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Nutritional Relationships Among Microorganisms in an Epilithic Biofilm Community

Thomas K. Haack and Gordon A. McFeters

Department of Microbiology, Montana State University, Bozeman, Montana 59717, USA

Abstract. Previous studies of an epilithic algal-bacterial community in a pristine mountain stream suggested that heterotrophic bacteria were responding to the metabolic activities of the phototrophic population. Subsequent studies were performed to follow the flow of labeled carbon, from its initial inorganic form, through the trophic levels of the mat community. A majority of primary production metabolites were excreted by the algal population during active growth; this shifted to an incorporation into cellular material as phototrophic activity declined. Results suggest that there was a direct flux of soluble algal products to the bacterial population, with little heterotrophic utilization of dissolved organics from the overlying stream water. Both phototrophic productivity and bacterial utilization of algal products peaked at approximately the same time of year. Activity of the diatom-dominated algal population declined as silica concentrations in the stream water dropped, leading to a situation in which the sessile bacteria were substrate limited. These events resulted in an almost complete disappearance of the community in early September.

Introduction

An important feature of many sessile microbial communities in natural aquatic environments exposed to light is the presence of phototrophic organisms, which are intimately associated with the bacterial population in a dense polymeric slime matrix. The ability of these algal-bacterial communities to proliferate even in the cold, nutrient-deficient waters of alpine streams has led to the conclusion that phototrophic CO_2 fixation by the algae may support heterotrophic bacterial growth [14].

The excretion of soluble organic intermediates of primary production by algae was first demonstrated by Tolbert and Zill [24] and subsequently confirmed in several laboratories [12, 23, 25, 26]. The discovery that bacteria cocultured with algae possess higher growth rates than those grown in pure culture [19] suggested that bacteria may possess a metabolism capable of using these extracellular compounds. A number of researchers [1, 17, 18, 22, 27] have demonstrated bacterial uptake of compounds regarded as algal excretion prod-

Send reprint requests to G. A. McFeters at the above address.

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ucts, and most of these reports also describe the kinetics of this utilization process.

Using radiotracer techniques, Nalewajko and Marine [23], McFeters et al. [21], and Bauld and Brock [3] were able to demonstrate the incorporation of ^{14}C-algal extracellular products into bacterial biomass in batch cultures. Derenbach and Williams [10] were the first to directly demonstrate bacterial uptake of ^{14}C-algal extracellular products without previous separation of the two organisms. However, these and other investigations of algal-bacterial nutrient relations were performed in the planktonic environment. There is a paucity of data describing these processes in microbial mat communities, primarily because the allochthonous nutrient input of most aquatic environments imposes a structural and trophic complexity upon biofilms that cannot be adequately resolved by present methods.

This report describes the nutritional relationships of a sessile microbial mat community in a more structured manner than has been previously attempted. The extremely oligotrophic character of the stream in which the mat was located reduced its trophic complexity to what appeared to be a delicate relationship between algal and bacterial cells. It was thus possible to separate and define these 2 categories of organisms for analysis of their activities and interactive processes.

Materials and Methods

Study Site

The epilithic algal-bacterial mat community chosen for this study was located in Pine Creek, an extremely oligotrophic stream located in a high alpine zone (2,740 m) of the Absaroka mountain range in southwestern Montana. Its general characteristics were described in a previous report [14].

Chemical Analyses

Several chemical properties of the stream water were monitored throughout the study period by previously described methods [14]. Samples for the determination of silica concentrations were collected in acid-washed Nalgene jars and analyzed by the heteropoly blue method [2], as outlined in the Hack Wastewater Analysis Handbook (Hack Chemical Co., Ames, IA). Total organic carbon in the stream water was measured on a Total Carbon Analyzer (Oceanography International, College Station, TX).

Carbon Flow Investigations

The general procedure for carbon flow experiments is outlined in Figure 1. A specific area of the mat community was removed from rocks in the streambed using a sterile scalpel and brush in combination. This procedure cleaned the rock surfaces of virtually all the mat material, which was transferred to a sterile Nalgene jar containing a small amount of filtered stream water (0.22 μm, Millipore Corp., Bedford, MA) and agitated by hand to make a uniform suspension. Aliquots of this suspension, equivalent to 1 cm^2 of mat material, were then added to several 250 ml media bottles (Wheaton Scientific, Millville, NJ) containing filtered stream water. Controls were killed

with the addition of formalin (1% final concentration). An untreated, foil-wrapped dark control also was prepared to check for dark CO_2 uptake by the heterotrophic population. The flasks were sealed by caps fitted with butyl septa (Wheaton). After 15 min in situ preincubation, 0.1 ml $NaH^{14}CO_3$ (10.0 $\mu g/\mu Ci$; New England Nuclear, Boston, MA) was added to each reaction vessel. After 3 hours in situ incubation, activity in half of the untreated replicates was stopped with formalin addition. The remaining vessels were wrapped in foil, returned to the laboratory at ambient temperature, and formalinized after an additional 21 hours of dark incubation at in situ temperature. Incubations were always at the same time of day under consistent illumination conditions. All samples were then acidified with 1 ml 50% HCl, and $^{14}CO_2$ was removed from solution by flushing the samples for 3 hours with nitrogen. The remaining suspension was homogenized with a Sorvall Omnimixer to dissociate clumps of cells. Duplicate aliquots (10 ml) of this suspension were separated into 2 size fractions using 5.0 and 0.2 μm membrane filters in series (Nuclepore, Pleasanton, CA). This process of differential filtration was previously used to separate phototrophic and heterotrophic populations arising from planktonic environments [5, 10]. Observations with epifluorescence microscopy indicated that the 5.0 μm filters succeeded in retaining virtually all of the phototrophic cells. Samples to which only L[^{14}C(U)]-glutamic acid (292.0 mCi/mmol, NEN) was added were processed in an identical manner to determine the percentage of heterotrophic activity retained on 5.0 μm filters. These values were used to correct for inefficiency of particulate separation, as microscopic observations indicated that approximately 15% of the bacterial cells were being retained by the large pore size filters. Each filter was rinsed three times with 10 ml portions of reagent-grade water, and the filtrate was collected to determine the concentration of labeled soluble organic compounds. The filters were placed in scintillation vials and 10 ml Filter Count scintillation cocktail (Packard Instruments, Downers Grove, IL) was added. One ml aliquots of the filtrate were added to 5 ml Aquasol (NEN). Activities of all samples were analyzed on a Packard Tri-Carb 560 CD scintillation counter. After correction for background, quenching, and errors related to the efficiency of separation of the algal and bacterial components, the radioactivity retained on 5.0 μm filters was recorded as phototrophic incorporation; that detected on 0.2 μm filters was recorded as heterotrophic productivity at the expense of soluble organic products excreted by the phototrophs. Radioactivity in the filtrate fraction was assumed to be algal products not utilized by heterotrophic processes, since the N_2 flushing procedure succeeded in removing 100% of the activity arising from dissolved $^{14}CO_2$.

Results

The carbon flow experiments were designed to allow a precise definition of the nutritional interactions occurring between the phototrophic and heterotrophic microorganisms in the Pine Creek mat community. Addition of ^{14}C-bicarbonate allowed for examination of carbon flow through the trophic levels of the mat, and size-differential filtration of the system after incubation separated the various labeled carbon pools into defined compartments.

The results of these experiments are outlined in Table 1, and are divided into several fundamental processes.

Carbon Conversion by Mat Phototrophs

Relative phototrophic production was calculated as the total radioactivity detected in all three fractions of the system (particulate on 5.0 μm and 0.2 μm filters and filtrate) following $^{14}CO_2$ removal from the reaction vessels. The total amount of inorganic carbon in the system, and consequently the specific activity of the added $^{14}CO_2$, did not vary significantly during the study period, making

Table 1. Results of carbon flow experiments performed on the epilithic algal-bacterial mat community of Pine Creek in 1981

Date	Incubation time	A. Total C transformation (dpm)[c]	B. Algal fixation (dpm)	C. Algal excretion (dpm)	D. Bacterial uptake (dpm)	E. % Algal extracellular products incorporated by bacteria
6/25	3 h	—	—	—	—	—
	24 h[b]	10,630	5,026	1,404	4,200	75.0
7/8	3 h	64,142	9,428	50,490	4,223	7.7
	24 h	69,250[a]	17,833	44,253[a]	7,164	13.9
7/21	3 h	98,554	44,760	49,104	4,690	8.7
	24 h	98,237[a]	65,170	25,047	8,020	24.2
7/28	3 h	81,712	24,740	45,656	11,312	24.8
	24 h	74,522[a]	33,250[a]	20,800	27,438	56.9
8/11	3 h	81,742	26,590	51,888	3,264	5.9
	24 h	71,460[a]	34,610	30,926	5,922[a]	16.1
8/21	3 h	6,248	5,582	0	666	100
	24 h	6,784[a]	6,117[a]	0	666[a]	100

[a] Values for 24 hour incubations were not significantly different from 3 hour samples at the 95% level.
[b] All samples received constant illumination for 3 hours. Half of the samples were subsequently incubated under dark conditions for an additional 21 hours.
[c] All values were calculated from triplicate samples; the average reported values are equivalent to 1 cm^2 of mat material.

phototrophic conversion rates accurate indicators of changes in the primary production. Phototrophic production (Table 1, column A) increased steadily and remained at high levels until the middle of August, when a sharp decrease led to nondetectable values in early September. Two sets of replicates were considered in these experiments: one set was incubated in the light for 3 h before formalin addition, the other set was dark-incubated for 21 hours subsequent to the light exposure. The values in column A did not differ appreciably between the 3- and 24-hour samples, which would suggest that inorganic carbon fixation was a light driven process. One limitation of the carbon flow system was the inability to evaluate $^{14}CO_2$ resulting from bacterial respiration of algal fixation products. Thus, it is probable that the values found in column A represent an underestimation of the total $^{14}CO_2$ fixation for the incubation period.

Fate of Photoassimilated Compounds

It was assumed that the fixation products of the phototrophs either were incorporated into cellular matter or excreted into the surrounding medium, the latter process being a characteristic feature of algal metabolism [11]. The seasonal values for these two fractions are presented in columns B and C of Table 1.

Table 2. Comparison of primary production rates and excretion processes by mat photographs, and coincident silica concentrations in Pine Creek

Date	Total $^{14}CO_2$ assimilation by phototrophs (dpm)	% Excreted as soluble ^{14}C compounds	Silica concentration in stream water (mg/l)
6/25	N.D.	N.D.	1.25
7/8	64,142	85.3	0.95
7/21	98,554	54.6	0.99
7/28	81,712	69.7	1.44
8/11	81,742	67.5	0.24
8/21	6,248	10.3	0.09
9/2	0	0	0.15

Both fractions show long-term seasonal trends similar to total fixation, but differ radically with respect to their short-term patterns. Radioactivity in the cellular fraction increased during the dark incubation (presumably from algal heterotrophy), but the labeled algal extracellular products in the diluent water decreased significantly in the same period, and were completely absent from the system after incubation for the last two sampling dates.

In Table 2, seasonal values for phototrophic production are compared with the fraction of assimilation products released as soluble organic compounds. Excretion values were above 50% during the period in which production rates were relatively high (June 25–August 11). In late August, production declined appreciably and only a small portion of assimilation products (10%) were released by algal cells. Table 2 also lists the concentration of silica in the stream water throughout the season. Silica was the most abundant inorganic compound monitored in June and July, but dropped suddenly in August to very low levels that continued into September.

Bacterial Uptake of Excreted Algal Products

The amount of soluble algal extracellular products incorporated into bacterial biomass was measured by the radioactivity retained by a 0.2 μm filter after adjustment for retention of bacterial cells on the 5.0 μm prefilter. Column D of Table 1 lists these results, which also exhibited a mid-season peak followed by a gradual decline characteristic of the phototrophic activity patterns. However, 24 hour samples were significantly higher than identical samples incubated only in the light on all but the last two sampling dates. Column E lists the fraction of algal extracellular products that was incorporated into bacterial biomass, and indicates that bacterial heterotrophic activity on excreted algal organic products was a significant process in the absence of light.

Heterotrophic uptake of inorganic carbon was found to be insignificant in the dark controls, and also in reaction vessels in which the phototrophic pop-

ulation was removed before addition of labeled bicarbonate. These data agree with the results of other investigators working with planktonic systems [5, 6, 10].

The rates of actual primary production in the mat was normalized to a specific chlorophyl *a* level and used to monitor the physiological state of sessile phototrophs during the study. Interestingly, the results (Fig. 2) show that maximal conversion rates by phototrophs (5.2 mgC/mgchl *a*/3 hours) occurred not when the stream water temperature was greatest, but rather at a point earlier in the season when the stream water was at 3°C and just beginning to warm. These rates remained relatively high as the mat biomass increased, but declined sharply while water temperatures were still rising. By early September, photosynthetic activity was virtually nonexistent, and algal biomass had decreased to the lowest levels of the season.

Figure 3 provides a graphic representation of phototrophic and bacterial metabolic activity patterns over the course of the study season. Phototrophic conversion rates were comparatively high (ca. 64,000–98,000 dpm/3 hours) and appeared to reach a threshold level during July, after which the decline was abrupt and rapid. Bacterial heterotrophic activity on algal products showed patterns similar to phototrophic production. Uptake rates of these products began to decline in early August at the same time that excretion processes were waning. After mid-August, algal excretion dropped to the point where algal nutrients were limiting to the heterotrophic bacteria, and activities in both populations soon became nondetectable.

Light vs Light-Dark Incubations

The data obtained on July 21 were selected for an illustration of the processes occurring in the carbon flow system over a 24-hour time course (Fig. 4). The labeled carbon accumulated in all three fractions very rapidly during the light incubation. Incorporation into bacterial and algal cellular components continued in the dark, but at rates considerably slower than those occurring in the light. As bacterial incorporation of algal extracellular products continued in the dark, a concurrent loss of radioactivity was seen in the filtrate of the experimental system.

Discussion

There were dramatic changes in the biomass and activity of the phototrophic population in the Pine Creek mat community during the study season. Both chlorophyl *a* levels and $^{14}CO_2$ conversion rates in the mat increased through the summer to maximum values in early August. This apparent climax was rapidly followed by abrupt declines in both parameters, with conversion rates dropping to essentially zero in 3 weeks.

Phototrophic excretion of soluble organics also was monitored throughout the season using the carbon flow system. Several researchers have demonstrated this process by phototrophic organisms in pure cultures [11, 12, 20, 24]. In the

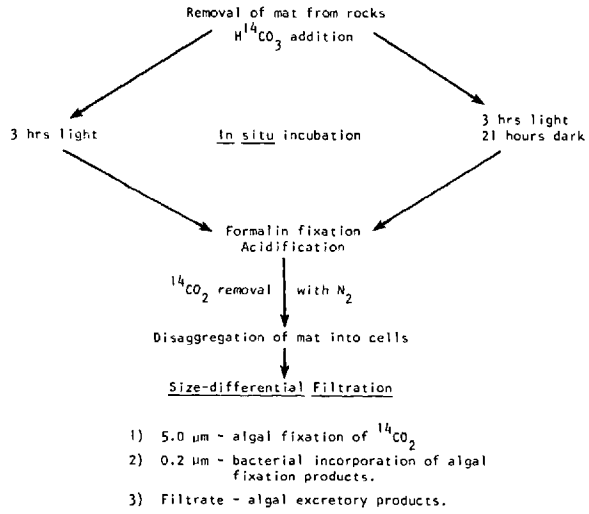

Fig. 1. Schematic of generalized procedure for carbon flow experiments.

complex environment of sessile stream communities, however, the behavior of algal cells with respect to this process is largely unknown. Previous publications [3, 12] have reported that aquatic phototrophs excrete between 3–40% of the total carbon fixed during primary production, but do not take into account changes that may occur with the physiological state of the organisms. Since the attached phototrophic population of Pine Creek exhibited such dramatic seasonal fluctuations, it provided an ideal natural system for the study of excretion processes.

Table 2 lists the percent of total phototrophic fixation that was excreted by algal cells as soluble organic products. These values were always above 50% (for 3 hour samples) in the period from 6/25–8/11, during which total production rates were also comparatively high. It is possible that the mechanical process of mat disaggregation may have resulted in the additional release of organic matter by the phototrophs, but it was assumed that prior fixation minimized this effect. After August 11, transformation rates of $^{14}CO_2$ by phototrophs declined appreciably, and only 10% of the process metabolites were excreted. This was precisely when primary production rates per unit of algal biomass decreased abruptly from the high rates observed in July (Fig. 2). These results suggest that the algal population excreted the greatest amount of metabolic intermediates when growth and production rates were highest. As cells became stressed and less productive, their metabolism was altered so that a majority of transformation products were being incorporated into cellular material.

Several chemical and physical parameters of the stream were monitored to determine a potential trigger for the decline of the phototrophic community. No correlations were found between phototrophic activities and either nitrogen or phosphorus concentrations in a previous study [14]. Stream water temperatures, which have been shown to influence algal growth rates [1], were still at their warmest of the season when the declines began in August. Light intensity

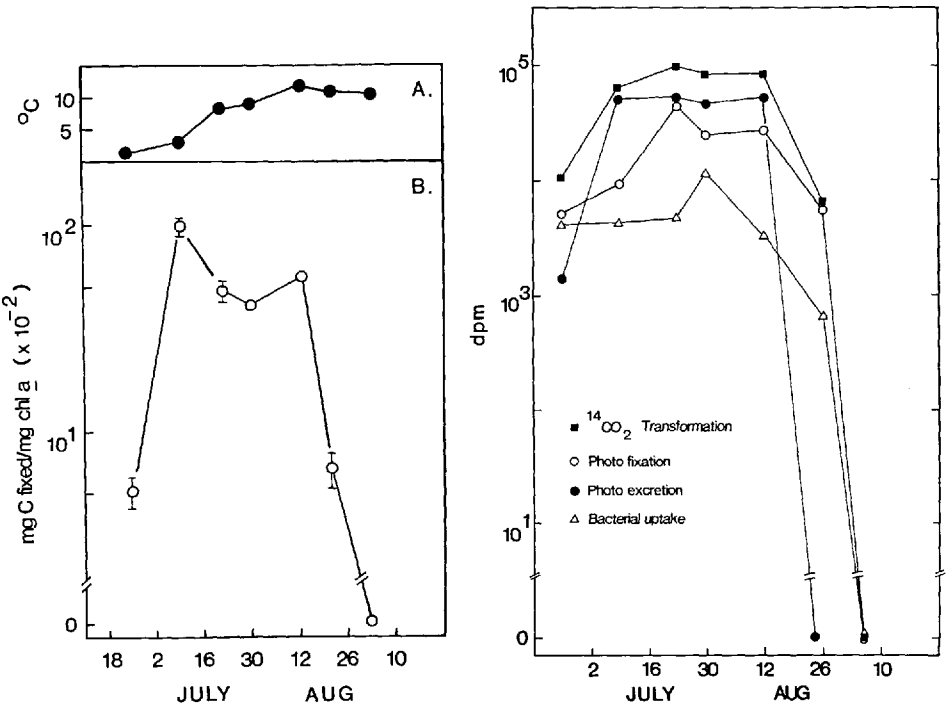

Fig. 2. A Stream water temperatures of Pine Creek during the 1981 study season. **B** Primary production per unit biomass by the mat phototrophs.

Fig. 3. Seasonal patterns of carbon flow in the Pine Creek algal-bacterial mat community. All values are reported as the radioactivity detected in each fraction of the experimental systems, and normalized to 1 cm^2 of mat material.

was also monitored throughout the season and did not vary appreciably between any of the sampling dates.

The observation that greater than 85% of the mat phototrophs were diatoms (unpublished results) suggested that silica (SiO_2) was an essential inorganic nutrient. Table 2 reveals that silica concentrations in the water dropped in early August to levels considerably lower than values reported to be the minimum concentration required for diatom growth [16]. This occurrence was coincident with the declines observed in both chlorophyl *a* levels and algal productivity rates of the mat community. It thus appears that silica was a nutrient essential to the growth and maintenance of the phototrophic population, and its virtual disappearance from the stream water may have led to conditions under which the diatoms could not survive in the Pine Creek system.

The carbon flux experiments performed in 1981 were the first to directly demonstrate bacterial utilization of extracellular algal products in a sessile community without prior separation of the two components of interest. Over the course of the season, the activity of the heterotrophic bacteria was greatest when $^{14}CO_2$ conversion rates were also at a maximum (Fig. 3). The reductions in activity of the two populations also appeared to coincide up to August 21,

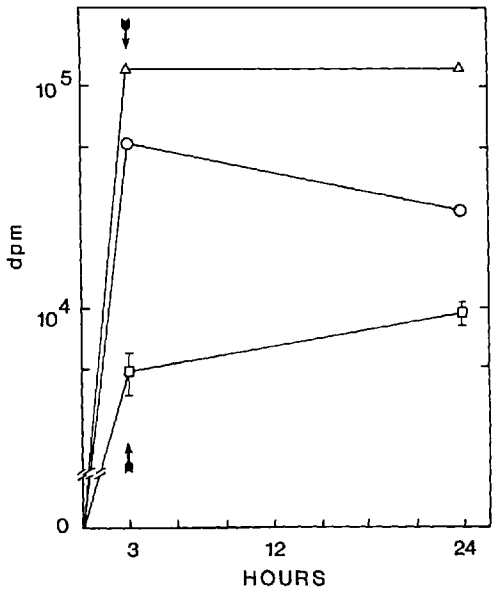

Fig. 4. Short-term carbon flow in the Pine Creek mat. Rates of phototrophic $^{14}CO_2$ transformation (△), ^{14}C-soluble product excretion by algal cells (○), and bacterial uptake of ^{14}C excreted compounds (□) in 1 cm^2 of mat material during the course of a single representative assay. Arrows indicate onset of dark incubation conditions.

at which point it appeared that algal products became limiting to the bacteria. These results support the theory that bacteria in close association with algae act as sinks for the extracellular intermediates of phototrophic production [3], and respond positively to the release of these compounds with higher metabolic activity. Correlations such as this have been observed before; Bell [4] found that bacterial uptake of algal extracellular products in the plankton of a lake was greatest during algal blooms. Others [6, 17, 25] have shown that bacteria respond positively to the release of extracellular metabolites by the phototrophs, using them as an additional nutrient source in otherwise dilute aquatic environments.

Nutrient Flux Within the Mat

Based on population and heterotrophic activity measurements of both sessile and planktonic bacterial populations, it has been suggested that the sessile group plays a much more active role in stream purification processes by removal of organics from the overlying water column [13, 15, 18]. The low levels of organic carbon in the stream water (less than 4 mg/l TOC) in Pine Creek, however, led to an early assumption that transport of organics from the bulk fluid into the mat was negligible, and that algal products were the main source of nutrition for heterotrophic bacteria within the mat community.

Results from carbon flow experiments demonstrated that bacterial uptake of soluble algal excretory products occurred during both the light and dark incubation periods (Fig. 4). During light incubation (0–3 hours), the phototrophs appeared to be actively excreting organic products, and bacterial uptake rates

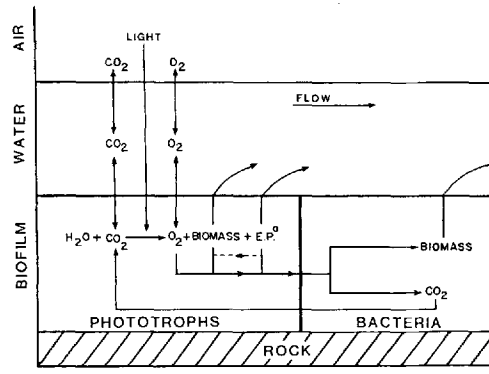

Fig. 5. Proposed model for carbon flow in the epilithic mat community of Pine Creek. *E.P. represents algal extracellular products.

of these compounds were relatively fast. As the samples were switched to dark conditions, phototrophic excretion rates declined appreciably. Bacterial cells, faced with the loss of these primary nutrients, turned to algal products that were present in the bulk fluid of the system. The uptake rates of this secondary nutrient source were considerably lower than those for light-induced excretion products, suggesting that the sessile bacteria of Pine Creek preferentially used algal products directly as they were excreted from the cells.

Previous microscopic examinations of the mat community support this hypothesis. Bacterial cells were primarily observed either on the surface of algal cells or enclosed within a dense polymeric slime matrix [14]. Such physical orientations would facilitate a direct intercellular flux of algal nutrients to the heterotrophic bacteria. Furthermore, the glycocalyx material could function as a diffusional barrier for nutrients external to the bacterial microenvironment and prevent the loss of those nutrients produced within the mat. The results of these investigations suggest that when phototrophs are an integral part of a sessile community, the efficiency with which organic material is removed from aquatic systems by bacteria will necessarily be reduced since these bacteria preferentially utilize the soluble metabolites of primary production.

Based on the results of these studies, a model for carbon flow among microorganisms in the Pine Creek mat is proposed (Fig. 5). CO_2 is transported into the mat by diffusion and reduced by phototrophs in the presence of sunlight. In addition to incorporation into algal biomass, much of the photoassimilated CO_2 is released within the mat as soluble organic compounds, along with oxygen. The sessile bacteria utilize both fixed algal material (in the form of lysis products) and excreted organics as a primary nutrient source. Bacterial uptake of these compounds leads either to an accumulation of bacterial biomass or satisfaction of bacterial maintenance energy requirements (resulting in the formation of CO_2). Algal extracellular products not utilized by the microbial population are transported outward into the stream water. Microbial cells are also continually removed from the mat by the fluid shear forces of the stream.

The ecological relationships among sessile aquatic microorganisms were examined in a highly pristine environment which allowed us to view its trophic structure in a less complex setting. However, the similarities in the basic struc-

ture of microbial films implies that many of the carbon flow features observed in this study could well be applied to mat communities that are trophically more complex. The definitive nature of the results also suggests that the experimental procedures used here could be adapted for studies of other biofilm systems.

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