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HCO_3^- Fixation by Naturally Occurring Tufts and Pure Cultures of *Thiothrix nivea*

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Naturally occurring tufts of the mixotroph *Thiothrix nivea* blanketed the East Everglades (Dade County, Fla.) Chekika artesian well and runoff areas. The rate of HCO_3^- fixation by these *Thiothrix* tufts was determined to be 14.0 ± 5.4 nmol of HCO_3^- per min per mg of dry weight, which reflected a growth rate of 5.0%/h. The addition of 10 mM glucose, ribose, acetate, or pyruvate or 0.05% Casamino Acids (Difco Laboratories, Detroit, Mich.) did not appear to alter the HCO_3^- fixation rate. Whereas 1 mM acetate or 10 mM lactate, ethanol, glycerol, α-ketoglutarate, succinate, fumarate, or citrate slightly stimulated HCO_3^- fixation, 5 to 10 mM malate inhibited HCO_3^- fixation by 90%. Pure *Thiothrix* cultures isolated from Chekika fixed HCO_3^- at rates as high as 29.9 ± 2.8 nmol of HCO_3^- per min per mg of dry weight in the presence of growth medium. Malate did not have a suppressive effect but rather slightly stimulated in vivo HCO_3^- fixation.

Winogradsky first described *Thiothrix nivea* in 1888 (20), and in conjunction with observations made from slide culture experiments with *Beggiatoa* species in 1887 (19), he formulated the concept of chemoautotrophy. Because of difficulties in culturing these sulfur-oxidizing bacteria and spontaneous reactions of sulfur with oxygen, Winogradsky subsequently demonstrated the existence of chemoautotrophs with pure cultures of *Nitrobacter* species in 1890 (21). Winogradsky's pioneering work with *T. nivea* in 1888 (20) showed that this organism was a sheathed, filamentous bacterium with the ability to attach to objects by means of a basal end holdfast which produced rosettes and gliding gonidia and deposited sulfur internally in the presence of H_2S. On the basis of these characteristics, which have since been confirmed (2, 12, 13), Winogradsky created the genus *Thiothrix*, in which *T. nivea* is currently the only accepted species (14).

T. nivea was found to dominate in certain spring-generated, flowing, H_2S-enriched waters (2, 11), and in addition *Thiothrix*-like filaments appeared to be a major component of the aphotic deep-sea hydrothermal vent environments (9, 16). Larkin obtained the first pure culture of *T. nivea* in 1980 (12). Growth of this isolate required a reduced sulfur source, an organic carbon source (acetate, malate, pyruvate, and oxaloacetate served as the sole organic carbon sources), and CO_2, thereby establishing the mixotrophic mode of nutrition for *T. nivea* (13, 17).

The objectives of this study were (i) to determine by $H^{14}CO_3^-$ uptake experiments the rate of HCO_3^- fixation by *T. nivea* in a spring containing H_2S, (ii) to isolate *T. nivea* in pure culture from the spring and to compare the rate of HCO_3^- fixation with that of the naturally occurring tufts, and (iii) to investigate the influence of various organics on the rate of HCO_3^- fixation by naturally occurring tufts and pure cultures of *T. nivea*.

MATERIALS AND METHODS

Field study site. Field studies were conducted in the Chekika State Recreation Area, a 640-acre park located in the East Everglades region of Dade County, Fla., and operated by the Florida Department of Natural Resources. The major attraction of the park is an artificial lake in a hardwood hammock that flows into a smaller pond and the surrounding lower sawgrass. At the time of this study the lake was supplied from a continuously flowing artesian well. The well was drilled in 1944 to a depth of 380 m, tapping the Floridan aquifer. The Floridan aquifer is 214 m below the overlying Biscayne aquifer, from which it is separated by the Floridan aquiclude (18). Floridan aquifer water contained a detectable hydrogen sulfide odor. The well casing extended above ground level approximately 1.5 m and was concealed by an encompassing rock structure. Water flowed from the well at a rate of 4,500 liters/min down a rocky runoff 2 m wide and 10 m long into the lake (Fig. 1). The entire well and runoff system was shaded by two large overhanging trees. The rocks surrounding the well and the runoff area were covered by a dense growth of *Thiothrix* tufts from which flocks continually broke off and were washed out into the lake. The gross appearance of the *Thiothrix* tufts in the runoff varied from a grayish off-white to chalky white. The off-white filaments were located in the interior of the tufts and were believed to be older than the whiter filaments which extended from the edge of a tuft (Fig. 2).

Measurement of pH, conductivity, concentration of dissolved gases, bicarbonate, and chloride in the Chekika water. The conductivity and pH of the water were measured with a standard Beckman pH meter and an Altex Conductivity Bridge (Beckman Instruments, Inc., Fullerton, Calif.). Dissolved oxygen was measured with an oxygen probe (YSI 5331; Yellow Springs Instrument Co., Yellow Springs, Ohio). The concentration of bicarbonate was determined potentiometrically (1) by using a pH meter while titrating the water with 0.02 N HCl. The concentration of chloride was determined volumetrically by titrating with 0.01 N mercuric nitrate (5). Hydrogen sulfide concentration was determined by the methylene blue method (1), using a Varian 635 Techtron spectrophotometer (Varian Associates, Palo Alto, Calif.).

Total direct counts. Total counts of the bacteria in the emerging Chekika well water were determined by staining a 50-ml sample with acridine orange (Sigma Chemical Co., St.

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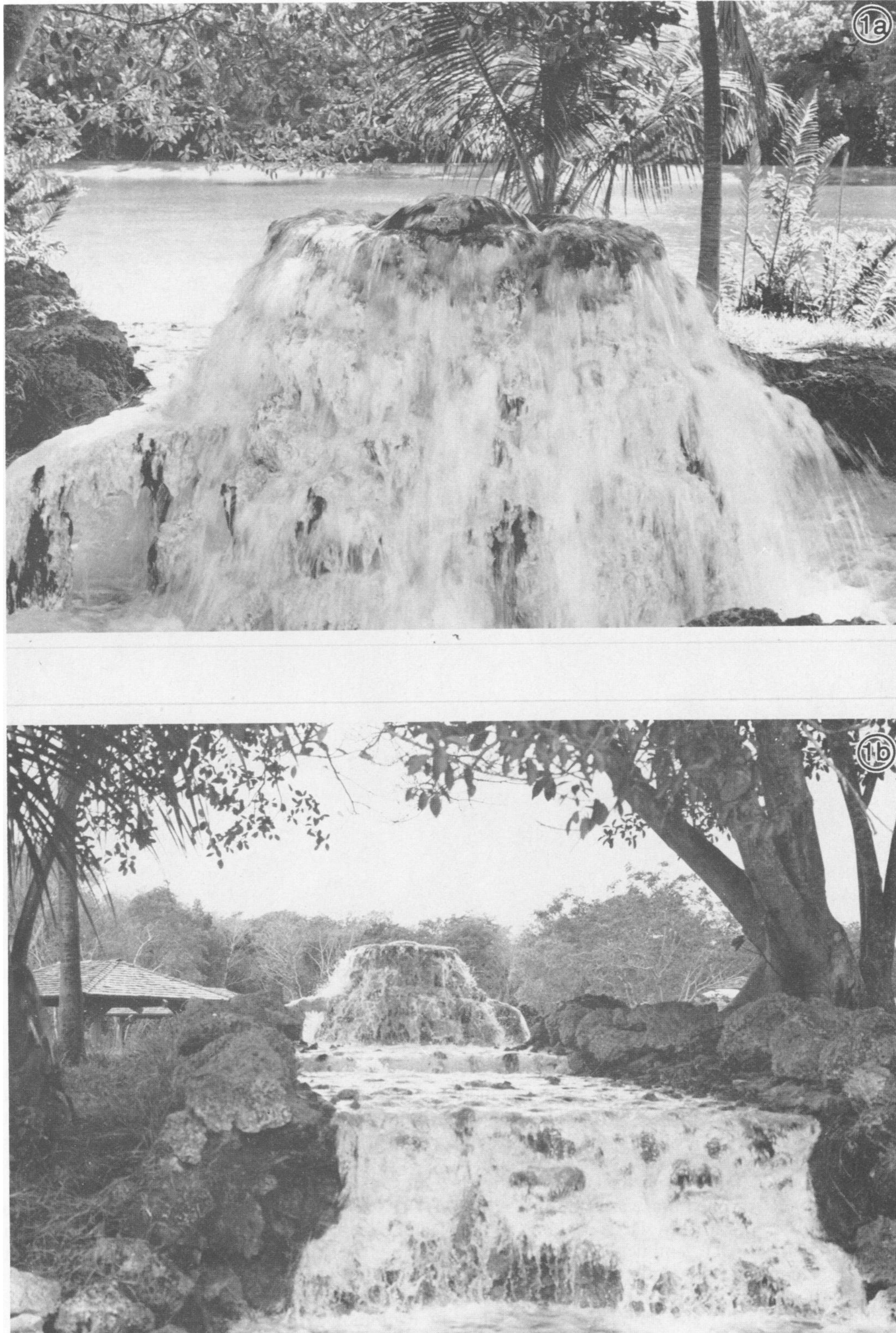

FIG. 1. Field study site. (a) Chekika artesian well and a portion of the lake. (b) Chekika well and runoff.

Louis, Mo.) and filtering the sample onto a 25-mm, 0.2-μm-pore-size filter (Nuclepore Corp., Pleasanton, Calif.) prestained with irgalan black (CIBA-GEIGY Corp., Greensboro, N.C.) by procedure of Hobbie et al. (6). A standard epifluorescence microscope (Carl Zeiss, Oberkochem Federal Republic of Germany) was used to count the fluorescing bacteria, and the total direct count was calculated from the cell count for 10 microscopic fields of view.

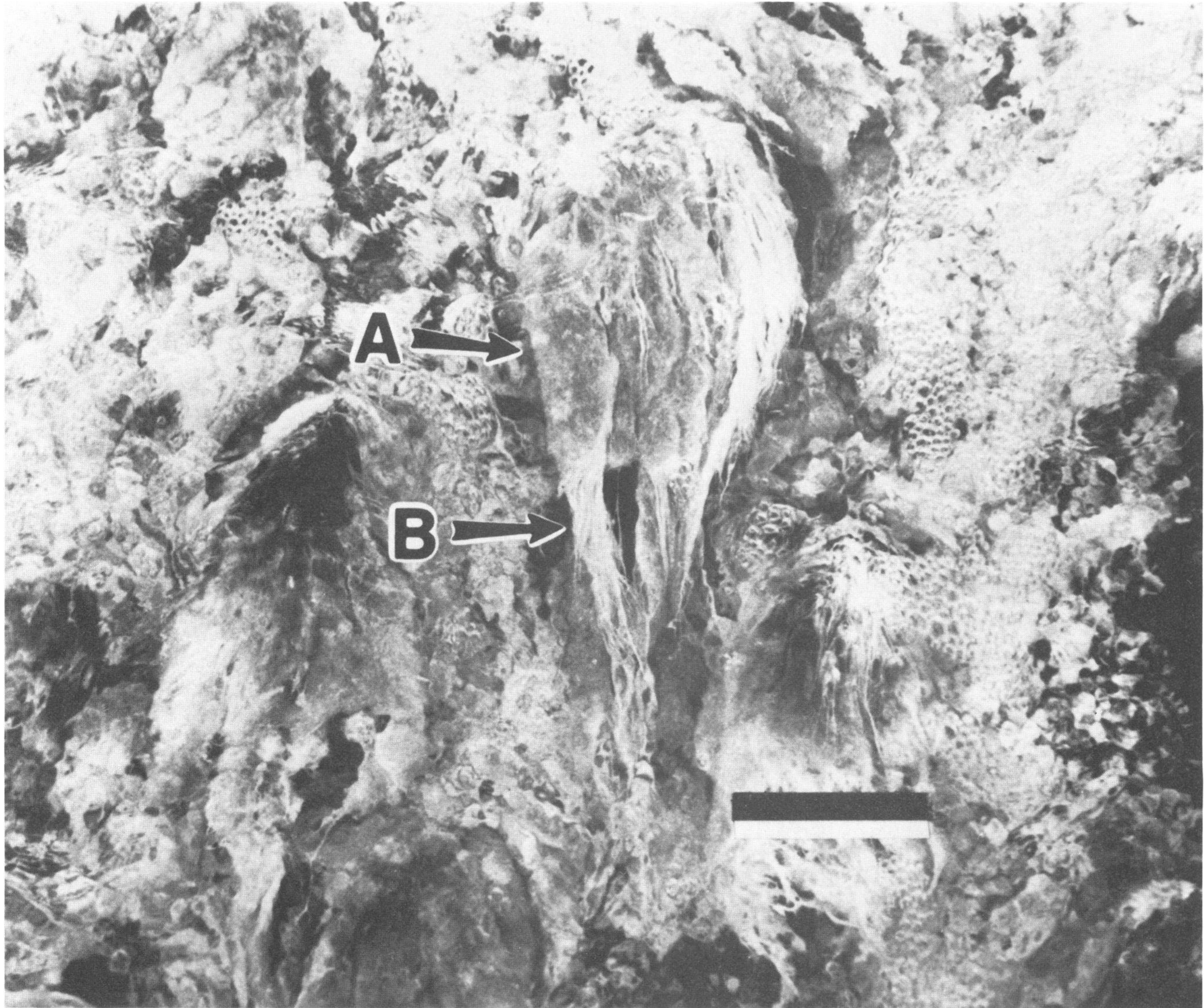

FIG. 2. Tufts of *T. nivea* attached to rocks in the Chekika runoff. A, Old-appearing filaments; B, young-appearing filaments. Bar, 10 cm.

Media and viable plate counts. Plate counts of the bacteria in the emerging Chekika well water were carried out by filtering 100-ml samples onto 47-mm, 0.2-μm-pore-size Metricel membrane filters (Gelman Sciences, Inc., Ann Arbor, Mich.) and placing one filter each onto tryptone glucose yeast agar (TGY) (4), 0.03% yeast extract agar (prepared with 10% Chekika water), agar only (prepared with 10% Chekika water), and MY Chekika water agar (see isolation and culture of *T. nivea*). Each medium was solidified with 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.). In addition, TGY spread plates with 0.1-ml water samples from the Chekika well were prepared. To enumerate the heterotrophs associated with *Thiothrix* tufts, a 1-ml sample of *T. nivea* filaments was aseptically glass homogenized, and serial dilutions of the homogenate were made by using sterile Chekika water. Spread plates were made by using 0.1-ml samples from the dilutions on 0.03% yeast extract agar and agar-only plates prepared with 10% Chekika water and Bacto-Agar. All plates were incubated at 25°C for 5 days before counting.

Percentage of *Thiothrix* and non-*Thiothrix* bacterial components of biomass of *Thiothrix* tufts. To determine the contribution to the biomass of *Thiothrix* filaments by the bacteria associated with these filaments, a wet weight of 1 pg per cell (8) was multiplied by the number of viable bacteria per ml (1 g) of tuft and a factor of 10^4 to account for the difference between the number of bacteria determined by the spread-plate technique and the total number of bacteria present in most aquatic samples (7, 10, 15).

$H^{14}CO_3^-$ uptake experiments with naturally occurring tufts and pure cultures of *T. nivea*. Field $H^{14}CO_3^-$ uptake experiments were conducted by placing *Thiothrix* tufts into scintillation vials containing 10 ml of Chekika well water and then adding 100 μl of $H^{14}CO_3^-$ with a specific activity of 8.4 mCi/mmol (Dupont, NEN Research Products, Boston, Mass.) and incubating the vials for 10 min in the stream. Dark vials were covered with aluminum foil. The reaction was stopped by the addition of 5 ml of 0.3% formaldehyde. Control samples had 0.3% formaldehyde added to vials containing *Thiothrix* tufts before the addition of $H^{14}CO_3^-$. *T. nivea* filaments from each vial were collected by filtration onto 25-mm Metricel membrane filters (pore size, 0.45 μm).

The excess radioactivity was removed by three washes (20 ml each) with Chekika water. In addition, any label which may have accrued on the filters by precipitation or adherence would have been accounted for in the control values and thereby subtracted out of the experimental values. Furthermore, to check for $H^{14}CO_3^-$ precipitation, $H^{14}CO_3^-$ was added to filter-sterilized Floridan aquifer water for 10 min. The water was then filtered, and the filters possessed less than 100 cpm above background. No reduction in counts per minute was observed for acid-fumed samples. This suggests that $H^{14}CO_3^-$ precipitation was not significant under these conditions and that any that could occur was corrected for by the Formalin-killed controls. To assess the effect of organic compounds on *Thiothrix* $H^{14}CO_3^-$ uptake, samples were incubated in the same manner with the addition of the test compound to the vial prior to the addition of $H^{14}CO_3^-$ label. The organics were added from stock 1 M (pH adjusted to 7.0) solutions in order that 10, 5, or 1 mM final concentrations were achieved in the vials.

Initial work showed that large amounts of filaments in the vials lowered the specific counts per minute taken up. In the experiments reported here, results are presented for sample weights less than 35 mg (wet weight) of tufts per vial (10 ml). Wet weights were converted to dry weights by using a wet weight-to-dry weight ratio of 8.65 ± 1.6 determined from the mean of four samples of tufts which were filtered and weighed wet and then dried to a constant weight overnight in a 55°C incubator.

$H^{14}CO_3^-$ uptake experiments with *T. nivea* pure cultures were done by using broth cultures that were grown for 48 h, after which 30-ml samples were centrifuged at $12,000 \times g$ for 10 min in a Sorvall RC5B centrifuge (Du Pont Co., Wilmington, Del.). The pellets were suspended in various growth media or buffers (pH 7.5), each containing 0.06% thiosulfate in a total volume of 10 ml. Experiments were initiated with the addition of 100 μl of $H^{14}CO_3^-$ to a vial containing a concentrated sample of *T. nivea*; the reaction was stopped after 10 min by the addition of 5 ml of 0.3% formaldehyde. The formaldehyde solution was added to control samples before the addition of label. The filaments were then collected and weighed as in the field experiments, with the exception that distilled water was used for the wash.

After the weight determination, the filters were placed into scintillation vials to which Hydromix liquid scintillation cocktail (Yorktown Research, Hackensack, N.J.) was added, and the ^{14}C was measured in a Beckman LS 9000 liquid scintillation counter.

Rates of HCO_3^- fixation and growth. The rate of HCO_3^- fixation was calculated by subtracting the counts per minute of the control from the counts per minute of the sample, correcting for quench, and dividing by the sample dry weight. This value was then divided by the specific activity of the label in the reaction mixture. The specific activity of $H^{14}CO_3^-$ was 12,400 cpm/μmol of HCO_3^- for experiments conducted in Chekika water and changed proportionally with the concentration of carbonate in the reaction mixtures used in pure culture incubations. Growth rate was expressed as percent increase in biomass per hour.

Isolation and culture of *T. nivea*. A modified MY medium (12) was used for the isolation and growth of *T. nivea* in pure cultures. The modifications were the use of Floridan aquifer water (Chekika or Key Largo-John Pennycamp well [referred to as C-MY and K-MY medium, respectively]) to prepare the medium and the increase in the concentration of sodium thiosulfate to 0.06%. The pH was 7.8. For solid media, 1.5% Bacto-Agar was added. For broth cultures, the well water was autoclaved and filtered through a Metricel membrane filter (pore size, 0.2 μm) to remove precipitates before medium preparation.

T. nivea was obtained in pure culture from the Chekika well runoff by the endpoint dilution technique after initial homogenization of a 2-cm-long *Thiothrix* tuft in sterile Chekika water. Samples (0.1 ml) from each dilution were spread onto C-MY agar plates and incubated at 25°C for 5 days. A filamentous colony which appeared was isolated from numerous other nonfilamentous colonies and streaked for further isolation. The isolate appeared to be pure, consisted of filaments filled with sulfur granules, and was identical in appearance to those seen in slide preparations made with impure *Thiothrix* tufts from the Chekika well (Fig. 3 and 4). The purity of the culture was confirmed by streaking colonies on nutrient agar and brain heart infusion agar. The pure culture was identified as *T. nivea* by the characteristics described by Brock (3), Larkin (12), and Larkin and Shinabarger (13).

Broth cultures were started by sterile loop transfer of an isolated *Thiothrix* colony into a screw-cap tube containing 4.5 ml of C-MY broth. The tube was incubated at 25°C on a Junior Orbit Shaker rotating at 80 rpm. After 48 h, the tubes contained a dense growth of *T. nivea*. Stock broth cultures were started and maintained by sterile transfers of 0.1-ml samples into new C-MY broth tubes every 48 h. Microscopic examinations of the broth cultures showed that they were pure and contained filaments of *T. nivea* in which there were numerous sulfur granules (Fig. 4B). To culture larger volumes of *T. nivea*, stock cultures were inoculated into 300 or 1,200 ml of either C-MY or K-MY broth. *T. nivea* stock tubes and flask cultures were checked for contamination by spread plating 0.1-ml samples onto nutrient agar, brain heart infusion agar, and C-MY agar plates.

RESULTS

Chekika well water chemical and physical data. The water emerging from the Chekika well had a constant temperature of 23°C and a pH of 7.65. The hydrogen sulfide concentration was 88 μM in the emerging water and decreased to 29 μM by the end of the race. Oxygen was near saturation (219 μM) throughout the race, although the emerging water was anaerobic. The concentration of bicarbonate in the water was 3.67 mM, and the specific conductance was 5,300 μS/cm at 25°C. The concentration of chloride was 17.2 mM.

Enumeration of bacteria in the water and tufts. Chekika well water emerging from the pipe had a total cell titer of 6.40×10^3 ($\pm 2.08 \times 10^3$) bacteria per ml (acridine orange direct count) (AODC), of which 76% fluoresced green.

Viable titers from emerging well and runoff water in addition to that from bacteria associated with *Thiothrix* tufts are given in Table 1. The greatest number of colonies occurred on C-MY medium, which indicated there were at least 13 viable bacteria per ml of emergent water. Low-nutrient well water agar and yeast extract agar, both of which contained 10% Chekika water, supported the growth of fewer bacteria, and the high-nutrient, TGY agar produced the lowest counts. At least 78 viable bacteria per ml were found in the runoff water; this is six times as many viable bacteria as in the emerging well water.

Numerous bacteria were observed associated with the *Thiothrix* tufts (Fig. 3). The viable titer of these tuft-associated bacteria was 3.8×10^6 ($\pm 2.0 \times 10^5$) viable bacteria per ml of tuft, which accounts for about 3.8% (±0.2%) of the biomass of the tufts.

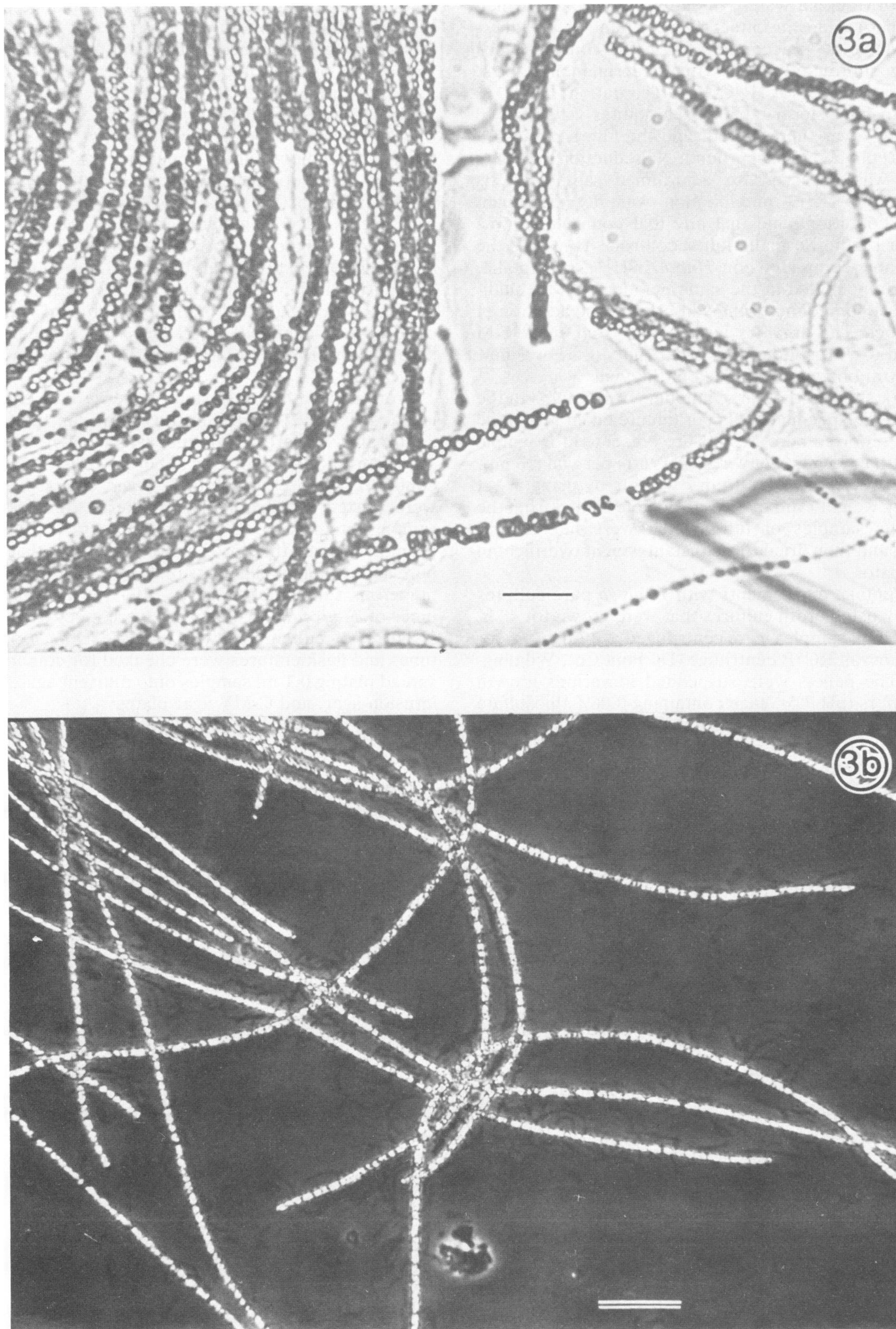

FIG. 3. Photomicrographs of *T. nivea* filaments from the Chekika runoff. (a) Filaments showing the accumulation of internal sulfur granules. Bar, 5 μm. (b) Phase-contrast photomicrograph of numerous unicellular bacteria associated with *Thiothrix* tufts. Bar, 10 μm.

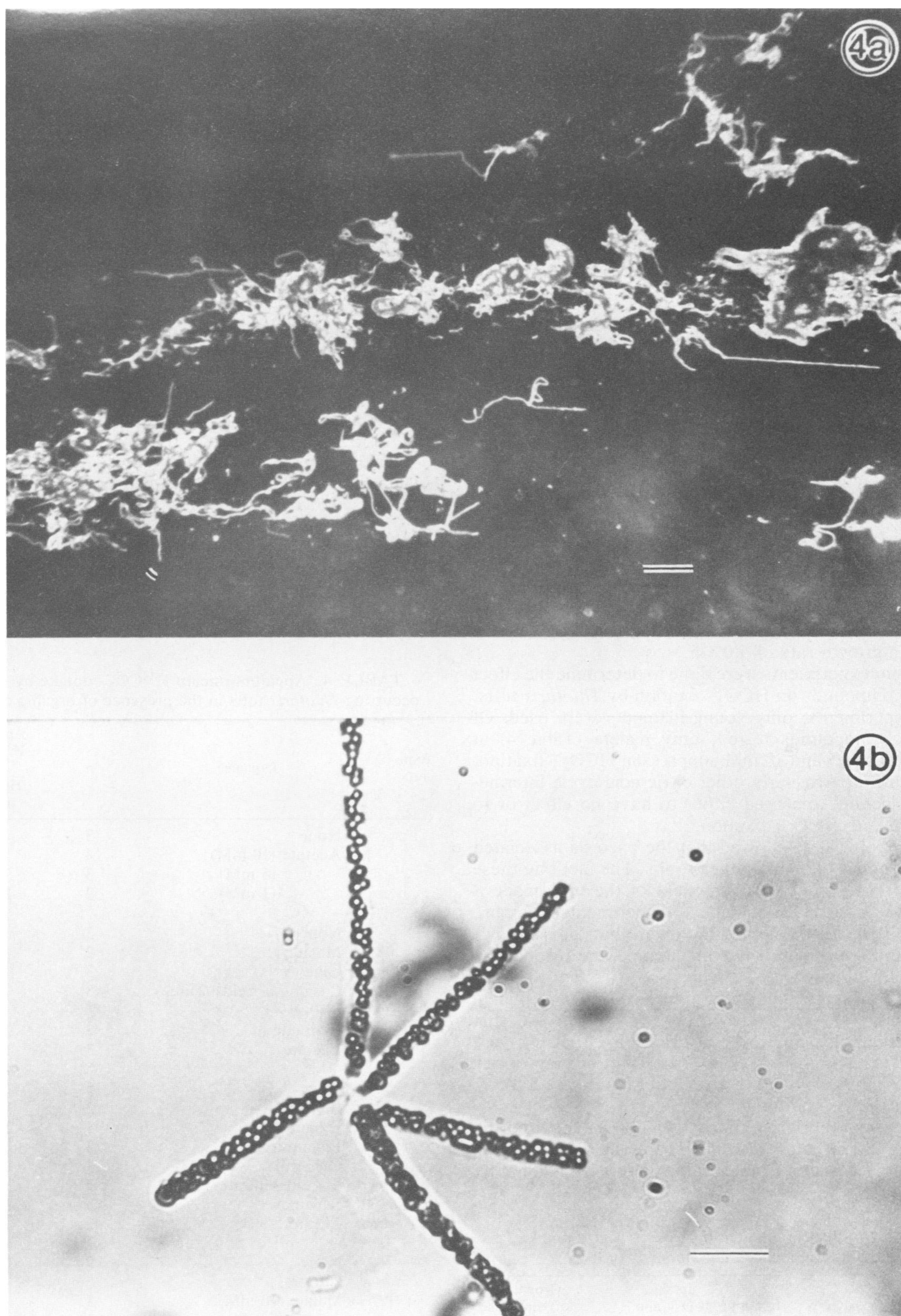

FIG. 4. Photomicrographs of *T. nivea* filaments in pure culture. (a) Phase-contrast photomicrograph of pure culture filaments growing as colonies on C-MY agar. Bar, 1 mm. (b) Pure culture filaments (rosette) grown in C-MY broth. Bar, 5 μm.

TABLE 1. Chekika well water bacteria

Source	Bacteria (CFU/ml) in[a]:				Counting method
	TGY	YE	C-MY	WWA	
Emergent water	1.1	7.0	13.0	10.3	Filter[b]
Runoff water	78.0	ND	ND	ND	Spread[c]
T. nivea tufts	ND	3.8×10^6	ND	3.7×10^6	Spread[d]

[a] TGY agar (4) was prepared with distilled water. YE agar contained 0.03% yeast extract. YE and well water agar (WWA) were prepared with 10% Chekika water. C-MY is described in Materials and Methods. ND, Not determined.

[b] Samples (100 ml) were filtered through sterile 47-mm membrane filters (pore size, 0.2 μm) (Gelman Sciences Inc.), and one filter each was placed on each of the different plates.

[c] Samples (0.1 ml) ($n = 5$) of water were spread plated; standard deviation was 31 CFU/ml.

[d] Samples (0.1 ml) from dilutions of homogenized tufts were spread plated in duplicate; CFUs between plates differed by less than 6%.

Labeled-bicarbonate uptake by naturally occurring tufts of *T. nivea*. The Formalin control value for field experiments was never greater than 50 cpm above the background value for the scintillation counter, and the counts per minute for experimental samples were at least 30-fold greater. Light did not stimulate HCO_3^- uptake (Table 2). Young filaments (Fig. 2) could fix HCO_3^- at a rate three times that of the older filaments (Table 3). By including data from other experiments (so that $n = 18$) and by using only samples incubated with label, the rate of HCO_3^- fixation for the naturally occurring tufts was determined to be 14.0 ± 5.4 nmol of HCO_3^- per min per mg of dry weight, which represents a growth rate of 5.0%/h.

Subsequent experiments were done to determine the effect of organic compounds on HCO_3^- fixation by *Thiothrix* tufts. In these experiments, only young filaments were used. Of the organic compounds tested, only malate (Table 4) at concentrations of 5 and 10 mM suppressed HCO_3^- fixation. Other organics, particularly other citric acid cycle intermediates and lactate, appeared either to have no effect or to slightly stimulate HCO_3^- fixation.

The plate count data showed that the bacteria associated with the *Thiothrix* tufts are heterotrophs. The fact that these bacteria were a significant component of the tufts made it necessary to do the $H^{14}CO_3^-$ uptake experiments with pure cultures of *T. nivea* to support the claim that the *Thiothrix* filaments, and not the associated bacteria, were responsible for the HCO_3^- fixation.

Labeled-bicarbonate uptake in pure cultures. Replicate pure cultures of *T. nivea* grown in C-MY broth and suspended in 10 ml of C-MY broth at pH 7.6 fixed HCO_3^- at a rate of 29.9 ± 2.8 nmol of HCO_3^- per min per mg of dry weight. This corresponds to a growth rate of 10.8%/h for *T. nivea* under these conditions.

HCO_3^- uptake by pure cultures of *T. nivea* required the presence of Florida aquifer water (Table 6) or MY media prepared with this water (Table 5); the cultures would not fix

TABLE 2. Effect of ambient light on $H^{14}CO_3^-$ uptake by naturally occurring *Thiothrix* tufts

Sample treatment	n	$H^{14}CO_3^-$ uptake (nmol of HCO_3^-/min per mg of dry wt)	Growth rate (% increase in biomass/h)
Light	7	7.0 ± 2.5	2.5
Dark	3	5.3 ± 1.1	1.9

TABLE 3. Mean wet weight and $H^{14}CO_3^-$ uptake by young and old naturally occurring *Thiothrix* tufts

Sample age	n	Mean wet wt (mg)	$H^{14}CO_3^-$ uptake (nmol of HCO_3^-/min per mg of dry wt)	Growth rate (% increase in biomass/h)
Young	4	34.8 ± 8.5	15.4 ± 6.1	5.5
Old	4	32.1 ± 5.1	5.5 ± 1.1	2.0

HCO_3^- when suspended in Tris buffer or phosphate buffer (both containing 0.06% thiosulfate) alone.

Malate did not have such a suppressive effect on HCO_3^- uptake by *T. nivea* in pure culture (Table 6), and in fact low concentrations of malate stimulated HCO_3^- uptake. The rate of HCO_3^- uptake by pure cultures in Chekika water (Table 6) was less than that in C-MY medium (Table 5) but was almost the same as that for the naturally occurring *Thiothrix* tufts.

DISCUSSION

Blanketed by *T. nivea* tufts, the Chekika artesian well system provided an interesting site for studies of this bacterium because >96% of the bacterial biomass consisted of *T. nivea* filaments. Thus, $H^{14}CO_3^-$ uptake experiments were initiated with the naturally occurring *Thiothrix* tufts. Because the experimental mixtures were contained in vials, the experimental conditions were such that they could only approximate in situ conditions since a direct in situ experi-

TABLE 4. Apparent (mean) $H^{14}CO_3^-$ uptake by naturally occurring *Thiothrix* tufts in the presence of organic compounds

Expt no.	Organic[a]	n	Apparent (mean) $H^{14}CO_3^-$ uptake (nmol of HCO_3^-/min per mg of dry wt)
1	None	3	6.8 ± 3.6
	Acetate (10 mM)	4	7.6 ± 2.3
	(5 mM)	3	8.5 ± 3.1
	(1 mM)	2	10.7 ± 1.0
2	None	3	7.7 ± 3.9
	Malate	2	0.5
	Glucose	3	5.3 ± 2.0
	Casamino Acids (Difco) (0.05%)	3	6.3 ± 1.5
	Pyruvate	3	6.6 ± 1.9
	Lactate	3	12.2 ± 7.1
3	None	5	17.9 ± 5.7
	Ribose	3	18.1 ± 2.7
	Ethanol	3	23.2 ± 1.5
	Fumarate	3	25.7 ± 12.5
	Glycerol	3	26.1 ± 17.6
	α-Ketoglutarate	3	26.3 ± 12.2
	Succinate	3	34.1 ± 15.6
	Citrate	2	35.5 ± 17.1
4	None	9	12.2 ± 3.6
	Citrate	5	9.9 ± 2.6
	Malate (10 mM)	3	1.3 ± 0.3
	(5 mM)	3	2.0 ± 0.2
	(1 mM)	2	12.5 ± 0.2

[a] Unless otherwise indicated, the concentration of the added organic was 10 mM.

TABLE 5. Effect of Tris and phosphate buffer (pH 7.6) on $H^{14}CO_3^-$ uptake by pure cultures of *T. nivea*

Incubation mixture[a]	$H^{14}CO_3^-$ uptake (nmol of HCO_3^-/min per mg of dry wt)	Growth rate (% increase in biomass/h)
Tris (10 ml)	None	
Tris (9.9 ml)–C-MY (0.1 ml)	None	
Tris (9.0 ml)–C-MY (1.0 ml)	None	
Tris (5.0 ml)–C-MY (5.0 ml)	11.3	4.1
C-MY (10 ml)	26.2	9.4
Phosphate (10 ml)	0.02	0.0
Phosphate (5.0 ml)–K-MY (5.0 ml)	19.5	7.0
Phosphate (2.5 ml)–K-MY (7.5 ml)	13.3	4.8
K-MY (10 ml)	23.1	8.3

[a] Thiosulfate was adjusted to 0.06% in all samples.

ment could not be carried out in the flowing water. To the best of our knowledge this is the first report on a rate of HCO_3^- fixation by *T. nivea* tufts in a naturally occurring sulfide spring.

Although CO_2-fixing enzymes were not assayed in this study, Strohl and Schmidt (17) have detected high phosphoenolpyruvate carboxylase activity in crude extracts made from a *Thiothrix* strain isolated from the Floridan aquifer at Key Largo. Consequently, it is noted that with the use of $H^{14}CO_3^-$ label the resulting HCO_3^- uptake is assumed to be synonymous with CO_2 fixation.

During the initial course of $H^{14}CO_3^-$ uptake experiments, a source of variation in rates of HCO_3^- fixation for *T. nivea* was observed. The whiter exterior filaments possessed higher rates of HCO_3^- fixation (Table 3) than the gelatinous inner filaments. This is possibly related to the age or position of the filament in the tuft. Therefore, experiments with natural tufts were done only with the outer filaments.

To support the claim that the *T. nivea* filaments and not other organisms were primarily responsible for the HCO_3 fixation, $H^{14}CO_3^-$ fixation experiments were conducted with *T. nivea* isolated in pure cultures from the Chekika system. The results of these experiments showed that pure cultured *T. nivea* in filtered Chekika water (Table 6) fixed HCO_3^- at a specific rate which was the same as that obtained with the naturally occurring tufts incubated only in the presence of label. However, when these experiments were repeated in C-MY medium, the rate of HCO_3^- fixation doubled. This could reflect an increased ability to fix HCO_3^- when nutrient enrichment (an organic nutrient, a coenzyme, or a trace element) is supplied in the medium. Further, the inability of *T. nivea* to fix HCO_3^- in Tris buffer (Table 5) and the extremely low rate of HCO_3^- fixation in phosphate buffer (Table 5) (both buffers contained thiosulfate) suggest that HCO_3^- fixation requires a component found in Chekika water. The fact that *T. nivea* did not grow in MY medium unless it was prepared with Floridan aquifer water suggests that this strain of *T. nivea* requires a component in the natural water not found in MY medium ingredients.

TABLE 6. $H^{14}CO_3^-$ uptake by pure *T. nivea* cultures in 10 ml of membrane-filtered Chekika water (pore size, 0.2 μm) containing 0.06% thiosulfate and various concentrations of malate at pH 7.6

Malate concn (mM)	*n*	$H^{14}CO_3^-$ uptake (nmol of HCO_3^-/min per mg of dry wt)	Growth rate (% increase in biomass/h)
0	2	13.0 ± 2.6	4.7
1.0	2	18.9 ± 9.0	
2.5	2	28.1 ± 9.8	
5.0	2	18.6 ± 0.5	
7.5	2	11.5 ± 5.4	
10	2	8.4 ± 1.6	

The suppressive effect of malate on HCO_3^- fixation by naturally occurring *Thiothrix* tufts needs further clarification, since HCO_3^- fixation was not suppressed but rather was stimulated in experiments with pure cultures of *T. nivea* (Table 6). The suppressive effect obtained during uptake experiments with the *Thiothrix* tufts may have resulted from interactions with the bacteria that were associated with the *Thiothrix* filaments. These associated bacteria may have taken up malate and metabolized it, releasing a product(s) which had an inhibitory effect(s) on HCO_3^- fixation. A further consideration is that the apparent HCO_3^- uptake obtained with naturally occurring tufts in the presence of organic compounds may be an underestimate due to isotope dilution resulting from respiration of the organic by either the *T. nivea* filaments or the bacteria associated with them. Therefore, the possibility remains that the organics could have stimulated growth of the tufts, and this is not reflected in the data. However, we feel that with an incubation time of 10 min a significant quantity of the organic would not have been converted to CO_2 and that the tufts primarily incorporated carbon from carbonate instead of the added organic. In addition, the uptake rates for the naturally occurring tufts (incubated with label only) and pure cultures (incubated in Chekika water) are within the range of the apparent rates for the naturally occurring tufts in the presence of the organics.

The naturally occurring *Thiothrix* tufts fixed HCO_3^- at a rate less than that of pure cultures in the laboratory. Nevertheless, we conclude that the *Thiothrix* tufts contributed to primary productivity in the Chekika system by utilizing carbonate and sulfide supplied by the Floridan aquifer artesian well.

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