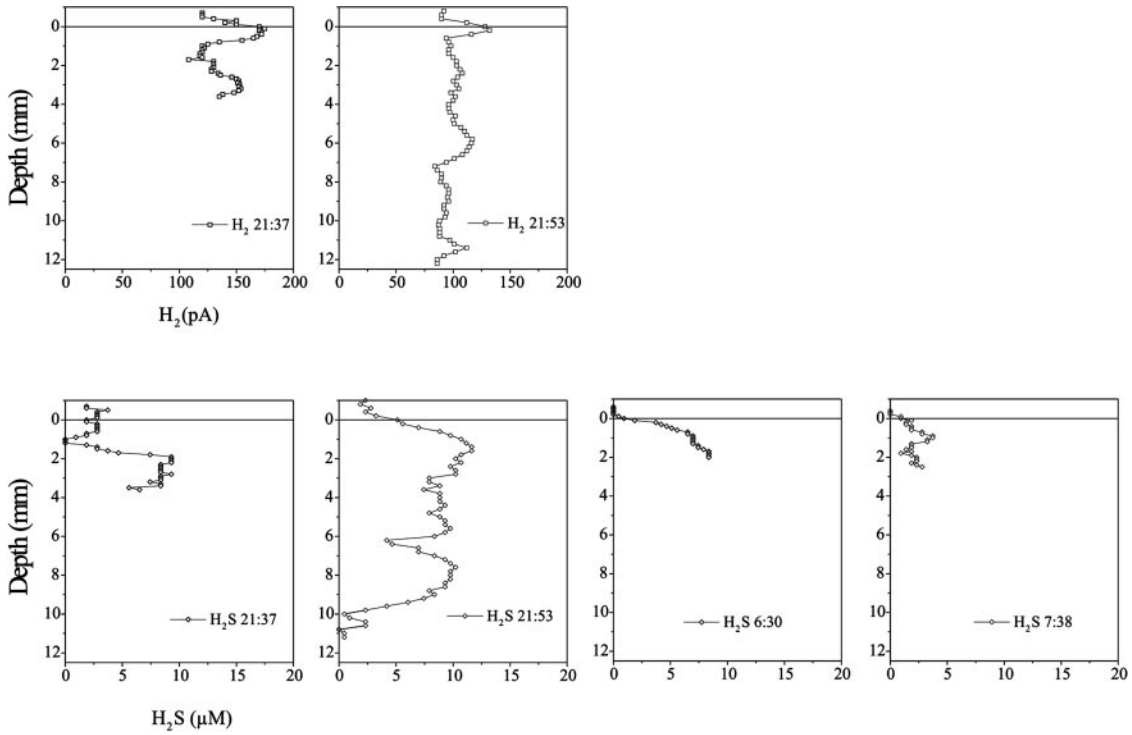

FIG. 1. H_2 and H_2S profiles from Mushroom Spring microbial mats measured in situ at different times around sunset and sunrise on 24 to 25 August 1999.

tration measured in Octopus Spring water [32]). The pH was set to the in situ value of approximately 8.3. By adding 1 ml of in situ Mushroom Spring water and 1 ml of Octopus Spring water containing labeled bicarbonate, the total amount of labeled substrate was approximately 50% of the indigenous inorganic carbon pool. The cores were incubated during the afternoon, starting at 1:15 p.m. (T0) and ending at 5:20 p.m. (T1), or during the morning, starting at 6:45 a.m. (T2) and ending at 10:55 a.m. (T3). The incubations were stopped by freezing on dry ice.

In order to evaluate the carbon transfer from cyanobacteria to GNSLB during the night (28), a pulse-labeling experiment was performed during the natural day/night cycle. The pulse-labeling experiment was performed by incubating mat samples in vials during the afternoon, as described above, except that the water containing the [^{13}C]bicarbonate that did not diffuse into the cores was removed at 5:20 p.m. (T1). The cores were then rinsed with spring water and then resealed and incubated overnight in fresh spring water. The incubations were stopped the next morning at 6:45 a.m. (T2) and 10:55 a.m. (T3). In one set of vials, unlabeled acetate (to 0.5 mM) and glycolate (to 0.5 mM) were added to attempt to trap labeled intermediates that might be transferred between organisms.

(ii) 1999 ^{13}C-labeling experiment. An experiment was designed to observe heterotrophy by GNSLB during the morning from 6:56 a.m. to 11:30 a.m. by labeling with [^{13}C]acetate. The experiment was performed by adding 100 μl of a 20 mM stock solution of labeled sodium acetate dissolved in Octopus Spring water to 4 ml of in situ spring water; the final concentration of labeled acetate in the incubation vials was 0.5 mM.

(iii) 2000 ^{13}C-labeling experiment. In 2000, a [^{13}C]bicarbonate labeling experiment similar to the 1997 experiment was designed, except that a 1-h preincubation period in the dark was included for most samples to allow the labeled substrate, sulfide, and hydrogen to diffuse further into the cores. Samples were incubated in the morning from 8:05 a.m. to 11:15 a.m. In this case, hydrogen was added by injecting 1 ml of pure hydrogen gas. Duplicate samples were incubated and analyzed.

Lipid analysis. Lyophilized cores were ultrasonically extracted, derivatized, and analyzed by GC, GC-mass spectrometry (MS), and isotope-ratio-monitoring GC-MS (see the work of Schouten et al. [42] for details). The stable carbon isotopic compositions of each individual lipid are expressed relative to their natural abundance isotopic composition ($\Delta\delta^{13}C$).

RESULTS AND DISCUSSION

Microsensor measurements performed on 24 to 25 August 1999 showed that during the day, H_2S and H_2 were not detectable in the Mushroom Spring mat but showed their presence in the evening (Fig. 1). Hydrogen accumulated in the upper 1 mm of the mat and at a depth of a few millimeters just as the light intensity dropped to below the compensation point for oxygenic photosynthesis (i.e., the mat became a sink rather than a source of oxygen [A. Wieland et al., unpublished data]). Shortly thereafter, H_2S was detected, and H_2S was present in the mat during the night and early morning, at least until 7:38 a.m. The H_2 electrode was poisoned by H_2S, and H_2 could therefore be measured only before H_2S concentrations increased; it may have been present during the night and early morning as well (Fig. 1).

We measured maximal H_2S concentrations of about 10 μM in the mats. However, the lack of simultaneously measured pH profiles prevented accurate determination of total sulfide microprofiles in the alkaline mat. Revsbech and Ward (37) reported pH levels of 7.8 to 8.4 during the early evening within Octopus Spring mats, which are very similar to the mats in Mushroom Spring. Using these pH values with our H_2S data, we estimated (23) a maximal total sulfide level of about 150 μM in the Mushroom Spring mat.

Labeling experiments without preincubation. ^{14}C-labeling experiments conducted in the morning of 24 August 1999 showed [^{14}C]bicarbonate uptake to be mainly light dependent (Table 1). The decrease in ^{14}C uptake after removal of infrared light suggests the involvement of GNSLB in the bicarbonate

TABLE 1. Radioactivity of bulk organic matter from hot spring microbial mats relative to the full-light incubation (%) after labeling with [^{14}C]bicarbonate

Condition	% of full light[a]	
	1999, nonpreincubated (±error)	2000, preincubated (±SD)
Dark	13 ± 3**	16 ± 10**
−IR[b]	78 ± 44	53 ± 5**
−VIS[c]	29 ± 6**	71 ± 7**
+Sulfide	159 ± 13*	125 ± 4**
+H_2	210 ± 1**	135 ± 19**
Formalin	0 ± 0**	1 ± 0**

[a] Error, range between minimum and maximum of duplicate samples; SD, standard deviation. *$P < 0.07$ and **$P < 0.05$ (one tail, unequal-variance t test).
[b] IR, infrared light.
[c] VIS, visible light.

uptake of the mat, since all bacteriochlorophylls absorb in the infrared region. The decrease in ^{14}C uptake after removal of visible light, which excites chlorophyll *a*, suggests the involvement of cyanobacteria in bicarbonate uptake. Both hydrogen and sulfide addition stimulated the label uptake.

The uptake of ^{13}C-labeled bicarbonate by cyanobacteria and GNSLB was studied by analysis of the ^{13}C content of their specific lipids (Table 2). Results for the 1997 afternoon [^{13}C]bicarbonate labeling experiment (Table 2, T1) showed that the cyanobacterial biomarker heptadecane (44) was heavily labeled with ^{13}C ($\Delta\delta^{13}C = 1{,}610‰$). GNSLB-specific lipids, i.e., wax esters (44, 47, 49), were hardly labeled during this experiment ($\Delta\delta^{13}C$ ranging from 2 to 14‰ [Fig. 2A and Table 2, T1]). The 1997 morning labeling experiment (Table 2) showed a different labeling pattern. Heptadecane was again heavily labeled but to a lesser extent than during the afternoon labeling experiment ($\Delta\delta^{13}C = 1{,}030‰$). In contrast, the GNSLB lipids were labeled to a larger degree than during the afternoon experiment ($\Delta\delta^{13}C$ ranging from 10 to 63‰ [Fig. 2A and Table 2]). Similar results were observed in another experiment performed in 1999, except that when incubation was terminated before there was direct sunlight on the mat, label incorporation into the cyanobacterial lipids was severely suppressed (i.e., >90%) relative to the full-morning incubation, suggesting less oxygenic photoautotrophy before direct illumination of the mat (data not shown). However, the labeling of the wax esters indicated that incorporation of inorganic carbon by GNSLB might have occurred largely before direct illumination of the mat.

Labeling experiments (Fig. 2A) suggest that during the afternoon, when oxygenic photosynthesis has oxygenated the mat and inorganic electron donors such as H_2 and sulfide do not accumulate in the photic zone, cyanobacteria appear to be mainly responsible for the uptake of inorganic carbon and that anoxygenic photosynthesis does not play an important role at this time. GNSLB appear to take up inorganic carbon mainly during the morning period, when the anoxic mat is dimly illuminated and reduced compounds that might serve as electron

TABLE 2. Increase in δ^{13}C values relative to the natural abundance isotope value of lipids extracted from hot spring microbial mats after different labeling experiments

Source and compound	[^{13}C]bicarbonate (‰)									[^{13}C]acetate (‰)
	25 August 1997[a]					10 October 2000[b]				25 August 1999[b]
	a.m.	p.m./pulse				a.m.				a.m.
							Preincubation			
		T1	T2	T3	T3[c]			+H_2S	+H_2	
Cyanobacteria heptadecane	1,030	1,610	2,470	2,420	2,510	180	180	320	170	140
GNSL B										
C_{32} wax ester	63	3	570	1,830	780	14	48 ± 1[f]	51 ± 5	76 ± 0**	180
C_{33} wax ester	24	2	600	2,180	890	8	32 ± 0	45 ± 2**	50 ± 2**	250
C_{34} wax ester	34	2	830	2,180	1,130	9	44 ± 0	54 ± 1**	75 ± 3**	170
C_{35} wax ester	10	14	650	2,160	950	7	23 ± 1	35 ± 2**	33 ± 3*	320
Mixed										
C_{15} FA[d]	56	NM[g]	260	NM	350	9	33 ± 5	38 ± 5	60 ± 2**	140
C_{16} FA[d]	76	290	530	950	580	32	33 ± 6	38 ± 6	35 ± 3	190
C_{17} FA[d]	39	NM	630	1,900	990	9	44 ± 3	60 ± 1**	82 ± 4**	240
C_{17} OH[e]	36	20	630	2,100	910	−1	28 ± 2	33 ± 2	49 ± 2**	120
C_{18} FA[d]	170	680	980	1,420	860	110	95 ± 13	110 ± 1	84 ± 9	240
C_{18} OH[e]	11	23	66	250	110	−7	−3 ± 3	−1 ± 1	0 ± 3	130

[a] 1997 from 6:45 a.m. to 10:55 a.m., p.m./pulse from 1:15 p.m. to 5:20 p.m. (T1) and then, following removal of ^{13}C in water and washing, to 6:45 a.m. (T2) and 10:55 a.m. (T3).
[b] 2000 from 8:05 a.m. to 11:15 a.m. *$P < 0.08$ and **$P < 0.05$ (one tail, unequal-variance t test).
[c] Unlabeled acetate and glycolate added at T2.
[d] FA, fatty acid.
[e] OH, alcohol.
[f] ±error (range between minimum and maximum). *$P < 0.08$ and **$P < 0.05$ (one tail, unequal-variance t test).
[g] NM, not measured.

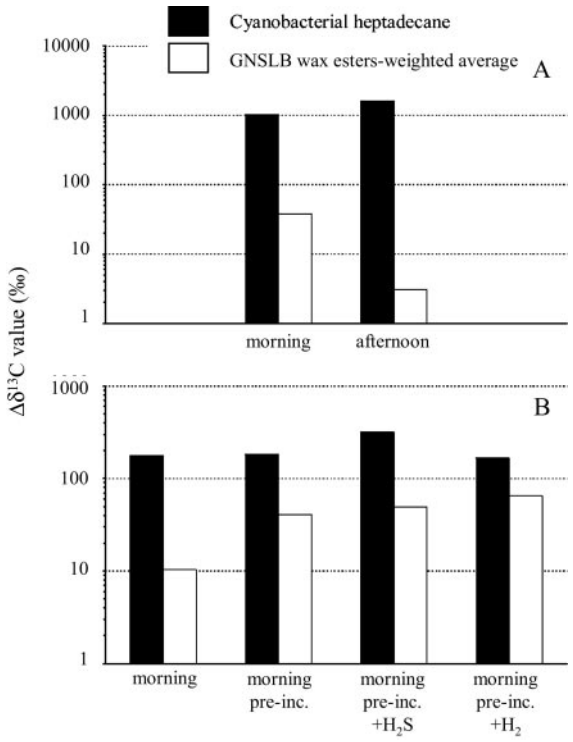

FIG. 2. The degree of labeling of specific lipids expressed in $\Delta\delta^{13}C$ (per mille) relative to natural-abundance stable carbon isotopic composition on a log scale. (A) 1997 afternoon and morning labeling experiments. (B) 2000 morning labeling experiments (error in wax ester data from duplicate cores of the preincubation [pre-inc.] experiments is too small to include in the figure [Table 2]).

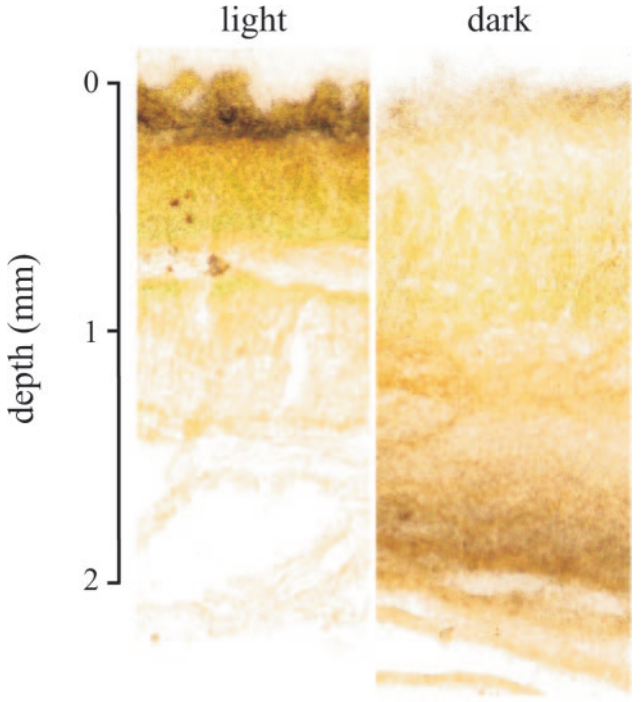

FIG. 3. ^{14}C-labeled acetate diffusion experiment into mat core in the light and in the dark followed by cryosectioning and autoradiography. Silver grains, which appear as tiny black dots, demonstrate where the labeled substrate has diffused during the incubation.

donors for anoxygenic photoautotrophy, such as hydrogen and sulfide, are still present.

Diffusion limitation. A possible explanation for the relatively low $\Delta\delta^{13}C$ values of GNSLB lipids could be diffusion limitation. There is evidence to suggest that GNSLB are more abundant below the mat surface (11, 35, 39, 54). Furthermore, in order to avoid isotopic contamination of the natural mat, the labeling experiments were performed in closed vials. As a consequence, mass exchange between the mat and overlying water was undoubtedly impeded relative to natural flow conditions by the thickening of the diffusive boundary layer under stagnant conditions.

Diffusion limitation of labeled substrates was tested in an incubation experiment using ^{14}C-labeled acetate followed by cryosectioning and autoradiography. These experiments showed that in the light, without preincubation, the ^{14}C was present only in the upper 0.5 mm of the mat, with the majority accumulated in the upper 0.2 mm (Fig. 3). In contrast, when the cores were incubated in the dark for 3.5 h with [^{14}C]acetate, there was a more homogeneous distribution of ^{14}C throughout the top 2 mm (Fig. 3). Basically, in the light, the labeled acetate was consumed at the surface of the core and did not diffuse further into the core during the rest of the incubation. We therefore hypothesize that diffusion limitation may have played an important role in our 1997 and 1999 experiments and may especially have had an effect on the availability of ^{13}C-labeled bicarbonate to GNSLB, which reside deeper in the mat.

Labeling experiments with preincubation. In an attempt to decrease the effect of diffusion limitation, samples in the 2000 experiments were preincubated in the dark after addition of label (and hydrogen or sulfide) for 1 hour to allow label and added electron donors to diffuse further into the cores. We can estimate a maximum distance the labeled substrate might have diffused in to the core before incubation in the light started by using the following equation (43):

$$L = \sqrt{\frac{4Dt}{\pi}}$$

where L is the diffusion distance, D is the diffusion coefficient, and t is the incubation time. This distance would be approximately 0.5 to 1 mm during a 1-h preincubation, large enough to reach more of the underlying GNSLB population and expose all cyanobacteria to the labeled substrate. ^{14}C-labeling experiments conducted in the morning with preincubation again showed that bicarbonate uptake by the mat was light dependent (Table 1). However, in this experiment, the removal of infrared light resulted in a larger decrease in label uptake than removal of visible light, suggesting that GNSLB had better access to the labeled substrate when the substrate was allowed to diffuse further into the mat. Thus, GNSLB appear to play an important role in bicarbonate uptake during the morning in these mats. Again, both sulfide and hydrogen stimulated bicarbonate uptake (Table 1).

In 2000, the morning labeling experiment (Table 2) with

[^{13}C]bicarbonate and without preincubation was repeated as a control for the experiments with preincubation. We found a similar labeling pattern as in 1997, a heavily labeled cyanobacterial biomarker (heptadecane, $\Delta\delta^{13}$C of 180‰) and only limited labeling of GNSLB lipids ($\Delta\delta^{13}$C ranging from 7 to 14‰ [Fig. 2B and Table 2]). The relatively low $\Delta\delta^{13}$C values in the 2000 experiment may have been due to low light intensities in October 2000 relative to August 1997, resulting in decreased cyanobacterial photosynthetic activity. There was a substantial increase in the $\Delta\delta^{13}$C values of GNSLB lipids when the cores were preincubated ($\Delta\delta^{13}$C values ranging from 23 to 48‰ [Table 2]). In contrast, the δ^{13}C value of the heptadecane remained identical to the value in the nonpreincubated cores ($\Delta\delta^{13}$C = 180‰ [Fig. 2B and Table 2]). These results suggest that there is indeed an effect of diffusion limitation on label incorporation and that a substantial amount of labeled bicarbonate is incorporated into GNSLB when the labeled bicarbonate is allowed to diffuse into the cores. After preincubation, both hydrogen and sulfide stimulated [^{13}C]bicarbonate incorporation into GNSLB lipids (Fig. 2B and Table 2) (wax esters, $\Delta\delta^{13}$C ranging from 33 to 76‰), suggesting that both inorganic electron donors can be used by GNSLB for photoautotrophy in situ. Interestingly, we also observed more ^{13}C in heptadecane when sulfide was added, suggesting that mat *Synechococcus* populations may have potential for anoxygenic as well as oxygenic photosynthesis.

Inorganic carbon uptake rates. The ^{13}C label incorporation into specific lipids during the preincubation experiments can be converted into inorganic carbon uptake rates by cyanobacteria and GNSLB when the ratio of biomarker to biomass for the different organisms is known (6). By assuming that heptadecane represents approximately 0.15% of the total cyanobacterial biomass (40) and that the C_{32}, C_{33}, and C_{34} wax esters represent approximately 0.44 to 0.55% of the GNSLB biomass (estimated based on the amount of wax esters in *C. aurantiacus* [49], recently isolated *Roseiflexus* strains [our unpublished results], and the mat itself [48]), the total ^{13}C uptake for cyanobacteria and GNSLB in this experiment can be roughly estimated. These calculations showed that during the morning incubation, when the cores were preincubated, the inorganic carbon uptake by GNSLB exceeded inorganic carbon uptake by cyanobacteria, ca. 0.9 and 0.5 mg C g mat^{-1} h^{-1}, respectively. Preincubation with sulfide resulted in carbon uptake rates of ca. 1 and 0.8 mg C g mat^{-1} h^{-1} for GNSLB and cyanobacteria, respectively. The addition of hydrogen had a large effect on the carbon uptake rate of GNSLB (ca. 1.5 mg C g mat^{-1} h^{-1}) but not on the uptake rate of cyanobacteria (ca. 0.4 mg C g mat^{-1} h^{-1}). Assuming that our estimated lipid concentrations are correct, these estimations show that the inorganic carbon fixation rate by GNSLB during the morning could be of the same order of magnitude as the cyanobacterial fixation rate. The addition of sulfide stimulated photoautotrophy by GNSLB and seemed to have a positive effect on photoautotrophy by cyanobacteria. H_2 only stimulated carbon fixation by GNSLB.

The [^{13}C]bicarbonate labeling results using a 1-h preincubation period might underestimate the total amount of inorganic carbon taken up by GNSLB since we do not know if the entire GNSLB population was exposed to labeled substrate after a 1-h preincubation period. Furthermore, due to the experimental approach, molecular diffusion is the only transport mechanism for inorganic carbon (including the labeled substrates), oxygen, and nutrients from the overlying water to the core and for the transport of metabolic products from the mat to the overlying water. Besides diffusion limitations on mass transfer, there was no continuous external supply of nutrients, and the balance between production and consumption of metabolic products may have been altered. Incubation conditions might have, for instance, resulted in even more extreme superoxic, CO_2-limited, and alkaline conditions than occur naturally within the mat and therefore enhanced photorespiration and formation of glycolate by cyanobacteria during the day. Enhanced formation of glycolate might then enhance heterotrophy by GNSLB. The incubation conditions could also have contributed to a pH-driven shift of the carbonate equilibrium towards carbonate. Both could have led to an underestimation of ^{13}C uptake by GNSLB during a [^{13}C]bicarbonate labeling experiment.

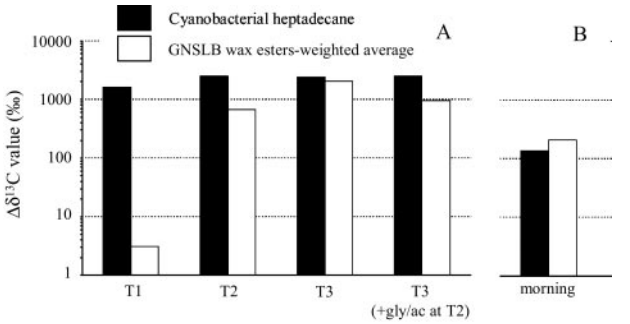

FIG. 4. The degree of labeling of specific lipids expressed in $\Delta\delta^{13}$C (per mille) relative to natural-abundance stable carbon isotopic composition on a log scale. (A) 1997 pulse-labeling experiment. (B) 1999 morning [^{13}C]acetate labeling experiment. T1, 5:20 p.m.; T2, 6:45 a.m.; T3, 10:55 a.m.

GNSLB photoheterotrophy. To assess the potential for heterotrophy by GNSLB, a pulse-labeling experiment with [^{13}C]bicarbonate (Table 2) was performed in 1997. It was impossible to perform this labeling experiment with preincubation since dark preincubation during the afternoon would only result in an artificial midday darkening followed by an abrupt unnatural exposure to very high light intensity. As mentioned above, during the afternoon, the cyanobacterial biomarker heptadecane was heavily labeled, while the GNSLB lipids were hardly labeled, suggesting inorganic carbon fixation mainly by cyanobacteria (Fig. 4A, T1). This was consistent with microautoradiographic results following $^{14}CO_2$-labeling experiments with homogenized mat material that showed *Synechococcus* to be the primary organism responsible for midday photoautotrophy (3). The [^{13}C]bicarbonate-containing water was removed, and the core was rinsed with fresh spring water so that we could focus on the fate of ^{13}C incorporated during the day. The $\Delta\delta^{13}$C values of all lipids, both cyanobacterial and GNSLB, increased overnight (Fig. 4A). The $\Delta\delta^{13}$C value of GNSLB lipids was higher later in the morning, while the $\Delta\delta^{13}$C value of the cyanobacterial heptadecane remained constant (Fig. 4A, T3). However, due to the stagnant conditions in the vial, the diffusion of ^{13}C-labeled cyanobacterial fermentation products from the core to the overlying water might be severely

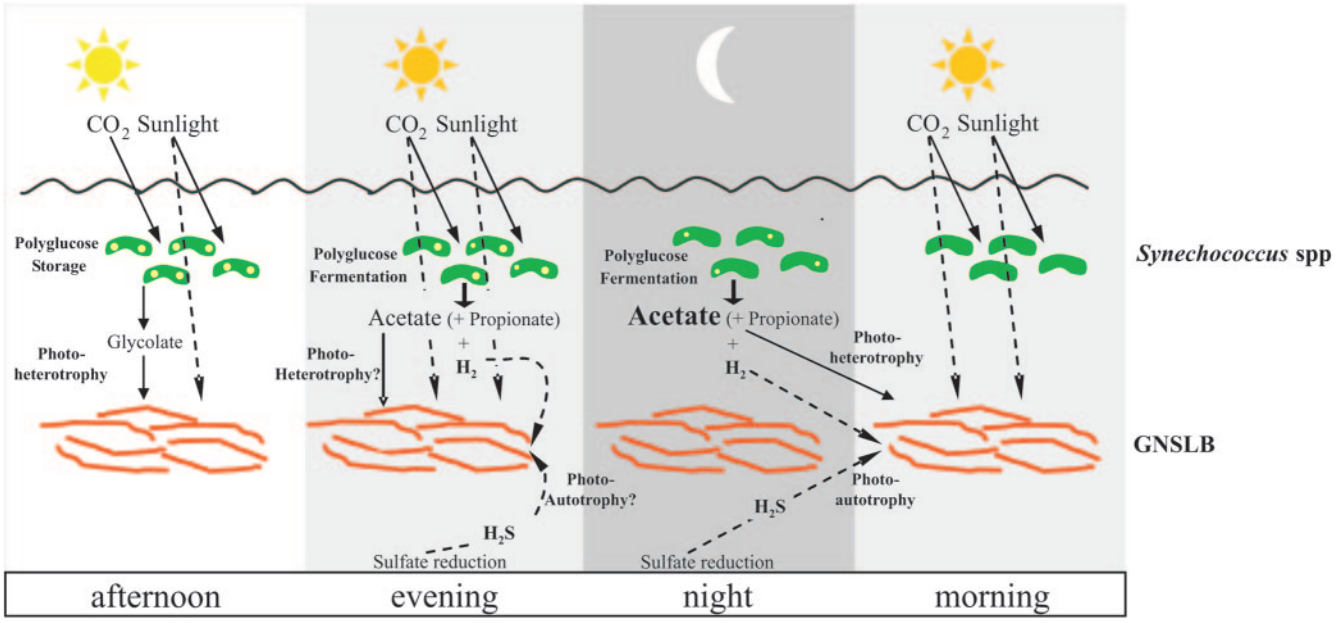

FIG. 5. Working model for carbon flow in an alkaline, siliceous hot spring microbial mat over a 24-h period. Only metabolic pathways thought to be relevant to photoautotrophy and photoheterotrophy are shown.

impeded, and the fermentation products that do diffuse into the overlying water are not transported downstream in vitro but accumulate in the vial. The accumulation of labeled cyanobacterial fermentation products during the night may result in an overestimation of GNSLB photoheterotrophy in the morning in vitro relative to in situ.

The increase in $\Delta\delta^{13}C$ values of the wax esters during the morning was not observed after the addition of a mixture of nonlabeled acetate and glycolate at T2, which presumably diluted the labeled intermediate pool. These observations are consistent with the results of Nold and Ward (28) and their interpretation that polysaccharides produced by *Synechococcus* are fermented during the night to intermediates (e.g., acetate) that are taken up photoheterotrophically by GNSLB during the morning (Fig. 4A and Table 2). However, since both cyanobacteria and GNSLB are able to produce polysaccharides (18, 20, 21) as well as other storage compounds (e.g., polyhydroxy alkanoic acids [2, 49]), it is possible that the overnight label transfer is not only from cyanobacteria to GNSLB but also from GNSLB storage products to GNSLB-specific lipids. In the pulse-labeling experiment, addition of glycolate and acetate might have interfered with the "natural" carbon flow within individual GNSLB cells or even between different GNSLB populations. For this process to be relatively important, more heavy labeling of GNSLB-specific lipids, and therefore GNSLB storage products, during the afternoon would be expected, but this was not seen (Table 2, T1). It can be argued that diffusion limitation and consequent oxygen and possibly glycolate buildup might have prevented us from observing much labeling of GNSLB lipids in the afternoon. However, the morning labeling experiment, which was comparable in incubation time, suggested that it should have been possible to observe more extensive labeling of GNSLB lipids with the high level of [^{13}C]bicarbonate added. We also cannot exclude the possibility that some of the labeled bicarbonate that diffused into the core during the afternoon was not removed by washing and might have been available for both cyanobacteria and GNSLB the next morning.

Uptake of organic substrates in the mat during the morning was demonstrated by labeling with [^{13}C]acetate (Table 2), which gave clear evidence of label incorporation in both cyanobacterial and GNSLB lipids (Fig. 4B and Table 2). The labeling pattern was different from a similar experiment with [^{13}C]bicarbonate (Table 2), as the $\Delta\delta^{13}C$ of the heptadecane was lower than that of the wax esters. The acetate uptake rates in milligrams C gram mat^{-1} hour^{-1} were calculated using the same assumptions as for bicarbonate uptake rates and amounted to ca. 0.05 and 0.8 mg C g mat^{-1} h^{-1} for cyanobacteria and GNSLB, respectively. If our assumptions concerning lipid concentrations in the cyanobacteria and GNSLB are correct and similar for both experiments, then these results suggest that acetate uptake by GNSLB in the morning might be of the same order of magnitude as that of bicarbonate fixation by GNSLB in the morning, even though several experimental biases might have been introduced. These experiments were performed without preincubation, and this would have resulted in greater availability of the labeled acetate to cyanobacteria than to GNSLB, leading to an underestimation of the relative importance of GNSLB. In contrast, we increased the acetate concentration, which might have led to a higher-than-natural level of photoheterotrophy. A previous study (1) has shown that a vial containing a no. 4 cork-borer-sized core of 1 cm in 2 ml of water contained only 0.95 ± 0.40 μmol acetate at 8:35 a.m. In our [^{13}C]acetate labeling experiment, we used no. 4 cork-borer-sized cores of the same length or a little less in 4 ml of water and added [^{13}C]acetate to a final concentration of 0.5 mM acetate or 2 μmol labeled acetate per vial. By increasing the acetate concentration in the vial by at least three times, our

results may overestimate the acetate uptake by both cyanobacteria and GNSLB in the morning.

Carbon flow model. Our labeling experiments provide the data for an updated working model for carbon flow in microbial mats in alkaline, siliceous hot springs (Fig. 5). During the day, when the mat is oxygenated, cyanobacteria appear to be the main inorganic carbon fixers producing mainly storage products (28) such as polyglucose and smaller amounts of glycolate, which is a carbon source for photoheterotrophy by GNSLB (3). During the low-light conditions of the evening, the cyanobacteria discontinue photosynthesis, oxygen production declines, and the mat turns anoxic (37, 38). Cyanobacteria initiate fermentation of their polyglucose into small organic acids, such as acetate and propionate (28), and H_2 (Fig. 1), some of which must diffuse to the overlying water and be transported downstream. Sulfate reduction becomes an important process resulting in sulfide production (16). The GNSLB population at that time might use the fermentation products for photoheterotrophy as well as the produced H_2 and sulfide for photoautotrophy, but light availability could be a problem since light intensity is waning and GNSLB populations appear to be more abundant below the mat surface (35). During the night, the cyanobacteria continue to ferment polyglucose producing acetate, propionate, and H_2, while sulfate reducers continue to produce sulfide. During the morning, the organic acids and inorganic electron donors are available for GNSLB for photoheterotrophy and photoautotrophy (Tables 1 and 2 and Fig. 2 and 3). Compared to the previous model (28), our results show that the inorganic carbon uptake by GNSLB is a relatively important process, especially during the morning and possibly during the evening as well. Hence, there appears to be a large diel variability in inorganic carbon uptake rates of GNSLB.

ACKNOWLEDGMENTS

We thank B. Lindstrom, Niels Ramsing, Thane Papke, Tony Scotti, Andy Gardner, and Rick Morgan for help with field work, Irene Rijpstra for analytical assistance, and Annie Glud for technical assistance and microsensor construction. The U.S. National Park Service is thanked for permission to conduct research in Yellowstone National Park.

This study was supported by the U.S. National Aeronautics and Space Administration (NASA) grants NAGW-2764 and NAG5-3652, the Danish Natural Science Research Council (M.K.), and a PIONIER grant awarded to J.S.S.D. by The Netherlands Organization for Scientific Research (NWO). NWO is also thanked for supporting travel of M.T.J.V.D.M. Shell International Petroleum Maatschappij BV is thanked for financial support for the GC-irMS facility.

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