The microbial dynamics of an epilithic algal-bacterial mat community in an oligotrophic, high alpine stream
by Thomas Kenneth Haack

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
Montana State University

Abstract:
An ecological study was performed to assess the microbial dynamics of an algal-bacterial mat community. The oligotrophic nature of the stream in which the mat existed made it possible to examine these processes in a more structured manner than has been previously attempted. The mat grew on the surfaces of rocks in the streambed, its initial formation resulting from the colonization of those surfaces by microorganisms transported from the feeder reservoir.

The activity and biomass of the diatom-dominated phototrophic population increased until an apparent climax stage was reached in mid-August. This proliferation was followed by an abrupt decline, apparently resulting from a sudden drop in silica concentrations in the stream water. The algae appeared to be actively excreting organic products of photoassimilation into their surrounding environment, with the actual percentage of total fixation released by the cells determined by the physiological states of the phototrophic population. Results demonstrated the utilization of algal extracellular products by the sessile bacteria. During the course of the season, bacterial activity with respect to algal products reached a maximum when phototrophic activity was highest. Time-course results suggested there was a direct flux of organic nutrients from algal to bacterial cells. Bacterial uptake rates of algal release compounds were very high in the light when the phototrophs were actively excreting. In the dark, these rates slowed considerably despite the fact that there was still an abundance of soluble organics in the surrounding medium. The bacterial population of the Pine Creek mat also possessed a high degree of specificity for algal products, and showed little metabolic activity on other soluble organic compounds during the time in which algae were actively excreting. With the decline in activity of the phototrophic population, the sessile bacteria became nutrient limited with respect to excreted organic compounds and possibly oxygen, leading to a situation in which algal lysis products became their major nutrient source. The continued heterotrophic activity of the bacteria may have then weakened the structure of the mat, facilitating its removal from the substratum by the shear force of the stream water.

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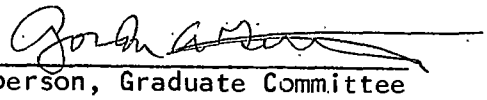
Approved:



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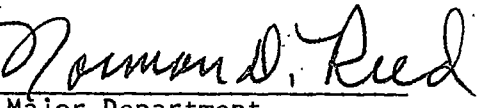

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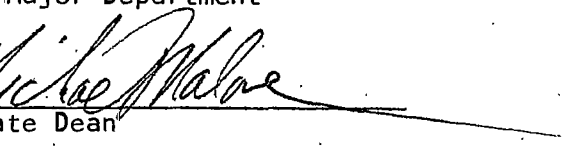

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MONTANA STATE UNIVERSITY
Bozeman, Montana

December, 1981

ACKNOWLEDGMENTS

I am grateful to Dr. Gordon McFeters for his guidance and assistance throughout the course of this project, as well as his advice on handling dangerous mountain rodents. I also thank the members of my advisory committee, Dr. Bill Characklis, Dr. Ken Temple, and Dr. Dave Ward for the special interests they took in my work.

Dr. Loren Bahls and Dr. Evan Smouse provided valuable assistance and consultation on some of the technical aspects of this work; their help is gratefully acknowledged.

Special thanks go to the department secretaries, Jerrie Beyrodt and Debbie Powell, for their enduring patience and friendly help over the last two years.

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ABSTRACT

An ecological study was performed to assess the microbial dynamics of an algal-bacterial mat community. The oligotrophic nature of the stream in which the mat existed made it possible to examine these processes in a more structured manner than has been previously attempted. The mat grew on the surfaces of rocks in the streambed, its initial formation resulting from the colonization of those surfaces by microorganisms transported from the feeder reservoir.

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INTRODUCTION

The ability of bacteria to adhere to surfaces has been appreciated since the early 1940's when Zobell (82) demonstrated their attachment to a wide variety of substrates. The potential importance of adherent populations was largely ignored however, owing to the fact that they could seldom be detected, observed or manipulated effectively using traditional microbiological methods. During the past decade a great deal of attention has been focused on this "alternative" microbial existence and has led to the development of many new analytical techniques for studying adherent microbial populations, increasing our knowledge of the processes leading to and occurring within these consortia.

Microorganisms attach to a solid substrate in response to the transport, adsorption, and accumulation of organic and inorganic compounds at the surface-water interface (49,50,59), and is enhanced in low nutrient environments (35,38,51). This "initial sorption" soon becomes what has been termed irreversible as the microbial cells produce copious quantities of extracellular polymeric material which binds them to the substrate (15,16,72).

Several researchers have obtained evidence which suggests that attachment to a surface can stimulate microbial activity and growth (35,38,48,82). This property has been exploited in development of wastewater treatment reactors utilizing microbial biofilms to remove organic matter from the water.

There are also serious consequences associated with the ability of microorganisms to immobilize themselves on a surface. These would include corrosion of pipes resulting from anaerobic sulfate reduction, fluid frictional resistance that occurs when a biofilm forms on ship hulls or in water distribution systems, accelerated decomposition of man-made objects in marine environments, and heat transfer resistance in the heat exchanger systems of power generating stations.

The efficiency with which biofilms can remove organics from water in wastewater treatment plants suggested that sessile microbial populations may also assume an active role in stream purification processes. This has been confirmed by Ladd, et al. (43) and Harrison, et al. (33) who have found that in addition to being numerically dominant, sessile bacteria are also much more active with respect to the removal of dissolved organic compounds from the water than their planktonic counterparts.

A key feature of 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 importance of this association is reflected in the fact that these algal-bacterial communities are able to flourish in extreme environments otherwise considered to be unsuitable for survival. Their presence has been demonstrated in habitats ranging from the hot springs of thermal areas (11) to the

cold, oligotrophic waters of mountain streams (54). Further, it has been shown that algal-bacterial mats are able to support the growth of fastidious organisms such as the coliform group (54), selected enteric pathogens (69), and even Legionella (67) in extremely dilute aquatic systems. These microorganisms are typically unable to survive in such environments; their proliferation in and release from microbial mats leads to unexpectedly high levels in the water column and could give false indications of poor water quality.

The relationships between phototrophs and bacteria that provide them with an apparent competitive survival advantage is a subject that has been examined using several different approaches. Microscopic examinations (58) have illustrated the close physical associations that can occur between algae and bacteria, with the latter microorganism often attached directly to the algal cells. Seasonal monitorings of various aquatic environments often reveal close correlations between the two types (1,27,39), with the magnitude of the bacterial population increasing in response to algal blooms.

The excretion of soluble organic intermediates of primary production by algae was first demonstrated by Tolbert and Zill (68) and subsequently confirmed in several laboratories (24,57,69,75). The discovery that bacteria co-cultured with algae possess higher growth rates than those grown in pure culture (46) suggested that bacteria may possess metabolic machinery capable of using these extracellular

compounds. A number of researchers (13,43,55,77) have been able to demonstrate bacterial uptake of compounds thought to be algal excretion products, and most of these reports also describe the kinetics of this utilization process.

Using radiotracer techniques, Nalewajko and Lean (57), McFeters, et al. (54), and Bauld and Brock (3) were able to demonstrate the direct incorporation of ^{14}C-algal extracellular products into bacterial biomass in batch cultures. Bell (4) showed that the rates of uptake and incorporation of these products by bacteria increased in response to algal blooms.

All of the studies just mentioned concerning algal-bacterial relationships were performed either in planktonic environments or in batch cultures under laboratory conditions. Their results suggest that there is a symbiotic relationship occurring among algae and bacteria in aquatic environments, with the bacteria using algal extracellular products of photoassimilation as a primary nutrient source. Such a symbiosis allows bacteria to proliferate in the commonly nutrient-deficient planktonic waters of aquatic environments.

The benefits ascribed to bacteria that associate with algae in a sessile mat community are probably even greater. The copious amounts of extracellular polymeric material in these systems not only provide an ideal attachment substrate, but may also serve as a means to concentrate and hold nutrients and provide a stable microenvironment for

both the algal and bacterial cells in a flowing environment.

Unfortunately, there is a paucity of data describing the ecological interactions occurring among phototrophic and heterotrophic organisms in a microbial mat community, primarily because the allochthonous nutrient input of most aquatic environments imposes a structural and trophic complexity upon them that cannot be adequately resolved by present methods. This requires that researchers performing studies in such environments view the sessile mat community in an unstructured manner, making numerous assumptions which simplify the system to allow reasonable analysis of the experimental results (43).

The study of microbial ecological dynamics between sessile algae and bacteria would be greatly facilitated by examining these processes in a mat community whose organismal and structural diversity is significantly reduced. Such communities are often found in extreme environments where low nutrient levels and adverse temperatures limit the number of organisms capable of survival and growth. Brock (11) used this rationale during his studies of algal-bacterial mat communities in the thermal springs of Yellowstone National Park. The major limitation to studies such as this is the constancy of the environments; they are models of extremely stable ecosystems which make it difficult to test the effects of changing environmental parameters on processes occurring within the mat.

The present study examined the ecological dynamics of a microbial

mat community that exists in a high alpine stream environment characterized by cold temperatures and very low nutrient loading. These extreme conditions reduced the trophic complexity of the mat community to what appeared to be a delicate relationship between the algal and bacterial components which comprised a majority of its biological composition. Further, due to the short duration of warm weather in this geographical setting, the mat existed in the streambed only for a few months of the year. During this time the community underwent a striking evolution, increasing in biomass and activity until an apparent climax stage was reached, and then losing this activity before its virtual disappearance in autumn. It was thus possible to follow the processes occurring within the mat from initial colonization to its eventual decline and release from rocks in the streambed, within the context of changing environmental parameters.

Objectives of research

1. To observe seasonal patterns of growth and activity among the constituent microorganisms of the Pine Creek algal-bacterial mat community.
2. To relate these patterns to changes occurring in the mat as a whole and in their physico-chemical environment.
3. To determine the ecological relationships that exist between algae and bacteria in the sessile mat community.
4. To develop a model of carbon flow through the trophic levels

of an algal-bacterial mat community.

MATERIALS AND METHODS

Study site. The site used for these investigations was the outlet stream of Pine Creek Lake, a cirque lake situated in an alpine environment at 9000 feet (2740m) in the Absaroka mountains, ten miles south of Livingston, Montana. The deep, oligotrophic lake was fed primarily from springs and snow runoff. There was little fluctuation in its levels from hydrologic events because of its large volume, and stream flow remained constant throughout the study periods. The drainage area surrounding the lake consisted almost entirely of rock with very little vegetative material, resulting in low levels of allochthonous organic input. The algal-bacterial mat community selected for studies existed in a V-shaped unshaded portion of the outlet stream with approximate dimensions of 6m x 2m. The community was entirely epilithic in nature, existing on the upper side of flat rocks which appeared to be uniform in composition and structure. The water column over the mat community varied from approximately 6-18 inches in depth. The only variation in the sunlight incident on the mat resulted from the angle of a given rock upon which the community existed. The overall gross appearance of the mat was not entirely uniform throughout the stream, with differences most likely the result of incident sunlight angles and variations of fluid shear stress.

Chemical determinations. Alkalinity and dissolved oxygen were both determined in the field with an accuracy of 0.05 mg/l using a hand-held digital titrator and reagents purchased from Hach Chemical

Company (Loveland, CO) according to their methods manual (32). pH determinations were also made on site to prevent carbon dioxide loss with change in elevation. Measurements were obtained electronically with a Model 609 digital field pH meter fitted with a 150C combination polymer electrode (EXTECH, Boston, MA). Samples for conductivity measurements were collected in acid-washed Nalgene bottles and analyzed at the laboratory with a Model MC-1 Lectro mho-meter (Lab-line, Melrose Park, IL). All conductivity measurements were adjusted to 25C, with an accuracy of 0.05 µmhos.

Water samples were collected from the stream for analysis of nitrates, nitrites, reactive phosphorous, and silica according to procedures outlined in Standard Methods for Water and Wastewater Analysis (2). Colorimetric procedures were used for analysis (31) using reagents purchased from Hach.

Collection and preparation of mat for analysis. Samples were taken from several rocks in the stream to ensure that a representative and uniform sample was obtained. The epilithic film was removed in part by scraping the rock surfaces with a sterile X-ACTO knife blade, using a 9-cm^2 plastic template to obtain a section of known area. Residual material was removed using a small nylon brush, followed by aspiration with sterile pasteur pipettes and small amounts of filtered stream water (0.22 µm, Type GSWP, Millipore Corp., Boston, MA). Visual inspection indicated that this method was reproducible and succeeded

in removing virtually all of the mat community from the surface of the rocks. Once removed, samples were placed in sterile polypropylene scintillation vials and stored at or below ambient temperature in the dark. After returning to the laboratory (3 hrs.) the cohesive mat material was diluted with filtered stream water and disaggregated using a Sorvall Omnimixer (Sorvall Inc., Newton, CT). Optimal disaggregation was achieved by agitating the samples at approximately 3/4 maximum speed for two minutes. The mixing chamber was immersed in an ice water bath to avoid frictional heating. The resulting suspension was then further diluted with filtered stream water to a final working concentration of about 6.3-7.0 ml H_2O per cm^2 of mat material.

Planktonic samplings for bacterial enumeration. Water samples were taken at points both upstream and downstream from the mat using 20 ml poly q scintillation vials (Beckman Instruments, Irvine, CA) and sterile 250 ml Nalgene bottles for total counts and viable counts, respectively. Samples for total counts were supplemented with formalin (2%) to fix the bacterial cells and prevent their attachment to the walls of the container. With this procedure, it was found that samples could be stored for two weeks without a loss in numbers. Collections were at approximately the same time of day for each sampling date to avoid any possible variations in the data resulting from daily bacterial release patterns associated with the mat.

Bacterial enumeration

1. Total bacteria. Epifluorescence microscopy (36) was used to enumerate the total number of bacteria present within the mat. Serial dilutions of the mat suspension were made in reagent grade water (Milli-Q; Millipore Corp.) supplemented with 2% formalin. This fixed the cells and brought them to a proper concentration for counting. Cells were enumerated using a Leitz Ortholux II universal microscope equipped with a 100 watt mercury lamp. A blank (filtered, formalinized water) was also prepared for each set of preparations and the counts from this filter, usually 1-2% of the replicate samples, were subtracted as background. Twenty fields were counted from a dilution that gave concentrations of 30-100 bacterial cells per field. The arithmetic means of these counts were used to determine the total numbers and 95% confidence intervals of bacterial cells present according to the following formulas:

Total bacteria = (Avg. #/field - avg. #/blank) x f.f. x d.f.

where f.f. = total number of fields per filter (11,942)
d.f. = dilution factor of suspension

Confidence interval = 2 SDm/ n

where SDm = standard deviation of the mean value
n = number of samples used to obtain the mean

This gave the total number of bacteria per ml in the planktonic samples; for epilithic samples the counts were adjusted to cells per cm^2 according to their initial dilutions with filter sterilized stream water. The dimensions of randomly selected bacteria on filters pre-

pared for epifluorescence microscopy were determined using a calibrated ocular micrometer. These measurements were used to determine volume, from which bacterial carbon was calculated using constants published by Geesey et al. (27).

2. Viable bacteria. Samples to be analyzed for viable bacteria were serially diluted with sterile peptone dilution broth (2) and collected onto Millipore 0.45 µm (Type HCWG) filters, which were then placed on Plate Count Agar (Difco) in 5 cm petri dishes. This procedure was sometimes repeated using 0.1 strength Plate Count Agar as the growth medium; incubation for all samples was six days at 14C or 25C. All visible colonies were counted, averaged, corrected for dilution, and reported as colony forming units per ml.

The same methods were used for enumeration of coliform bacteria, but in this case the growth medium consisted of m-Endo broth (Difco) added to filter pads. After 24-48 hrs incubation, those colonies possessing a green metallic sheen were enumerated and recorded as coliform bacteria.

Chlorophyll determinations. The planktonic phototrophic population was collected by passing 2 liters of lake water through Millipore 0.45 µm (Type HAWP) filters under vacuum. The filters were then placed in 5 cm plastic petri dishes and immediately wrapped in aluminum foil to prevent photodecomposition of the chlorophyll pigments. This filtration process was also used in collecting the phototrophic popula-

tion of the mat after its initial suspension in filtered stream water. Since analysis of the chlorophylls was not immediate, samples were stored at -80C and subsequently thawed at 5C in a desiccator containing silica gel. After the dried filters were placed in 15 ml centrifuge tubes and dissolved in 5 ml 90% acetone, the algal cells were broken by sonication with a Model W-225R cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY). Maximum energy was used with an 80% pulse for 30 minutes; the water bath temperature was maintained at 10C with a refrigerated circulating pump (Haake, Berlin, Germany). After disruption, the samples were adjusted to a final volume of 8 ml with 90% acetone and steeped at 4C for 48 hours. All procedures were carried out in the dark. When extraction of the chlorophyll pigments was complete, the samples were centrifuged for 15 minutes at 500g. Aliquots of the supernatant solution were analyzed spectrophotometrically (Varian Techtron 635) at 750 and 663 nm. The remaining solution was then acidified with HCl to 0.03N and centrifuged as before. Aliquots were again analyzed at 750 and 663 nm. Total chlorophyll _a_ content of the samples, corrected for pheophytin, was calculated according to formulas published in Standard Methods (2). Algal carbon was calculated by multiplying chlorophyll _a_ values by 60 (28).

Total organic carbon (TOC). Stream water was collected in acid-washed glass bottles for analysis. Organic carbon concentrations were

determined from triplicate samples using the Total Carbon System, Model 0524B (Oceanography International, College Station, TX). All glassware used in the procedures was washed in chromic acid and rinsed with organic-free water. Mat suspension aliquots were also analyzed as above and converted to values of organic carbon per unit area of mat surface.

Primary productivity by mat phototrophs. Aliquots of mat suspensions (10 ml) were dispensed into sterile glass vials, and 1 ml $NaH^{14}CO_3$ (sp. act. 10 μCi/ml, 10 μg/μCi, New England Nuclear, Boston, MA) was added to each of the triplicate samples and one foil wrapped control. The samples were incubated for 12 hours at temperatures identical to that of the stream at the time of collection under constant illumination (65 $\mu E \cdot m^{-2} \cdot sec^{-1}$). Incubations were terminated with the addition of 0.2 ml H_2SO_4, and $^{14}CO_2$ was driven off by bubbling air through the suspension for 30 minutes. Fractions (0.25 ml) of this reaction mixture were removed from the vials and added to 10 ml Aquasol scintillation cocktail (New England Nuclear). This method detected both ^{14}C fixed into cellular material of the phototrophs and that excreted into the culture medium. Samples were analyzed with a Packard Tri-Carb 460CD scintillation counter. Counts were converted to dpm by either external standard or channels-ratio methods and corrected for machine efficiency, background and controls. The fraction of $^{14}CO_2$ converted biologically was adjusted to total carbon fixation

by calculating the ratio of the ^{14}C added to the system as bicarbonate to the total inorganic carbon pool; determined from alkalinity, pH, and temperature measurements for each sampling date.

Bacterial metabolic activity assays. The metabolic activity of the sessile bacterial populations was assayed by previously described heterotrophic potential methods (43,79,80). Aliquots of mat suspension (10 ml) were dispensed into 25 ml Erlenmeyer flasks. A serum stopper was used to seal each reaction vessel; these were fitted with a plastic stem and cup assembly (Kontes Glass, Vineland, NJ) that held a fanned piece of filter paper inside each vessel. L-[^{14}C(U)]-glutamate (292.0 mCi/mmol, New England Nuclear) was added to duplicate suspensions and poisoned control ($HgCl_2$, 50 mg/l) in five concentrations ranging from 0.127 µg/l to 2.54 µg/l. Experiments were also performed using 1-^{14}C-glycolate (sp. act. 5 mCi/mmol; ICN Pharmaceuticals, Inc.) over a similar range of concentrations. Dark incubations were carried out for 12 hours at a temperature identical to that of the stream at sampling time. Reactions were terminated with the addition of 0.2 ml 50% H_2SO_4 to the suspensions, and β-phenethylamine (0.15 ml) was injected through the stoppers onto the filter paper wicks to trap $^{14}CO_2$ resulting from bacterial metabolic activity. After agitation on a rotary shaker for one hour, the paper wicks were removed and placed in 10 ml scintillation cocktail (Toluene with 5g 2,5-diphenyloxazole (PPO) per liter). Cells from

the suspension were collected onto Millipore 0.22 μm (Type GSWP) membrane filters which were subsequently dissolved in Cellosolve (2-ethoxyethanol, Sargent-Welch) and added to 10 ml Aquasol. The activity of each fraction was determined in the same way as that for primary productivity measurements. The mathematical operations involved in conversion of the raw data to estimates of rates of bacterial metabolic activity follow.

Heterotrophic potential theory. It has been suggested (43,79,80) that the rate of uptake of a given substrate by a heterogeneous population of microorganisms is describable in terms of Michaelis-Menten kinetics:

$$v = \frac{Vmax \cdot S}{K_t + S} \qquad (1)$$

Where v = the velocity of uptake at substrate concentration S

Vmax = the maximal uptake velocity attainable; i.e., when all the enzymes of the rate-limiting process are saturated

S = the substrate concentration of the system

K_t = the transport constant, or the substrate concentration when uptake velocity is exactly one-half of maximum

Using this equation, a plot of v vs. S gives a saturation limited, hyperbolic function when the cells actively transport the substrate in essentially one direction across the membrane.

Additional information is obtained by performing a Lineweaver-Burke transformation of equation 1:

$$\frac{S}{v} = \frac{K_t}{Vmax} + \frac{1}{Vmax} \cdot S \qquad (2)$$

This equation is in slope-intercept form and plots as a straight line with ascending slope when the data conform to kinetic assumptions. Equation 2 can be expanded to include both the unknown natural concentration (Sn) and known substrate (A) added to the system:

$$\frac{Sn + A}{v} = \frac{K_t + Sn}{Vmax} + \frac{1}{Vmax} \cdot A$$

The turnover time (Tn) of a given substrate is a term which describes the time required for the substrate to be completely removed from the system by the natural population. It is described by the equation:

$$Tn = \frac{Sn + A}{vn} = t/f$$

Where f = the fraction of isotope added to the experimental system that is assimilated or mineralized during the incubation period

t = the incubation time in hours

Equations 3 and 4 are then combined to give:

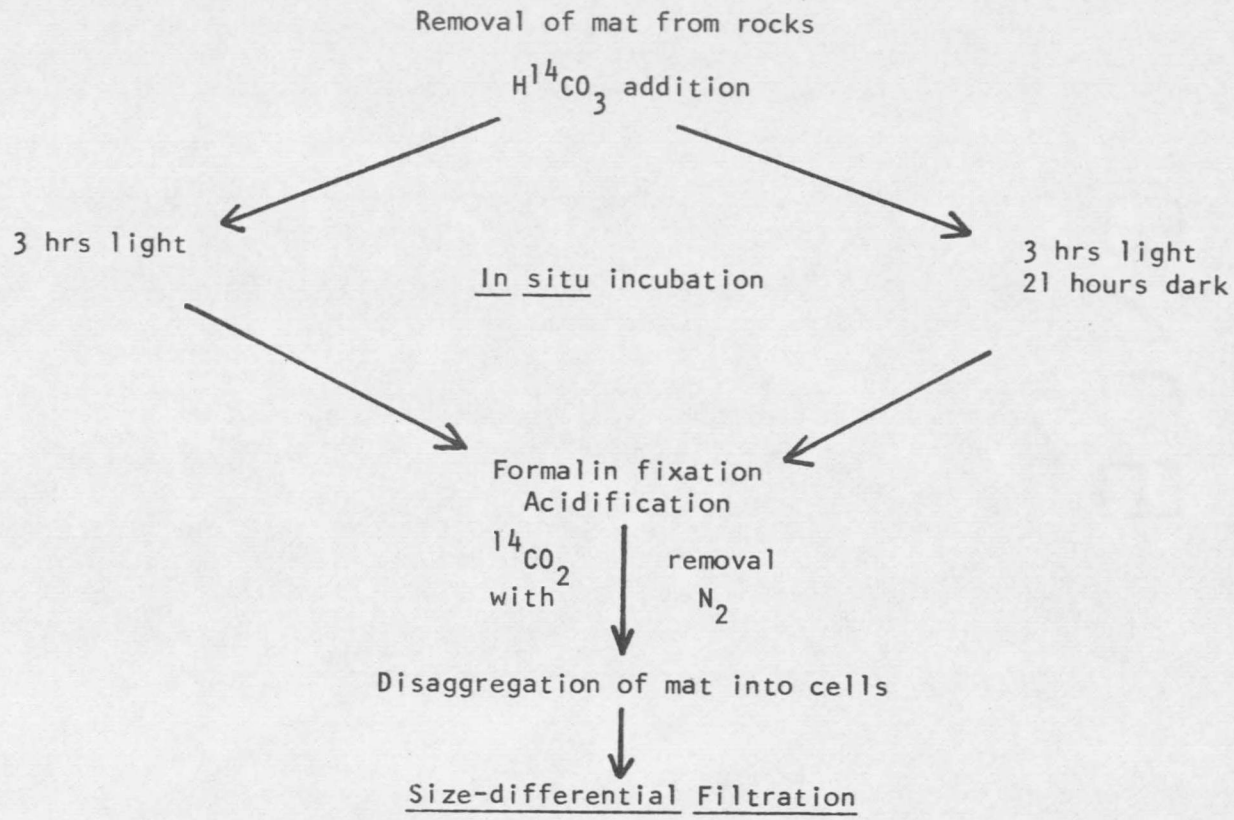
$$t/f = \frac{K_t + Sn}{Vmax} + \frac{1}{Vmax} \cdot A$$

In the methods used here, ^{14}C-glutamate was added to samples of mat homogenate in varying concentrations. The ratios of t/f were determined after termination of the experiment and plotted against A. This plotted as a straight line under the conditions mentioned previously, with the x-intercept as -(Kt + Sn) and the slope as 1/Vmax. The y-intercept, where A = 0, represents the turnover time of the

natural glutamate pool (Sn).

Mat structure analysis. The structure and composition of the epilithic mat was examined by scanning electron microscopy (SEM) using several strategies. Fully established communities were obtained by removing small chips from rocks existing in the streambed. Chips were also taken from terrestrial rocks of an identical nature, attached to a plexiglass slide with the newly exposed surface facing up, and placed in the stream to allow colonization by microorganisms in the stream. The last method employed a polycarbonate membrane as the colonization substrate. This material was held between two sheets of plexiglass, one of which was cut out to expose the membrane to the flowing water. Samples from both types of colonizing substrates were periodically removed using a scalpel and forceps, and immediately placed in 0.067M cacodylate buffer containing 10% EM grade glutaraldehyde for fixation. They were later transferred to an acetone series for dehydration, followed by critical point drying and sputter coating. The instrument used for observation was a JEM 100CX Electron Microscope with an ASID-40 scanning attachment.

Carbon flux experiments. The general procedure for these experiments is illustrated in Figure 1. Mat material, removed from rocks as described previously, was placed in a sterile Nalgene jar containing a small amount of filtered stream water and agitated by hand to make a thick suspension. Aliquots (7 ml) of this suspension were



1) 5.0μm - algal fixation of $^{14}CO_2$
2) 0.2μm - bacterial incorporation of algal fixation products.
3) filtrate - algal excretory products.

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

then added to each of several 250 ml media bottles (Wheaton Scientific, Millville, NJ) containing 193 ml filtered stream water. Controls were killed with 7 ml formalin. A live, foil wrapped dark control was also prepared to check for dark CO_2 uptake by the heterotrophic population. The flasks were sealed with caps fitted with butyl septa (Wheaton). After 15 minutes *in situ* preincubation, 0.1 ml $NaH^{14}CO_3$ (as before) was added to each reaction vessel. After 3 hours *in situ* incubation, half of the live replicates were terminated with 7 ml formalin. The remaining vessels were wrapped in foil, returned to the laboratory at ambient temperature, and formalinized after an additional 21 hours of dark incubation. All samples were then acidified with 1 ml 50% HCl and flushed for 3 hours with nitrogen. The CO_2 in the gas headspace was trapped in 5 ml Oxifluor-CO_2 (New England Nuclear). Activity of the samples was analyzed, corrected for efficiency, and converted to dpm as before. The remaining suspension was homogenized with a Sorvall Omnimixer to break up the cohesive, closely associated organisms of the mat. 10 ml duplicate aliquots of this suspension were separated into two 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 phototorphic and heterotrophic populations arising from planktonic environments (6,19). Observations with epifluorescence microscopy indicated that the 5.0 µm filters succeeded in retaining virually all of the

phototrophic cells. Samples to which a labelled organic substrate was added were processed in an identical manner to determine the percentage of heterotrophic activity retained on 5.0 µm filters, as microscopic observations indicated that approximately 15% of the bacterial cells were being retained by the large pore size filter. The filtrate was collected in order to analyze the concentration of labelled soluble organic compounds, and each filter was rinsed three times with 10 ml portions of Milli-Q water. The filters were placed in poly q scintillation vials to which 10 ml Filter Count scintillation cocktail (Packard Instruments, Downers Grove, IL) was added. 1 ml aliquots of the filtrate were added to 5 ml Aquasol. All samples were analyzed for activity as before. After correction for errors related to the efficiency of separation of the algal and bacterial components by the filter system employed, the disintegrations retained on 5.0 µm filters were reported as phototrophic incorporation; those detected on 0.2 µm filters were reported as heterotrophic productivity at the expense of soluble organic products excreted by the phototrophs. Disintegrations in the filtrate fraction were assumed to be algal products not utilized by the bacterial population, since the flushing procedure succeeded in removing 100% of the activity resulting from dissolved $^{14}CO_2$.

Bacterial identification and temperature relations. Several different bacterial colonies from Standard Plate Count and m-Endo

plates were isolated on tryptic soy agar (Difco) supplemented with glucose (0.05%) and yeast extract (0.3%) at 25C and maintained on Stock Culture Agar (Difco) slants. Identifications were made according to the scheme of LeChevallier et al. (44). Isolates were grown in mechanically mixed TGY broth cultures at temperatures of 5C, 15C, and 25C to determine their optimal growth temperatures and growth rates at suboptimal temperatures. Growth was monitored with a nephelometer (Turner Designs, Mt. View, CA), and generation times were calculated by:

$$\mu = \frac{\ln 2}{gt}$$

Where μ = the slope of the semilog plot

gt = the generation time in hours

ATP measurements. Fractions of the mat homogenate were collected onto polycarbonate filters (Nuclepore, 0.45 µm, 3.0 µm, 5.0 µm) and allowed to equilibrate on the filters for 15 minutes at ambient temperature, after which they were stored at -80C. The frozen samples were placed in boiling 0.02 M Tris (hydroxymethylaminomethane) buffer for 5 minutes to release ATP. ATP concentration was determined by reaction with firefly luciferin-luciferase enzyme measured on a Model 760 Luminescence Biometer (DuPont, Wilmington, DE). All ATP reagents were purchased from Packard (Downer's Grove, IL.). Values obtained in this manner were then converted to values for a specific area of mat

material for comparison with chlorophyll *a* measurements.

RESULTS

Environmental Parameters of Pine Creek.

Several chemical and physical properties of the stream water, monitored over the course of two years, are summarized in Table 1. Alkalinity levels were quite steady throughout the season, with values near 10 mg/l as $CaCO_3$. Dissolved oxygen was likewise present at values approaching 9 mg/l throughout the study seasons. The oxygen values represent full saturation for all dates except those where the temperature of the stream water fell below 7C. Conductivity values showed a rising trend as water temperatures increased, but never exceeded 35 µmhos. The pH of the stream water was always above neutrality and rose steadily to a maximum value of 8.60 in August.

In addition to these parameters, the levels of nitrates, nitrites, and reactive phosphorus in the stream water were evaluated periodically. All three species were consistently present at very low concentrations (0.1 mg/l) throughout the season, with no apparent fluctuations.

The biological measurements were always performed on clear, sunny days to reduce the variability of light in the experimental systems. As a result, light intensity was reasonably steady throughout the season, varying less than 10% throughout the study periods.

Only two of the measured parameters appeared to vary substantially over the course of the season. The stream water temperature rose from 1.5C at the start of the study periods to a maximum of 12-13C before dropping again in late summer. Silica concentrations in the stream

Table 1. Summary of several chemical and physical properties of Pine Creek during the course of a study season.

Date	Water Temperature (°C)	pH	Specific Conductivity (μmhos)	Alkalinity (mg/l)	Dissolved O_2 (mg/l)	NO_3-NO_2 (mg/l)	Reactive P (mg/l)	Silica (mg/l)
6/25	3.0	-	20.4	-	-	< 0.5	< 0.1	1.25
7/2	4.5	-	24.0	9.9	9.2			
7/9	6.0	7.8	30.0	9.5	7.5			0.95
7/16	7.0	8.2	30.0	9.9	8.7			
7/23	10.5	8.3	30.2	10.1	8.7			0.99
7/30	13.0	8.1	30.2	10.3	8.6	< 0.1	< 0.1	1.44
8/6	10.5	8.2		9.7	8.6			
8/12	13.0	8.4	31.5	10.5	8.4			0.24
8/18	11.0	8.6	33.2	10.7	8.3			0.092
8/26	10.0	8.4	32.4	10.4	8.1			
9/17	8.0		31.5	10.4	8.7	< 0.1	< 0.1	0.150
10/7	6.0	7.8	34.6	10.8	8.6			

water during 1981 were comparatively high in June and July, but began to decline in August, reaching extremely low levels that persisted through the rest of the sampling season.

Mat Structure and Composition

It was necessary to determine the physical structure and composition of the epilithic mat community in order to accurately assess the ecological relationships that occurred among the constituent microorganisms. These determinations were made by 1) characterization of the phototrophic population, 2) scanning electron and phase contrast microscopic observations, and 3) total organic carbon (TOC) analysis of the mat.

1. Characterization of the phototrophic population.

The abundance and composition of the phototrophic population for selected dates of the 1980 season are presented in Table 2. The percent volume values represent the estimated contribution of each group to the total algal volume in the sample. Living algae accounted for 80-90% of the volume of the mat, the remainder consisted of diatom stalks, detritus, and exopolysaccharide material. The Shannon-Weaver diversity index (76) values were moderately low, indicating that the number of algal species present in the mat was limited to a few major types. The organisms which were identified are considered to be indicators of cool, high elevation waters of moderate hardness.

2. Total Organic Carbon (TOC) Analysis.

Table 2. Organismal structure of the phototrophic population in the Pine Creek mat community for selected 1980 sampling dates. Data compiled by Dr. L. Bahls, Montana Department of Health and Environmental Sciences.

DATE	TOTAL TAXA	TAXA COUNTED	DIVERSITY ($\bar{d}$)	TAXA	DIVISION	% VOLUME
8/12/80	30	17	2.56	diatoms	Bacillariophyta	85
				Mougeotia	Chlorophyta	5
				Microcoleus	Cyanophyta	5
				Stigeoclonium	Chlorophyta	5
8/19/80	24	16	2.57	diatoms	Bacillariophyta	94
				Microcoleus	Cyanophyta	6
				unknown	Cyanophyta	1
8/26/81	28	17	2.46	diatoms	Bacillariophyta	99
				Microcoleus	Cyanophyta	1

The approximate composition of the mat community was determined assuming that there were three major components - algal cells, bacterial cells, and extracellular polymeric material. Algal carbon was estimated from chlorophyll a values, bacterial carbon was calculated from biomass determinations with epifluorescence microscopy, and extracellular polymeric material was taken to be the difference between cellular biomass and total organic carbon levels. The means used in these calculations were shown to be accurate estimations of the data from 95% confidence intervals. The results listed, in Table 3, indicate that the extracellular material is the largest organic component of the mat community. Algal biomass was second in magnitude, although significantly less than the polymeric material, and bacteria contributed less than 2% of the total mat carbon, despite being numerically dominant. These values are similar to those reported by Geesey et al. (27) for a woodland stream community. The general structure of epilithic biofilms in streams suggested by these data is supported by measurements of laboratory biofilms, despite the fact that the latter systems have no phototrophic component. In a continuous culture apparatus, Trulear (unpublished data) found that the extracellular glycocalyx material produced by bacteria in a laboratory grown film accounted for approximately 80% of the total carbon present in the film.

Table 3. Breakdown of the Pine Creek mat community into algal, bacterial, and extracellular slime polymer carbon, and the contribution of each component to total mat carbon during 1981.

	ALGAL CARBON		BACTERIAL CARBON		EXTRACELLULAR POLYMER CARBON	
DATE	$mg \cdot cm^{-2}$	% total	$mg \cdot cm^{-2}$	% total	$mg \cdot cm^{-2}$	% total
6/25/81	0.218	31.6	0.008	1.2	0.464	67.2
7/8/81	0.104	17.6	0.009	1.5	0.477	80.8
7/21/81	0.296	42.3	0.010	1.5	0.394	56.3
7/28/81	0.285	25.2	0.003	0.2	0.842	74.5
8/11/81	0.222	22.0	0.005	0.5	0.782	77.5
8/21/81	0.177	19.0	0.004	0.4	0.749	80.5
9/2/81	0.058	10.0	0.001	0.2	0.521	89.8

The TOC levels of the mat during the 1981 season are illustrated in Figure 2, along with the results of chlorophyll a measurements. The trends observed for these two parameters were nearly parallel, and support the idea that a close relationship exists between the algal population and the total biomass of the mat.

3. SEM analysis.

The mat intact community of Pine Creek was examined with scanning electron microscopy to determine a) colonization sequences, b) successional patterns, and c) attachment mechanisms. These processes were observed on both natural (denuded rock chips) and artificial (polycarbonate filter material) substrates placed in the streambed parallel to the flow. Although initial colonization was more easily observed on the artificial substratum, the process was quite slow and non-uniform relative to the natural substratum. This was most likely due to the hydrophobic properties of the filter material (82).

a. Colonization sequences.

Figure 3 shows photomicrographs contrasting the Pine Creek mat at early and late stages of development. Diatoms appeared to be the primary colonizing organism; bacteria were also found in early colonization stages, but were most often associated with a diatom cell rather than individually sorbed to the substratum. After the initial colonizers became attached, the biomass and diversity of the mat community proliferated rapidly. The surface of the colonizing

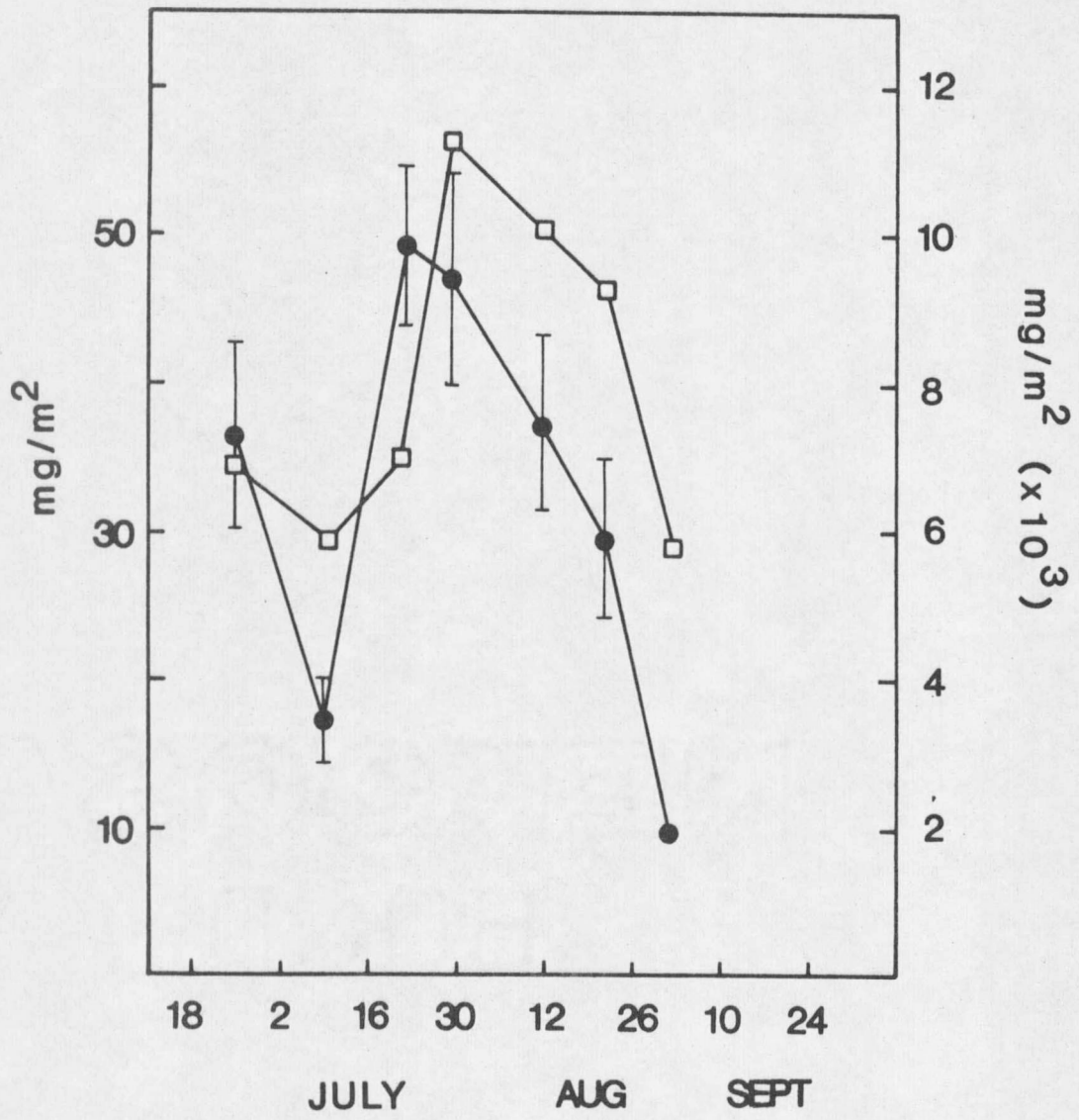
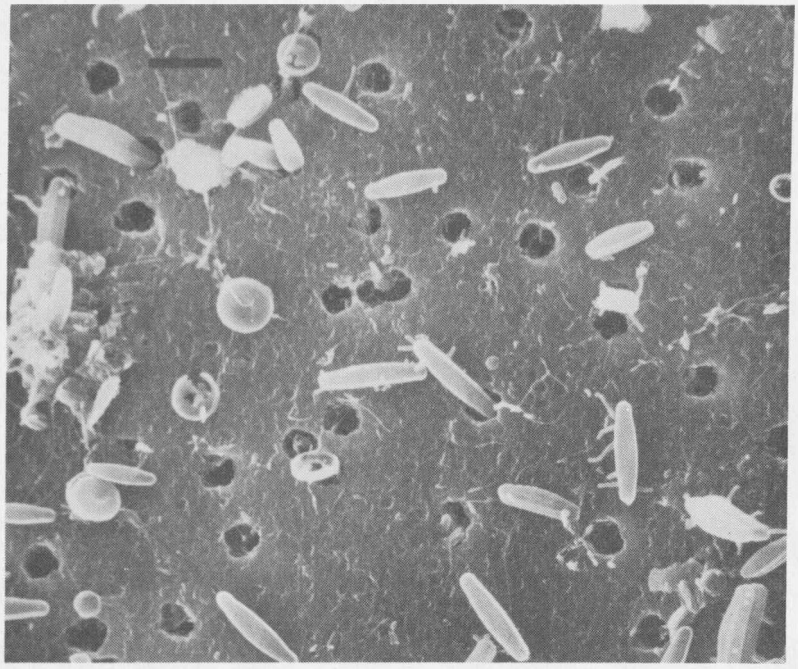


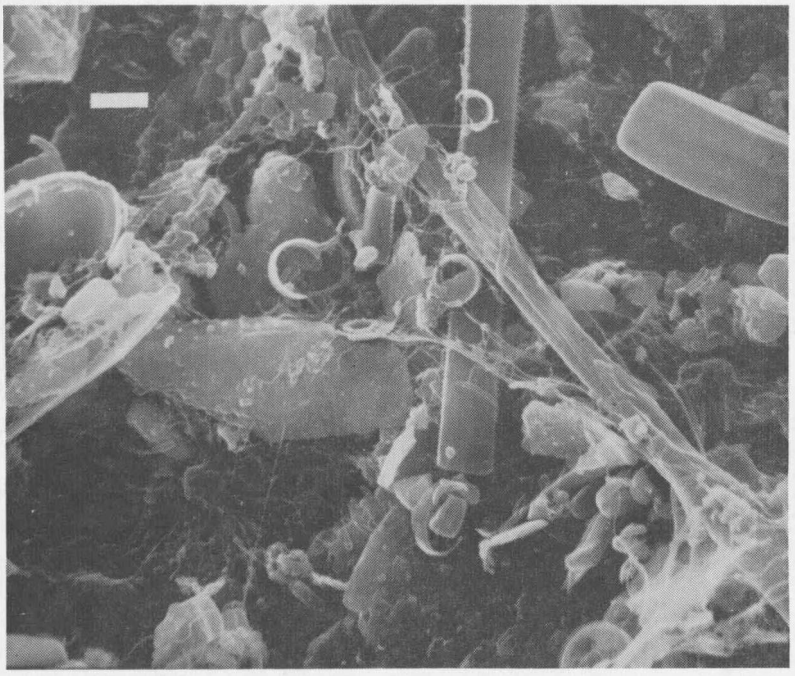
Figure 2. Total organic carbon (□) and chlorophyll a levels (●) of the Pine Creek mat during 1981. Bars represent 95% confidence intervals and, where not shown, are continued within the data points.

Figure 3. Scanning electron micrographs of the Pine Creek mat community contrasting A) early colonization stages and B) structure at climax of mat's development. Bars represent 10 μm.

A.



B.



substratum became covered with a dense polymeric material apparently produced by the sorbed organisms. At the peak of the mat's growth the organisms, especially bacteria, were deeply embedded within this extracellular material (Figure 4).

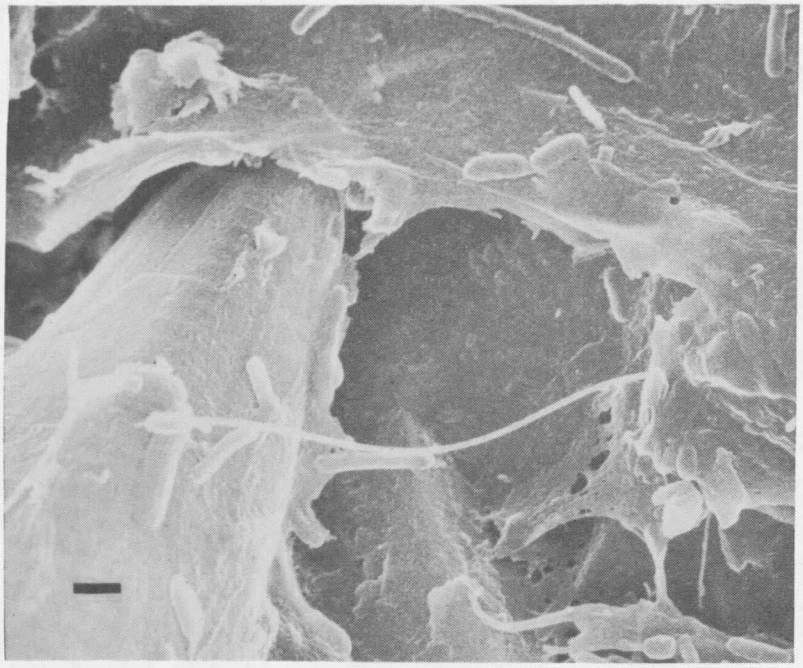
b. Successional patterns.

The visual data obtained from these SEM observations did not allow for any systematic determinations of successional patterns among phototrophic organisms within the mat, but seasonal trends were readily apparent. Cyclotella cells were observed in low magnitude throughout most of the season. Cells resembling Navicula and Cymbella were the most abundant organisms during intial colonization (Figures 3a, 5a). Their prominence gave way to a bloom of cells tentatively identified as Fragilaria (Figure 5b), which were prominent during the latter part of July. Stalked diatoms appeared suddenly in August as a dense bloom, but disappeared shortly thereafter as the mat began to release from the rock substratum. Beyond this point the attenuated mat community took on an organismal structure resembling that observed in June.

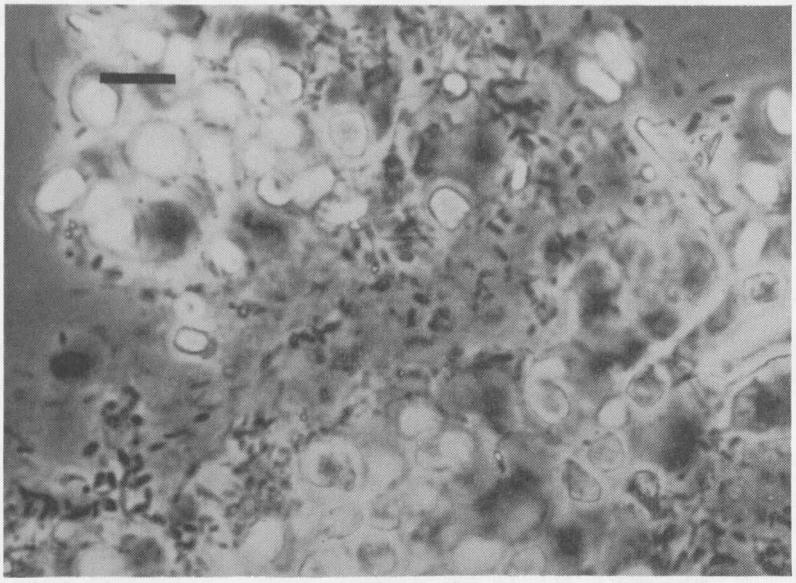
c. Attachment processes.

The attachment of microorganisms to surfaces has received a great deal of attention in recent years and the mechanisms involved in this process, though not completely delineated, have been extensively studied (49,51,59). It was therefore not the intent of

Figure 4. Orientation of microorganisms within the extracellular polymeric slime matrix of the Pine Creek mat. A. SEM; bar = 1.0 µm. B. Phase-contrast micrograph; bar = 10 µm.



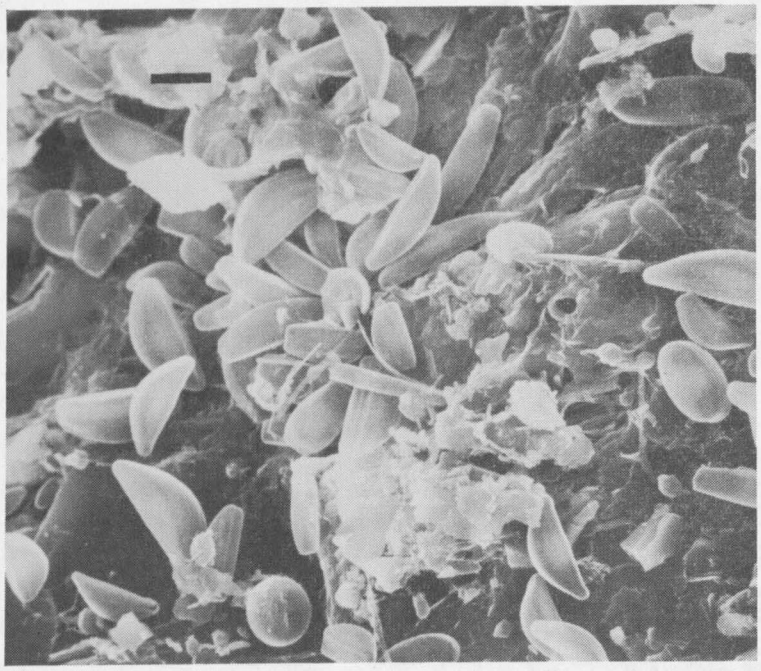
A.



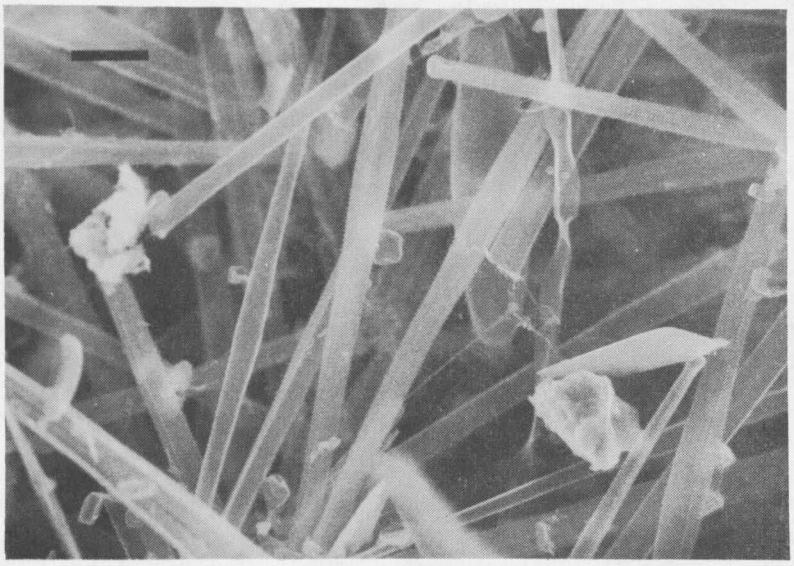
B.

Figure 5. Seasonal succession of phototrophs within the mat. A. Sample obtained on 6-25-81 demonstrating abundance of Cymbella and Navicula. B. Sample taken on 8-21-81 illustrating a bloom of organisms believed to be Fragilaria. Bars = 10 µm.

A.



B.



this study to give a detailed analysis of attachment processes by microorganisms in Pine Creek, but rather to examine adhesion as one step in the complete cycle of colonization, proliferation, and removal of a sessile microbial community.

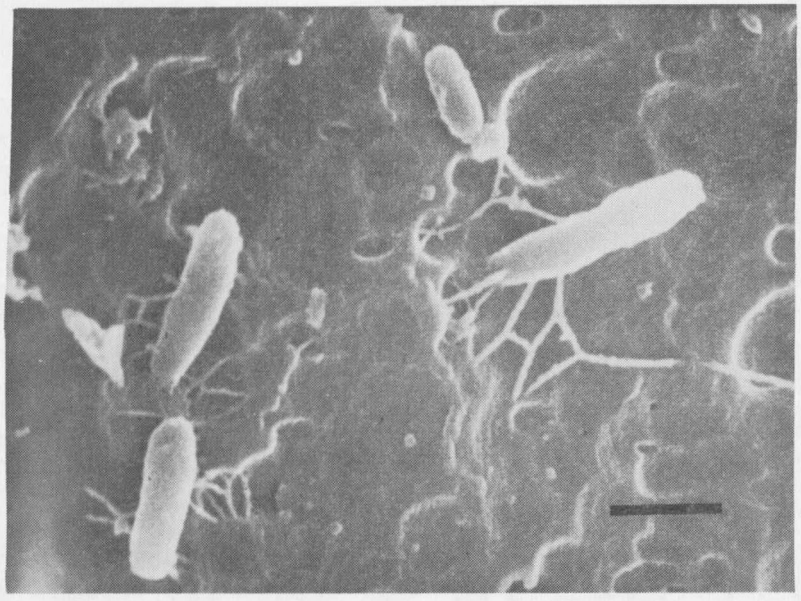
There were no apparent holdfast structures mediating diatom adsorption to the surfaces. In fact, there was seldom any extracellular material associated with these organisms, leaving the details of their siliceous outer structures readily resolvable. Whether this bears any significance or was merely an artifact of the dehydration processes used in sample preparation is not known. Methods in which the extracellular material is left largely intact for microscopic observation have been described (47), but these were not attempted in this investigation. However, bacteria sorbed to the surfaces of substrates did possess "structures" which appeared to be mediating attachment. These were visible as finger-like projections of material extending from the cell to the substrate surface (Figure 6). Similar structures were apparent on bacteria sorbed to surfaces other than rock, such as detrital matter or phototrophic organisms. Bacteria not existing directly on a surface, but rather within the complex structure of the mat were usually completely embedded within this extracellular material.

Bacterial enumerations.

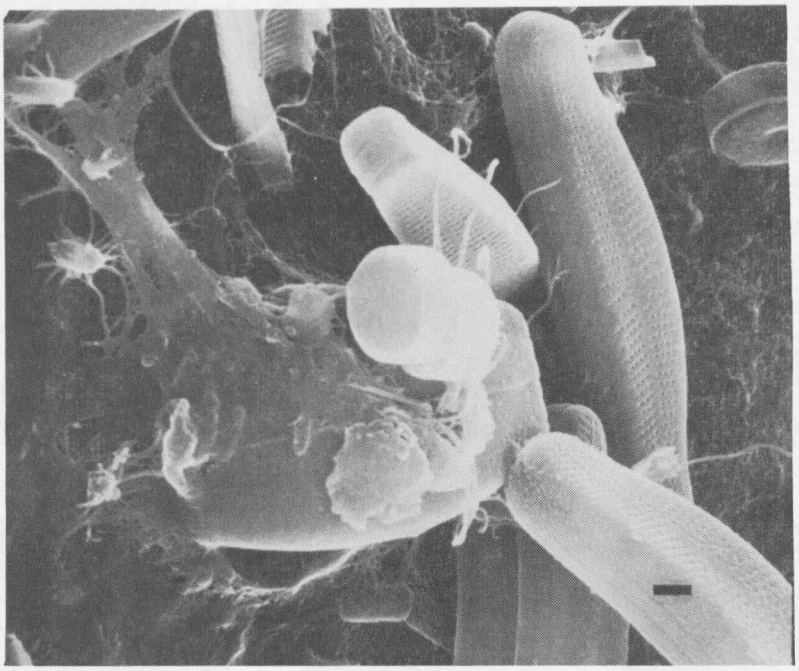
1. Planktonic bacteria.

Figure 6. Attachment mechanisms of mat microorganisms. Bacteria appeared to be immobilized by means of either exopolysaccharide fibrils or complete burial within the slime matrix of the mat. No attachment structures were apparent for the diatoms. Bars = 1.0 µm.

A.



B.



The number of viable bacteria present in the Pine Creek system were examined by plating aliquots of both stream water and mat suspensions onto Standard Plate Count agar and incubating plates for six days at 25C. The results for the planktonic population are summarized in Figure 7, and are contrasted with results obtained by direct microscopic observations (Figure 8a). The viable count results are characterized by two major peaks occurring during the season, while direct counts show no such pattern. One common feature of the two data sets is that the number of bacteria in water samples taken downstream from the mat were consistently higher than those taken from points upstream. These results can be justified on the basis that sessile bacteria grew in large numbers in the mat and were then released into the flowing water column. Several of these differences were not significant ($p < 0.05$), but this may have been due to the fact that the volume of water flowing over the mat community was sufficiently large to dilute those organisms when released into the water column.

2. Epilithic bacteria.

The population trends for viable bacteria present within the mat are illustrated in Figure 9, along with numbers of total bacteria throughout the season. Although numbers obtained by direct fluorescence microscopy were consistently 2 orders of magnitude higher than viable counts in the mat (as well as in planktonic samples),

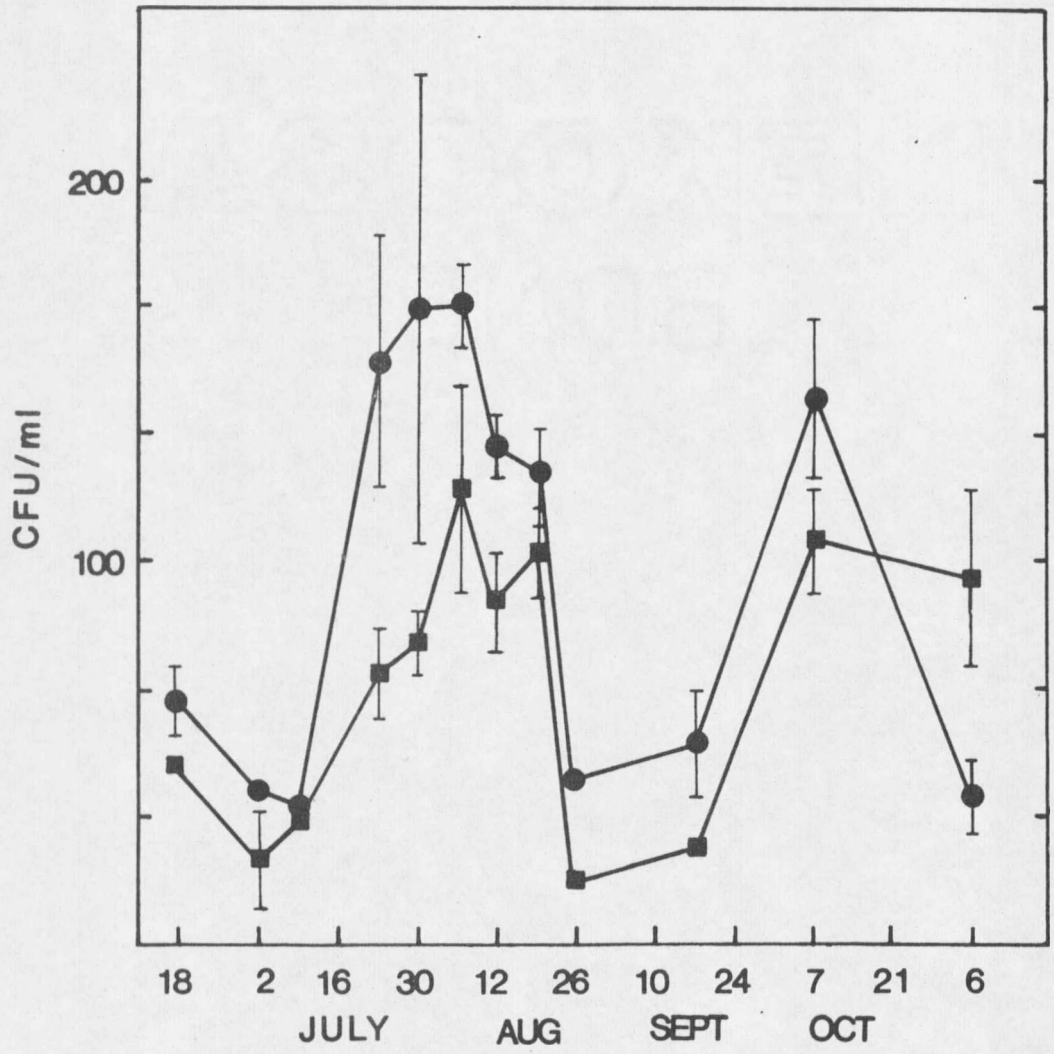


Figure 7. Concentrations of viable bacteria in the stream water of Pine Creek for 1980. ■- Samples taken upstream from the mat. ●- Samples taken downstream from the mat.

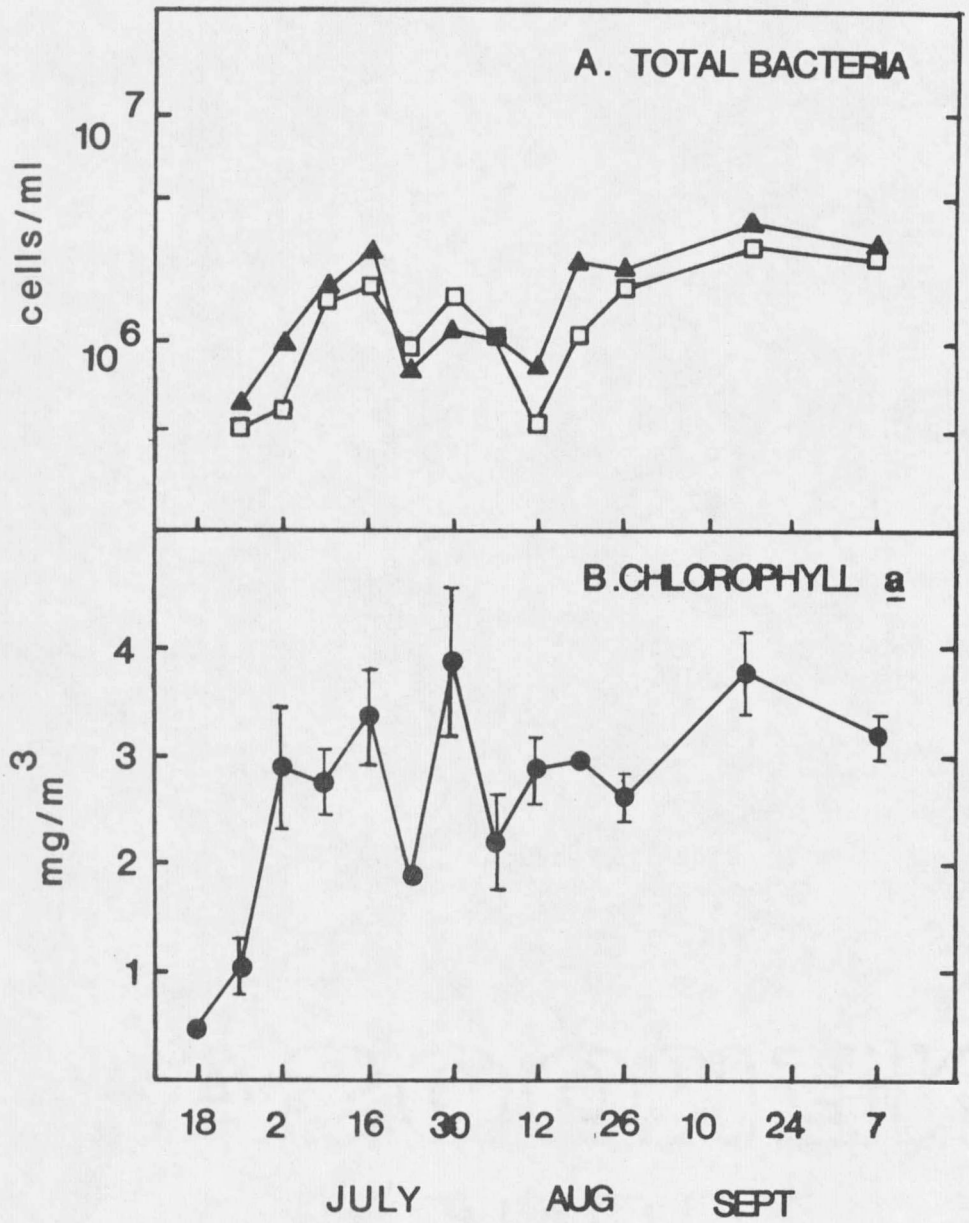


Figure 8. A. Seasonal population patterns of total bacteria in samples taken upstream (□) and downstream (▲) from the mat, as determined by epifluorescence microscopy. Confidence intervals are not included. B. Chlorophyll a levels of the stream water directly upstream from the mat.

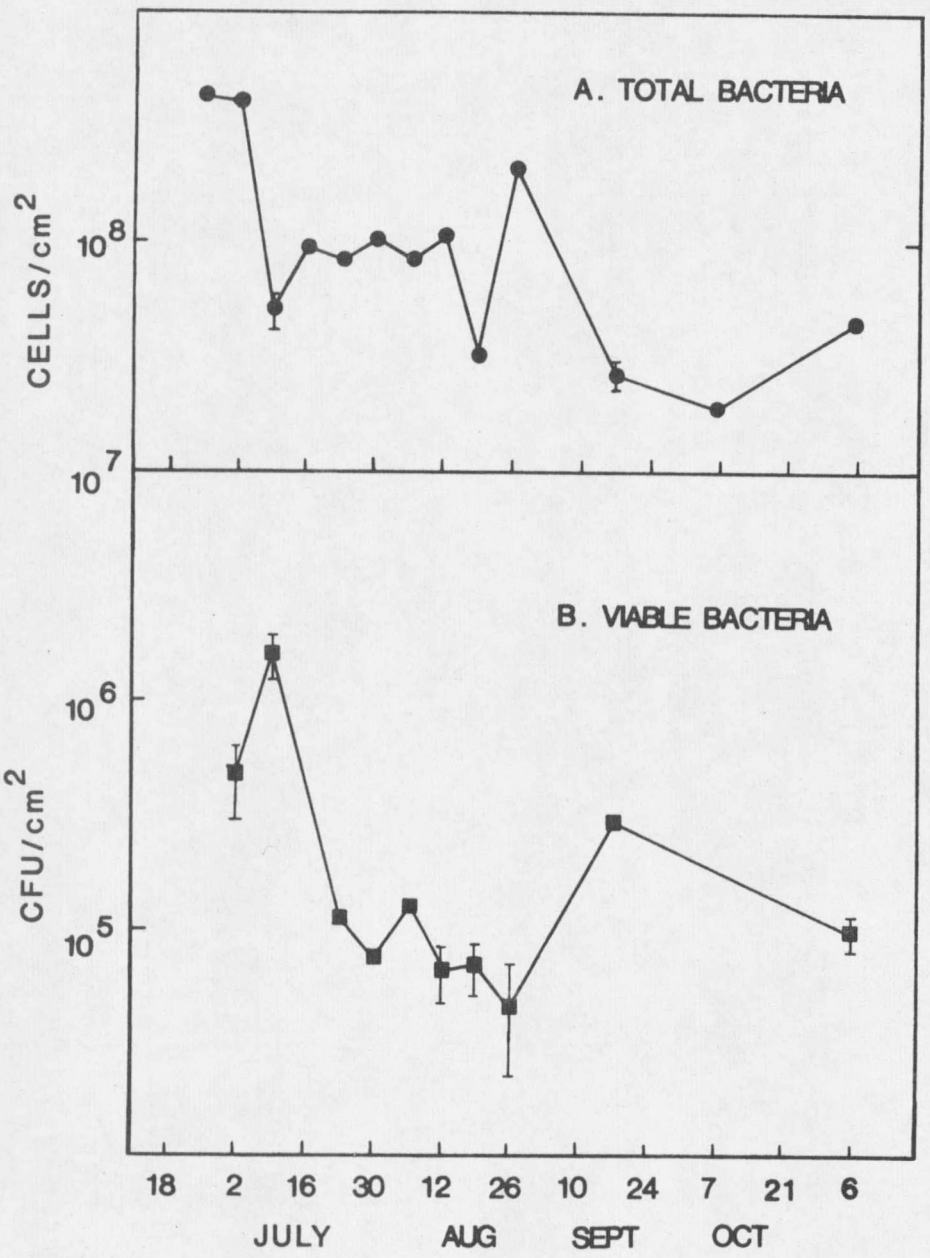


Figure 9. Bacterial populations in the Pine Creek mat during 1980.

both enumeration methods appeared to indicate a reduced bacterial population in the mat for most of July and August, with a moderate late-season surge.

3. Coliform Bacteria.

The numbers of coliform bacteria in Pine Creek were monitored by the membrane filtration method, using m-Endo broth as a selective differential growth medium. Coliforms were taken to be those colonies that formed a metallic green sheen after 48 hours incubation at 37C; the results of these investigations for the 1980 season are illustrated in Figure 10. Very few coliforms were detected for the majority of samplings throughout the season. One major surge in the population occurred in the middle of June however, with coliform numbers both in the mat and downstream from it rising from essentially zero to approximately 150 bacteria$\cdot cm^{-2}$ and 100$\cdot ml^{-1}$, respectively. This peak was not accompanied by a concomitant rise in coliform numbers upstream from the mat, and within two weeks the numbers from all sampling sites were once again at insignificant levels.

Planktonic chlorophyll levels.

The planktonic chlorophyll *a* levels in the outlet stream of Pine Creek Lake were monitored at a point directly upstream from the mat, and were used to represent the biomass of the planktonic phototrophic population throughout the 1980 season. As seen in

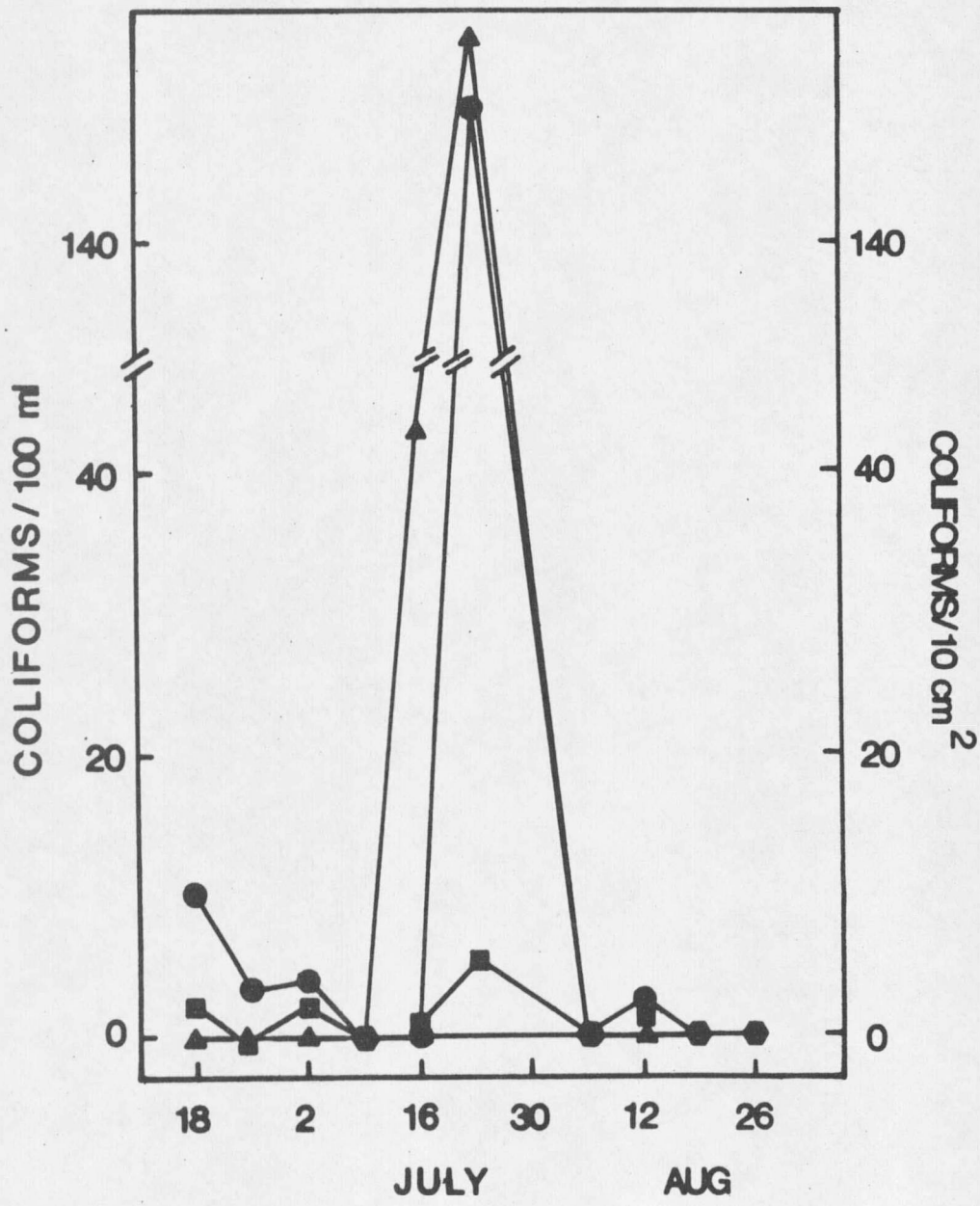


Figure 10. Levels of coliform bacteria in Pine Creek during the summer of 1980. Data shown represent samples taken upstream from (■), downstream from (●), and directly from (▲) the mat community.

Figure 8b, these levels were extremely low - <4 mg/m^3 for all samplings - and serve to illustrate the extreme oligotrophic nature and low productivity of the system. The phytoplankton population increased steadily as stream water temperatures approached their highest levels, and remained high as temperatures declined in late summer. These two parameters had a correlation (r) value of only 0.48, indicating that other factors were probably influencing phytoplankton growth in Pine Creek Lake. An unexpected peak in chlorophyll a levels occurred near the end of the season which may have been due to a successional change in the phytoplankton, but such a change was not documented.

Bacterial identification and temperature relations.

All organisms isolated from the Pine Creek system, with one exception, were gram negative rods; of these, the majority were oxidase negative. No filamentous bacteria were isolated. Sizes ranged from 0.7 µm to 5.7 µm and 0.2 µm to 1.1 µm for length and width, respectively, with an average cell volume of 0.65 μm^3. One feature of particular interest in these investigations was the fact that several of the isolates from both the mat and the stream water produced a sticky, cohesive material even when grown in pure culture using a complex medium.

The isolates and their respective generation times at various temperatures are presented in Table 4. In all cases the isolates

Table 4. Temperature relations of bacteria isolated from Pine Creek

Organism	Isolation Date	Sampling Location	Isolation Temperature (°C)	Generation Time (hrs) 5C	15C	25C
S. liguefaciens	7-23	D[b]	25	7.70	1.16	0.83
E. coli	11-6	M[a]	25	13.82	0.83	0.62
Ent. cloacae	7-16	M	25	4.12	2.03	1.14
S. liguefaciens	9-17	D[b]	25	12.04	2.02	1.10
S. liguefaciens	8-6	M[b]	25	13.67	2.24	1.48
Gp. IIK, biotype 1	8-6	U[c]	25	14.92	-	2.49
Micrococcus	7-16	M	25	-	2.78	2.02
Gp. IIK, biotype 1	7-23	D	25	4.60	3.31	2.34
Gp. Ve, biotype 2	8-26	M	25	-	3.11	2.41
Moraxella	6-25	M	5	8.66	-	2.15
Chromobacter	6-25	U	5	7.30	-	1.16
Pseudomonas/Alcaligenes	8-11	D	5	3.33	-	2.70
Pseudomonas/Alcaligenes	7-21	M	5	7.00	-	1.91

[a]Isolated from the mat [b]Downstream from the mat [c]Upstream from the mat

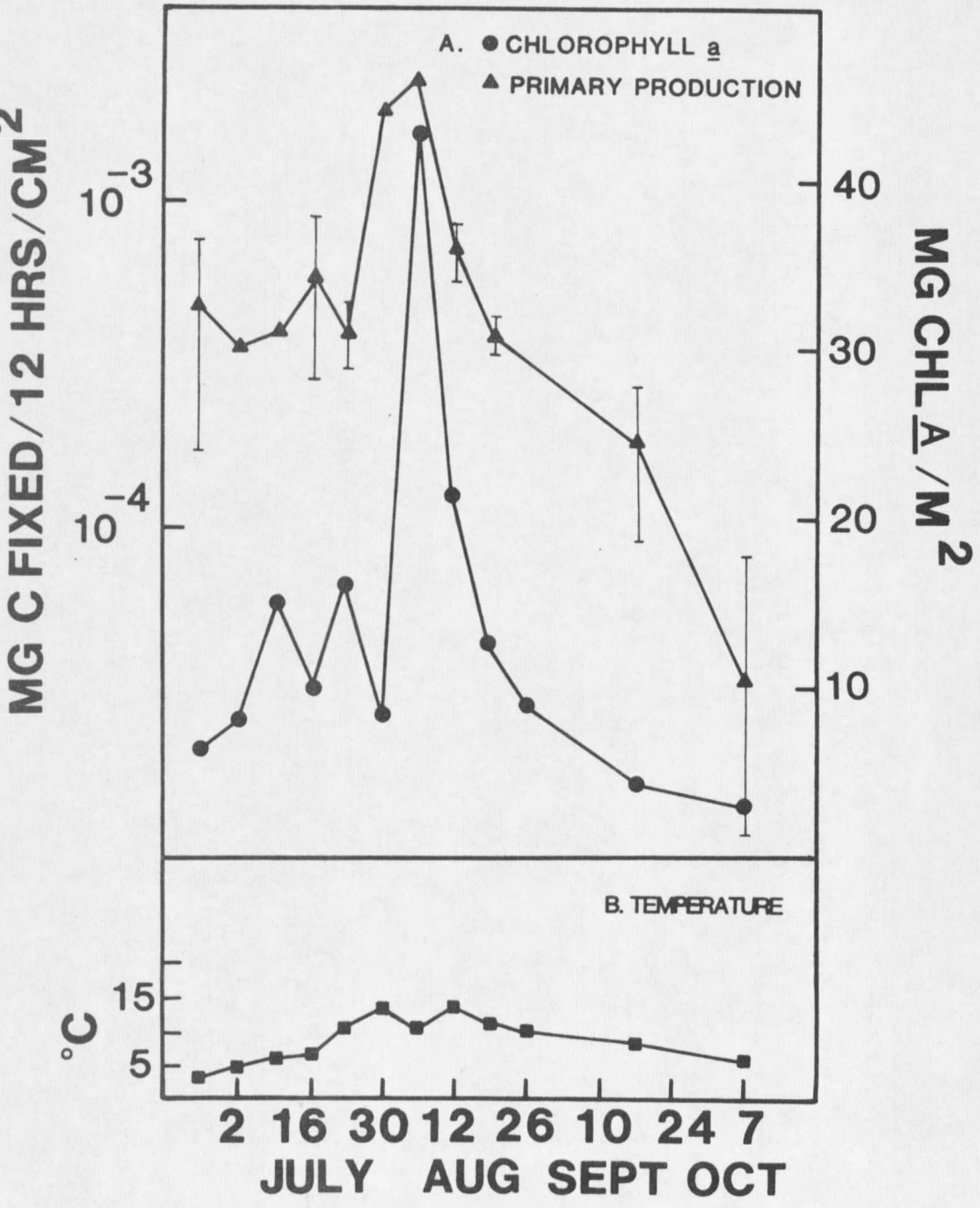
grown at 25C had growth rates which were highest at 25C and decreased steadily with temperature. Lag times were also consistently longer at lower temperatures. Another point of interest made evident by this table is that organisms isolated from the mat community did not possess higher growth rates than their planktonic counterparts. In addition, the time of season and the stream temperature at the time of sampling had no apparent effect on the optimal temperature range of the Pine Creek isolates.

Several organisms were isolated from the stream at 5C during 1981, their temperature relations are also given in Table 4. Despite their isolation and maintenance at 5C, their response to a wide range of temperatures appeared to be similar to those isolated at 25C, possessing significantly faster growth rates and shorter lag times at 25C than at 5C. These properties were also not affected by isolation sites or dates. Although a more active assessment of bacterial temperature relations may have been obtained by measuring metabolic activity instead of growth (66), the results of these investigations indicate that the bacteria isolated from Pine Creek were facultatively psychrophilic, and their growth and metabolic activity may have been limited by the cold temperatures of the stream water.

Phototrophic growth and metabolism in the mat.

Two parameters of the phototrophic population are summarized for the 1980 season in Figure 11. Chlorophyll a levels of a known

Figure 11. Seasonal dynamics of the Pine Creek mat phototrophs in 1980. Values for chlorophyll a were determined from single analyses. Panel B illustrates the stream water temperatures throughout the sampling period.


A. ● CHLOROPHYLL a
▲ PRIMARY PRODUCTION
MG C FIXED/12 HRS/CM2
10-3
10-4
40
30
20
10
MG CHL A /M2
B. TEMPERATURE
°C
15
5
2 16 30 12 26 10 24 7
JULY AUG SEPT OCT

area of mat material removed from rock surfaces were used for comparison of the algal population at the various sampling dates. These levels were low and reasonably steady in the early part of the season, increasing dramatically to values greater than 40 mg/m^2 in early August, and then rapidly declining to minimal levels below 15 mg/m^2 in a matter of only two weeks. The rates of primary production by the phototrophs showed similar patterns; peak values occurred at the same time of the season as did the chlorophyll _a_ maximum, and were significantly ($p < 0.05$) greater with respect to other times of the season. The bottom panel of Figure 11 shows the temperature pattern of the stream water during the 1980 season. The highest values of both chlorophyll _a_ and primary production in the mat community occurred at a time when the stream attained its warmest temperatures. However, both parameters decreased substantially when the stream water was at or near its maximum temperature of the season, and chlorophyll _a_ values had dropped approximately 5-fold while the stream water was near 10C.

The seasonal patterns for chlorophyll _a_ and total organic carbon in the mat during 1981 (Figure 2) resemble those of the previous year, but differ appreciably with respect to stream water temperatures. The mat reached its peak biomass in 1981 while the water was relatively cold (8.5C), and had almost completely disappeared by the time temperatures reached a maximum (12C) in late August.

The rates of primary production per unit chlorophyll a were calculated for 1981 to obtain an efficiency measurement for inorganic carbon conversion by phototrophs within the mat. Interestingly, the results (Figure 12) show that maximal conversion rates by phototrophs occurred not when algal biomass in the mat was greatest, but rather at a point earlier in the season when the water was at 3C and just beginning to warm. These rates remained relatively high as the mat biomass increased, but declined sharply while water temperatures were still rising. As conversion efficiency decreased, so did the biomass of the phototrophs within the mat. By early September, activity was virtually non-existent, and algal biomass had decreased to the lowest levels of the season.

Bacterial metabolic activity.

Heterotrophic potential studies were performed in 1980 to determine not only the seasonal patterns of bacterial metabolic activity in Pine Creek, but also the potential for removal of organic matter by sessile bacteria in a pristine environment. Least squares regression techniques were used to obtain Vmax values for ^{14}C-glutamate where the data points demonstrated linearity at the 95% level.

A major assumption of heterotrophic potential investigations is that algal utilization of the labelled organic substrate in the dark is negligible. This assumption is supported by the fact that phototrophs have a very low affinity for these compounds requiring much

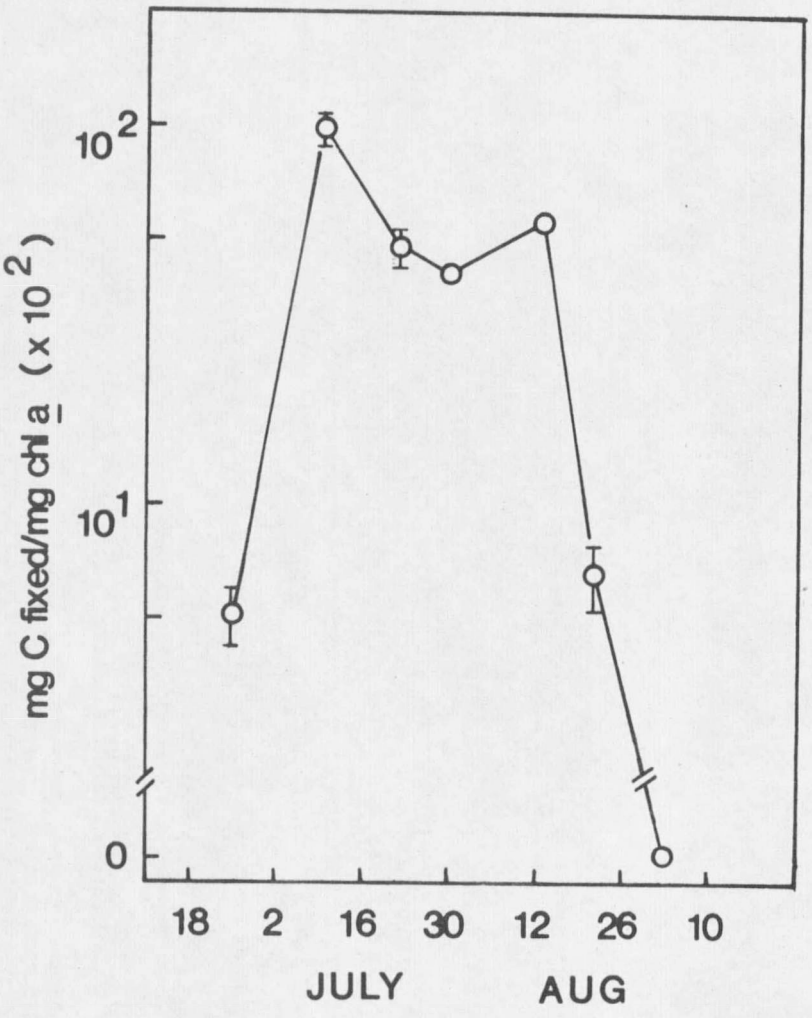


Figure 12. Primary production per unit biomass by the mat phototrophs in 1981.

higher concentrations than bacteria before uptake occurs (56,80). In addition there appears to be a lag period of several days before the phototrophs adapt to dark heterotrophy (5,8). Thus the utilization of ^{14}C-glutamate in these experiments is considered to be the result of bacterial metabolic activity, the seasonal patterns of which are summarized on a unit area basis in the second column of Table 5.

1. Glutamate utilization rates (Vmax).

Potential glutamate utilization rates by the bacterial population increased to a maximum in mid-September, and then dropped off gradually. On the other hand, the total number of bacteria present within this area decreased as Vmax was accelerating. In the third column (Table 5), Vmax values have been adjusted for the bacterial population at each date to obtain specific activity indices. This had the effect of accentuating the surge in metabolic activity by the heterotrophic bacteria when glutamate was employed as the substrate. The values seen in the months of July and August were extremely low, representing an almost complete lack of uptake of the labelled substrate. Values in September and October, on the other hand, were higher than those reported for systems which presumably contained higher concentrations of dissolved organic matter (13,30, 33,43).

Table 5. Algal primary production rates and heterotrophic uptake rates of glutamic acid by the epilithic mat community of Pine Creek from July-November, 1980. The specific activity index is adjusted for bacterial numbers in the mat at each sampling date.

Date	Bacterial Direct Counts ($cm^{-2} \times 10^{8}$)	Vmax Glutamate ($\mu g \cdot cm^{-2} \cdot hr^{-1}$)	Vmax Specific Activity Index ($\mu g \cdot cm^{2} \cdot hr^{-1} \cdot cell^{-1} \cdot l^{-1} \cdot 10^{-12}$)	Phototrophic Primary Production ($\mu g \cdot cm^{-2} \cdot hr^{-1}$)
7/2	4.35 ± 0.38	6.76×10^{-2}	$1.55 \times 10^{2} \pm 1.34 \times 10^{1}$	2.98×10^{-2}
7/23	0.86 ± 0.09	1.90×10^{-3}	$2.22 \times 10^{1} \pm 2.32 \times 10^{0}$	3.22×10^{-2}
7/30	1.08 ± 0.08	1.39×10^{-3}	$1.29 \times 10^{1} \pm 1.02 \times 10^{0}$	1.52×10^{-1}
8/6	0.83 ± 0.05	1.80×10^{-2}	$2.16 \times 10^{2} \pm 1.52 \times 10^{1}$	1.92×10^{-1}
8/26	2.23 ± 0.02	1.86×10^{-1}	$8.34 \times 10^{2} \pm 9.80 \times 10^{0}$	9.58×10^{-4}
9/17	0.26 ± 0.04	1.50×10^{0}	$5.66 \times 10^{4} \pm 7.75 \times 10^{3}$	1.28×10^{-2}
10/7	0.19 ± 0.01	7.21×10^{-1}	$3.27 \times 10^{4} \pm 2.52 \times 10^{3}$	2.86×10^{-3}
11/6	0.47 ± 0.03	1.20×10^{-1}	$2.53 \times 10^{3} \pm 1.49 \times 10^{2}$	---

2. Turnover rates.

Turnover times, which were also obtained from heterotrophic potential calculations, are a measure of the half-life in the environment of the substrate in question. These values are the inverse of heterotrophic uptake and are therefore difficult to use as an index of bacterial metabolic activity (78). In Figure 13 the inverse of turnover times have been adjusted for bacterial numbers within the mat to give a plot of turnover rate indices for the 1980 season. This graph illustrates the progressive rise in metabolic activity of the heterotrophic population beginning in mid-summer and continuing into autumn.

3. Relative heterotrophic activity.

The results made evident by the heterotrophic potential data were supported by a separate analysis. The uptake and turnover of a single concentration of ^{14}C-glutamate (added to the sample at zero time) within the incubation period showed patterns similar to those of Vmax and turnover rate indices. If adjusted for the bacterial population at each sampling date as before, a measure of the relative heterotrophic activity is obtained. These data, presented in Figure 14 along with the plot of chlorophyll a content of the mat, indicate that bacterial utilization rates of glutamate increased significantly as the season progressed. This trend appeared to follow the decline of the phototrophic population, and suggests that changes in the

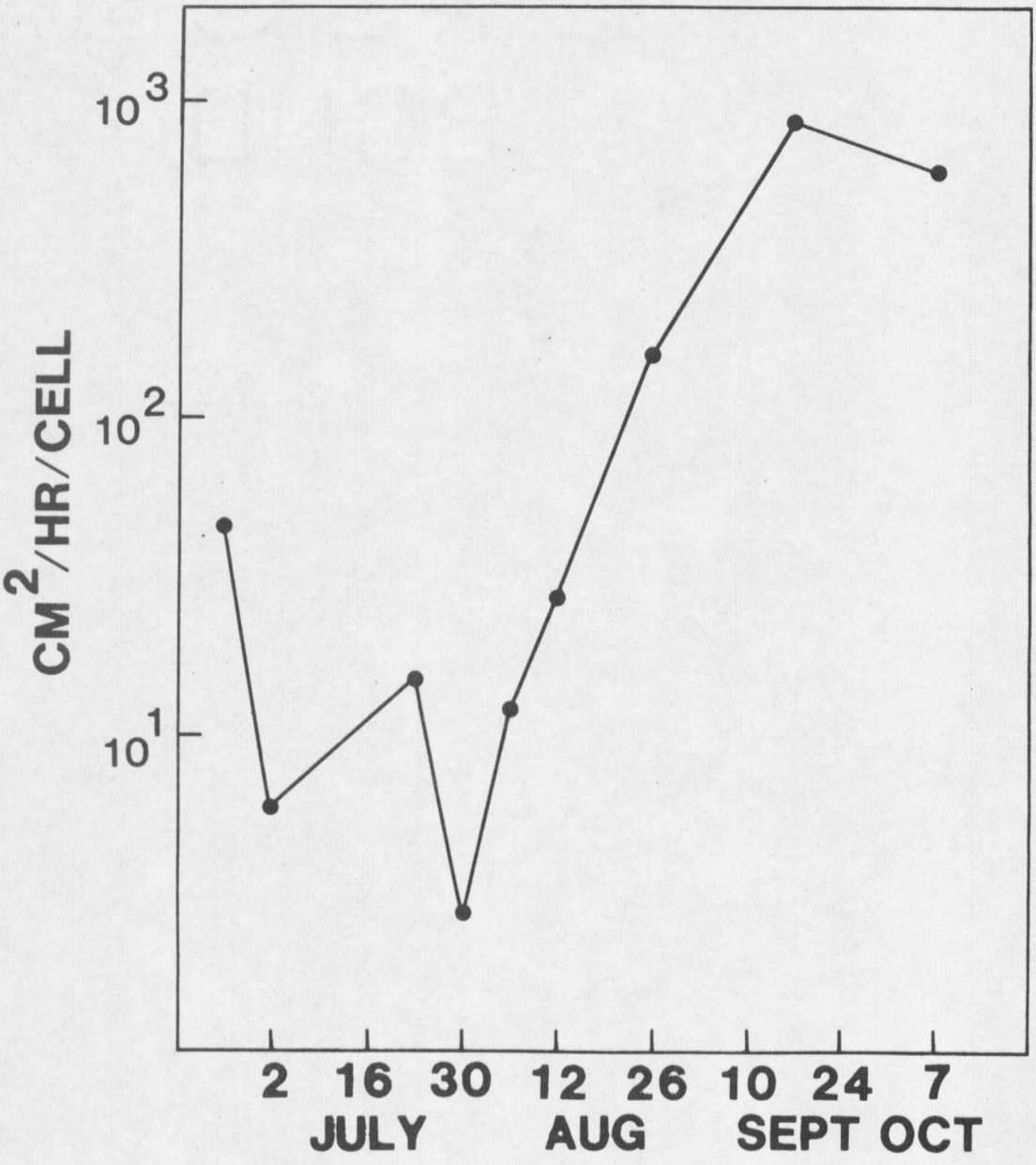


Figure 13. Turnover rate indices of glutamate in the Pine Creek mat during 1980, as determined from heterotrophic potential data.

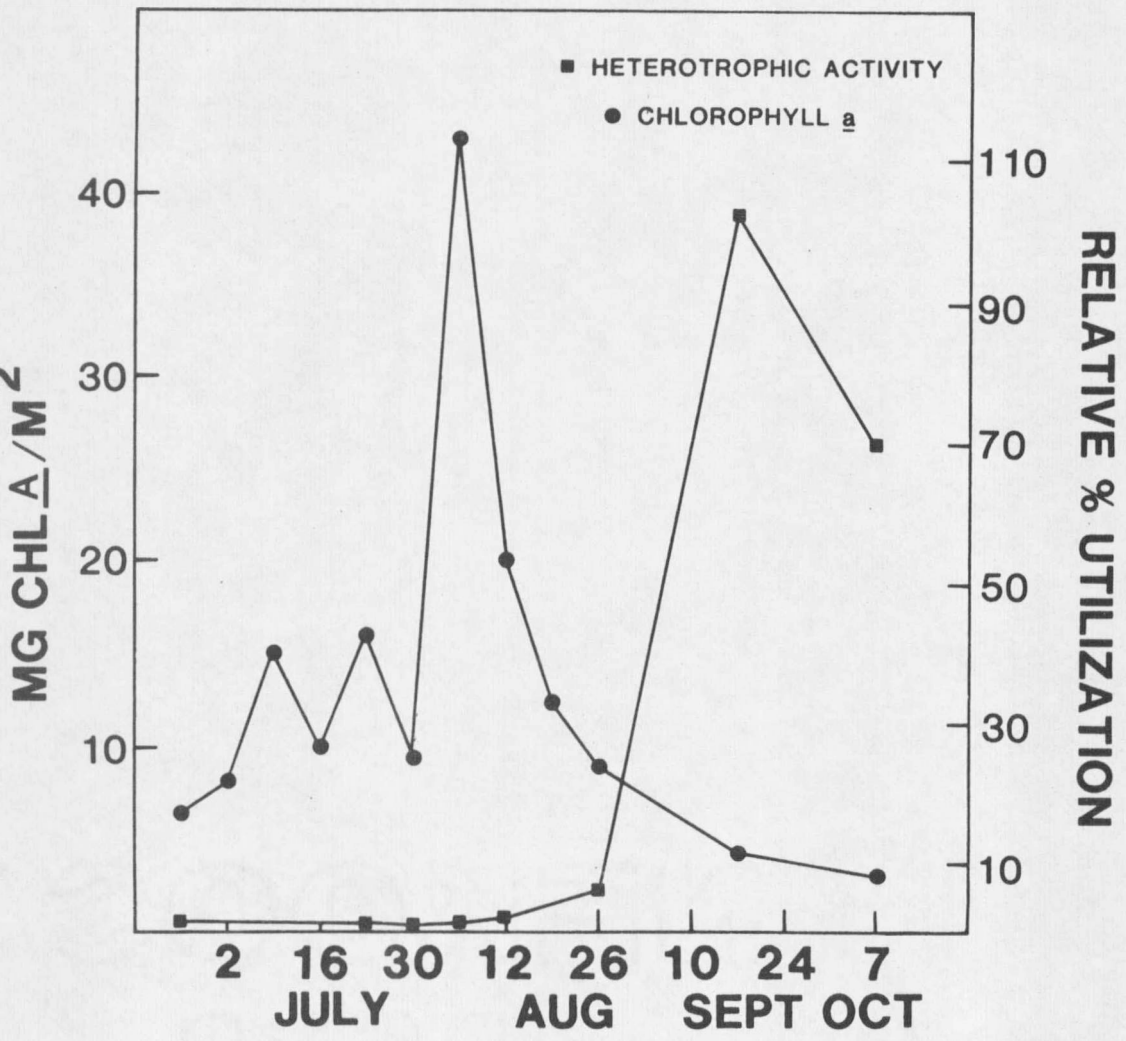


Figure 14. Dynamics of mat microorganisms. Comparison of the phototrophic biomass and bacterial heterotrophic activity on glutamate during the 1980 sampling period.

algal component concurrent with their decline stimulated the metabolic activity of the bacterial population.

4. Bacterial utilization of glycollate.

Heterotrophic potential studies were also performed in 1981 using both ^{14}C-glutamate and ^{14}C-glycollate as organic substrates for the sessile bacteria. The results from tests performed on August 21 are listed in Table 6. On this date the maximum uptake rates (Vmax) of glycollate were approximately 10 times higher than that for glutamate. The turnover time of the natural substrate pool was also substantially lower, indicating a shorter residence time for glycollate in the mat.

Carbon flux investigations

These experiments were designed and performed during the 1981 season to more precisely define the nutritional interactions occurring between the phototrophic and heterotrophic microorganisms in the Pine Creek mat community. Addition of ^{14}C-bicarbonate allowed a complete flow of carbon through the trophic levels of the mat, and size-differential filtration of the system after termination separated the various labelled carbon pools into defined compartments. The results of these experiments, outlined in Table 7 and Figure 15, reveal several interesting patterns among both algal and bacterial populations.

Heterotrophic uptake of inorganic carbon was found to be in-

Table 6. Maximal utilization rates of two organic substrates by the heterotrophic bacteria in the Pine Creek mat on 8-21-81.

Substrate	Vmax $\mu M \cdot hr^{-1}$	Turnover Time (hrs)
^{14}C-Glutamate	1.56×10^{-4}	148.6
^{14}C-Glycollate	2.89×10^{-3}	10.7

Table 7. Pathways of carbon flow in the algal-bacterial mat of Pine Creek for 1981. Compartmentalization of ^{14}C into discrete pools after $H^{14}CO_3^-$ addition is also contrasted between light and light-dark incubations.

DATE		A. ALGAL FIXATION (dpm)	B. ALGAL EXCRETION (dpm)	C. BACTERIAL UPTAKE (dpm)	D. TOTAL C TRANS-FORMATION (dpm)	% ALGAL EXTRACELLULAR PRODUCTS INCORPORATED BY BACTERIA
6/25	3 hrs					
	24 hrs	5026	1404	4200	10630	75.0
7/8	3 hrs	9428	50490	4223	64142	7.7
	24 hrs	17833	44253[a]	7164	69250[a]	13.9
7/21	3 hrs	44760	49104	4690	98554	8.7
	24 hrs	65170	25047	8020	98237[a]	24.2
7/28	3 hrs	24740	45656	11312	81712	24.8
	24 hrs	33250[a]	20800	27438	74522[a]	56.9
8/11	3 hrs	26590	51888	3264	81742	5.9
	24 hrs	34610	30926	5922[a]	71460[a]	16.1
8/21	3 hrs	5582	0	666	6248	100
	24 hrs	6117[a]	0	666[a]	6784[a]	100
9/2		0	0	0	0	

[a] Values for 24 hour incubations were not significantly different from 3 hour samples at the 95% level.

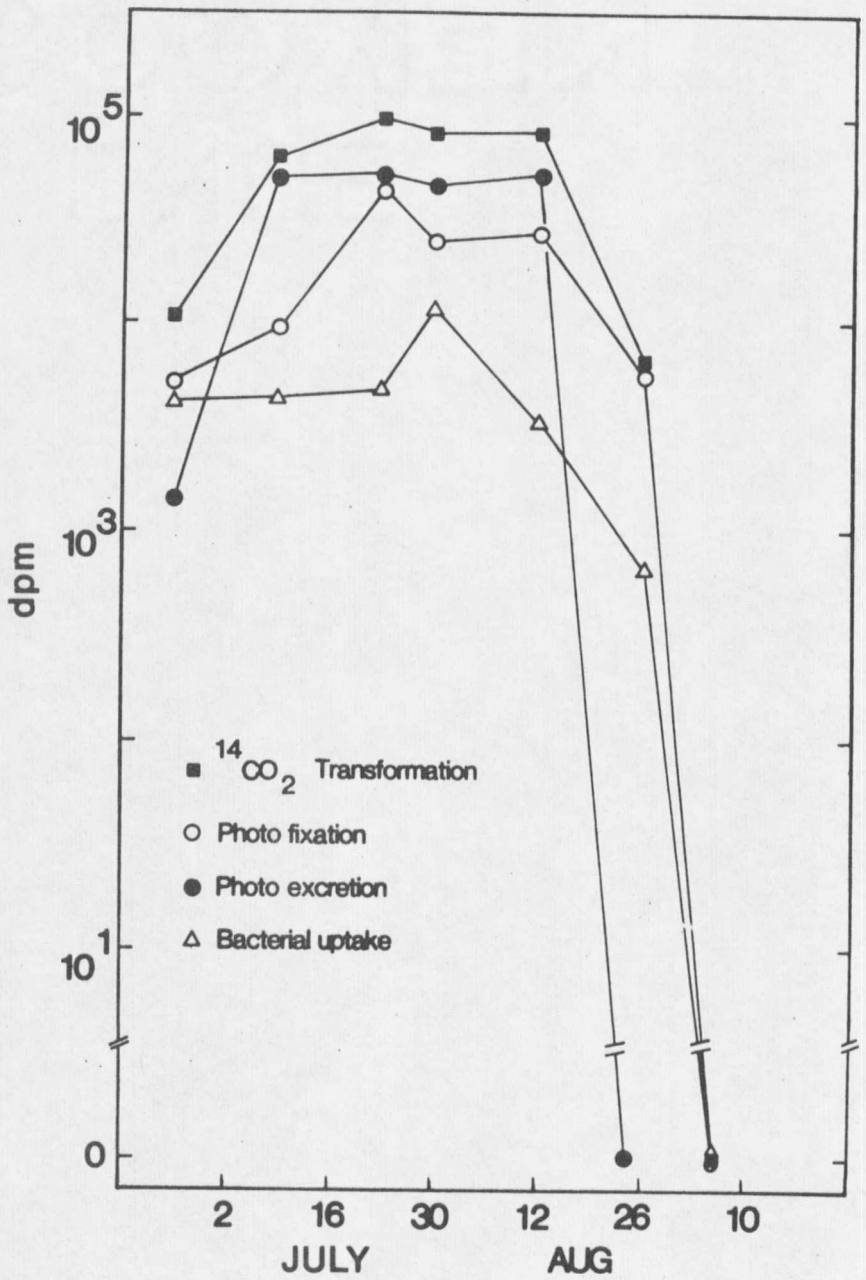


Figure 15. Seasonal patterns of carbon flow in the Pine Creek mat. All values are reported as the radioactivity contained in each fraction of the experimental systems in 1981.

significant in the dark controls, and also in reaction vessels that had the phototrophic population removed prior to addition of labelled bicarbonate. These data agree with the results of other investigators working with planktonic systems (6,7,19).

1. Primary production by mat phototrophs.

Phototrophic production was taken as the total radioactivity detected in all three fractions of the system following $^{14}CO_2$ removal from the gas headspace of the reaction vessel (particulate on 5.0 μm and 0.2 μm filters and filtrate). The values for this fraction (Column 4) 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 three hours before formalin addition, the other set was dark-incubated for 21 hours subsequent to the light exposure. The values in column 4 do not differ appreciably between the 3- and 24-hour samples, which would suggest that the inorganic carbon fixation was a light driven process, with insignificant amounts of carbon transformed by phototrophs in the dark. However, the radioactivity seen in the 5.0 μm particulate fraction (algal fixation) increased in all cases during dark incubation, indicating that algal heterotrophy was probably occurring in the dark. One limitation of the carbon flow system was the inability to accurately evaluate $^{14}CO_2$

resulting from bacterial respiration of algal fixation products, nor in this case could these values be reasonably estimated. Thus, it is probable that dark fixation was occurring to some degree in this system, and the values found in Column 4 represent an underestimation of the total carbon fixation for the incubation period.

2. Fate of photoassimilated compounds.

It was assumed that the fixation products of the phototrophs were either incorporated into cellular matter or excreted into the surrounding medium, the latter process being a characteristic feature of algal metabolism (23). The seasonal values for these two fractions are presented in Columns 1 and 2 of Table 7. Both fractions show long-term seasonal patterns similar to total fixation (Figure 15), but differ radically with respect to their short-time course results. Radioactivity in the cellular fraction increased during the dark incubation, but the labelled algal extracellular products in the diluent water decreased significantly in the same period, and were completely absent from the system for the last two sampling dates.

3. Bacterial uptake of excreted algal products.

The amount of soluble algal extracellular products incorporated into bacterial biomass was taken to be the radioactivity retained by a 0.2 µm filter after adjustment for retention of bacterial cells on the 5.0 µm prefilter. The third column of Table 7

lists these results, and once again the seasonal trend 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 for only 3 hours for all but the last two sampling dates. Column 5 lists the fraction of the total pool of algal extracellular products that was incorporated into bacterial biomass. Again these values do not take into account the algal products that were mineralized to $^{14}CO_2$ by the bacterial population, but the data still indicate that a significantly greater portion of excreted algal organic products were utilized by the heterotrophic bacteria as time progressed.

4. Light vs light-dark incubations.

The data obtained on July 21 were selected for an illustration of the processes occurring in the carbon flux system over a 24 hour time course (Figure 16). The labelled 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 exhibited in the light. As bacterial incorporation of algal extracellular products resulted in an increase in radioactivity detected on the 0.2 μm particulate fraction, a concurrent loss of radioactivity was seen in the filtrate of the bulk fluid.

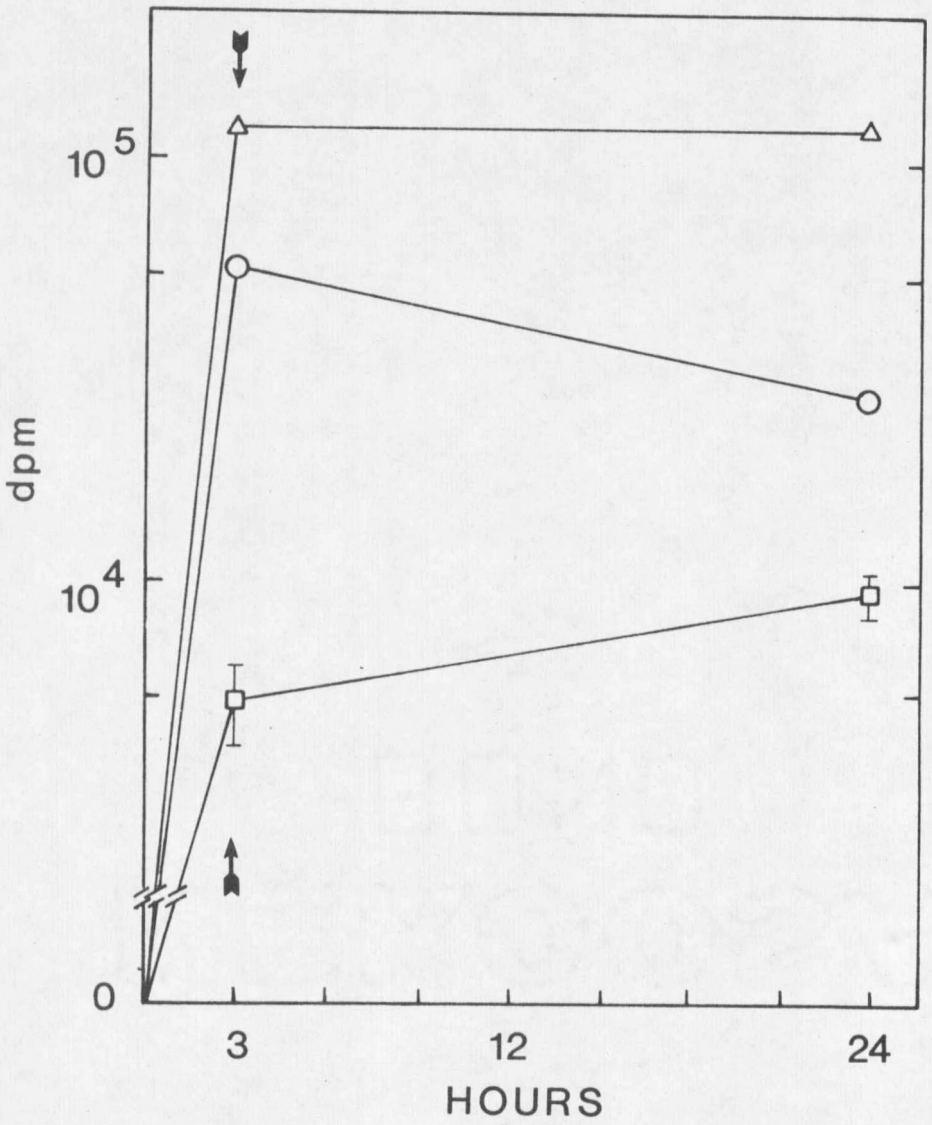


Figure 16. Short-term carbon flow in the Pine Creek mat. Rates of phototrophic $^{14}CO_2$ transformation (△), ^{14}C-soluble product excretion by algal cells (○), and bacterial uptake of ^{14}C excreted compounds (□) during the course of a single representative assay. Arrows indicate onset of dark incubation conditions.

DISCUSSION

Based on the results of these studies, the following model for carbon flow among microorganisms in the Pine Creek mat is proposed (Figure 17). CO_2 is transported into the mat by diffusion and reduced by phototrophs in the presence of sunlight. In addition to incorporation into algal cells, 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 to either 1) an accumulation of bacterial biomass or 2) satisfaction of bacterial maintenance energy requirements (resulting in the formation of CO_2 within the mat). Algal extracellular products not utilized by the bacterial 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.

This model forms the basis for much of the discussion in this dissertation. In the following sections, the processes outlined above will be analyzed in greater detail. The determination of environmental pressures responsible for the observed seasonal trends will also be emphasized.

Origins of mat community.

The epilithic mat of Pine Creek existed most prominently in the mouth of the outlet stream of the lake. Because of its close proxi-

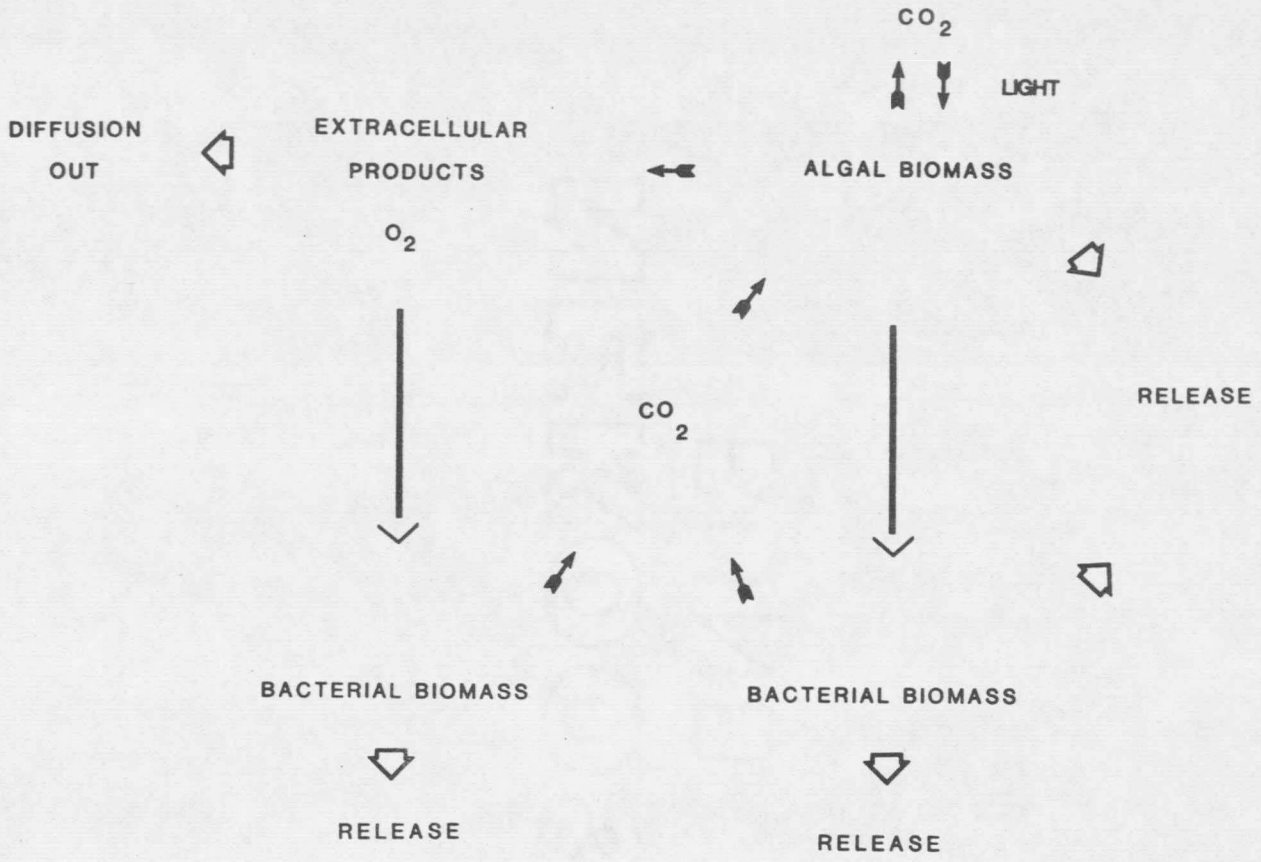


Figure 17. Proposed model for carbon flow in the epilithic mat community of Pine Creek.

mity to the lake, it was originally assumed that the sessile organisms had a planktonic origin. This assumption was confirmed by two observations: 1) Species diversity of the phototrophs, 2) Characterization of bacterial isolates.

The Shannon-Weaver diversity index for phototrophic organisms was approximately 2.5, indicating a low species diversity in the biofilm. This is normally considered to be characteristic of phytoplankton populations in lentic (lake) systems. Aquatic environments which are strictly lotic in nature (streams and rivers) typically have much higher diversity with respect to the phototrophic populations (76, L. Bahls, personal communication).

The second confirmation of the original assumption lies in the identification of bacterial isolates. Although a systematic investigation allowing statistical validation of the results was not attempted, identical organisms were isolated from both the planktonic and sessile environments. Further, they shared many phenotypic characteristics, including growth rates and temperature ranges in pure culture, and the ability to produce extracellular polymeric material that was supposedly used in mediating attachment to the rock surfaces of the stream benthos.

It thus appears that the sessile organisms of Pine Creek were derived from the overlying stream water as it left the lake. The mechanisms involved in transport and adhesion of microorganisms to

surfaces have been described elsewhere (50,59). Despite this conclusion, the data make it evident that the mat community was not a transient phenomenon of transport from the stream water. Comparison of phototrophic and bacterial population patterns from both environments (Figures 2,8 and 9) demonstrates the fact that both chlorophyll a levels and total bacterial numbers in the planktonic environment remained reasonably steady throughout the season, while these same parameters in the mat community were undergoing significant fluctuations. The conclusions drawn from these data suggest that growth of microorganisms in the mat was independent of population patterns in the stream water, and that mat proliferation was not the direct result of increased transport of organisms from the stream water. Other investigators have published results which suggest that sessile populations are more active and possess faster growth rates than their planktonic counterparts (35,38,48,82), and Bott and Brock (10) performed a study which supports the conclusion that the increases in bacterial numbers seen on artificial substrates was due to actual growth rather than transport to the surfaces.

Empirical observations suggest that attachment by microorganisms to the rocks in Pine Creek may occur only under certain conditions. The mat community was always well established by the time it was possible to reach the study site in mid-June. But once this community was removed from a section of rock, re-colonization was very slow if

not entirely absent, despite the fact that the intact mat material was rapidly proliferating. In addition, colonization of substrates for SEM observation appeared to vary quantitatively depending on what time of season they were placed in the stream. The specific chemical and physical factors necessary for attachment of microorganisms are not known, and probably vary widely from system to system. Some progress has been made toward this goal in marine environments (14), but it is an area that deserves more attention in the future.

Bacterial enumerations

1. Viable counts.

The results of viable bacterial enumerations from both the water column and the epilithic mat community showed patterns that were quite different from those of direct counts by epifluorescence microscopy. In addition, the absolute numbers of viable bacteria were consistently 3-4 orders of magnitude lower than total numbers, indicating that only a small percentage of the total population was detected by plate counts. The use of a single growth medium (Standard Plate Count Agar) at one incubation temperature undoutedly selected for a few specific bacterial groups capable of growth under those conditions, and the patterns seen reflected the relative abundance of these groups, rather than the entire bacterial population. This was illustrated by the observation that bacterial counts varied

for any given sampling date when the growth medium and incubation temperatures were changed. Consequently, this method of enumeration imposes serious limitations on any conclusions that may be drawn from the results.

2. Direct counts.

a. Planktonic bacteria.

Direct microscopic counts, despite their inability to distinguish between active and inactive cells, provided a superior analytical method with which to analyze the population dynamics occurring in the Pine Creek system and are used for all subsequent references to bacterial populations.

The low concentrations of inorganic nutrients, and the apparent lack of any appreciable fluctuations in most of these parameters is indicative of the oligotrophic nature and low productivity of Pine Creek. But despite this, the magnitude of the bacterioplankton population was similar to numbers reported by researchers (10^5 - 10^7 bacteria/ml) studying considerably more eutrophic environments (17,22,26).

The seasonal pattern for the bacterial population in the stream water was one of steady increase throughout the season, with a slight depression in numbers in late July and early August. Bacterial numbers did not decline as the water temperature dropped during the latter part of the year, and were higher in November than in June,

despite the fact that the water temperatures for both dates were nearly identical. This is in contrast to results published by Jones (39), who observed that aquatic bacterial populations are positively correlated with temperature.

Several researchers have noted that planktonic bacterial populations respond to fluctuations in the levels of soluble organic carbon (1,26), which are often the result of abnormally high runoff during the periods of heavy rainfall or other major hydrographic events. The levels of total organic carbon for Pine Creek, besides being quite low, did not experience the fluctuations characteristic of streams in lower and more complex life zones, as there was little allochthonous carbon input into the system. There was instead a steady increase to a maximum concentration of ca. 4.5 mg/l in August of 1981, followed by a moderately sharp decline to minimal levels near 1.5 mg/l. Although there was no correlation with organic carbon levels in Pine Creek, the seasonal pattern of the bacterioplankton bore some similarity to that of chlorophyll *a* in the stream water. Both parameters (Figure 8) fluctuated mildly throughout the season, but in general exhibited steadily rising trends with slopes that were approximately the same. A plot of total bacteria against chlorophyll *a* levels in the stream water (Figure 18) serves to illustrate the correlation observed between the two populations ($r = 0.68$). This trend has been observed in other aquatic systems (1,9,25,27) and

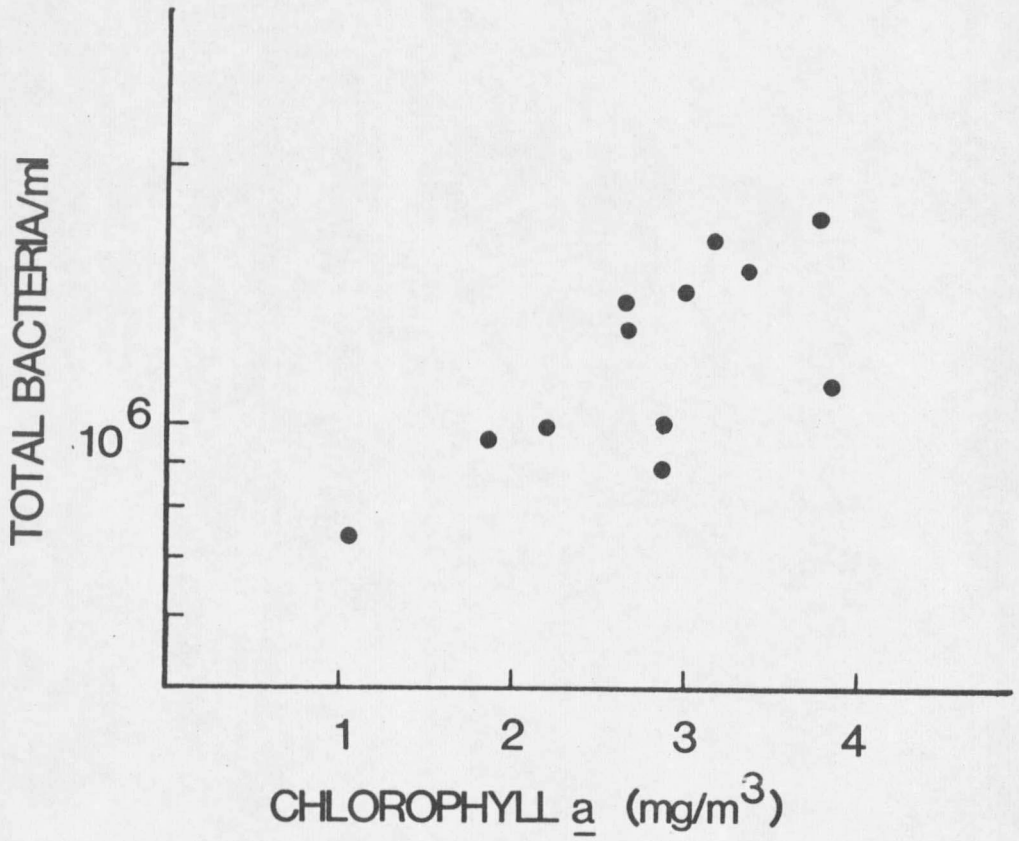


Figure 18. Correlation of total bacterial concentrations and chlorophyll a levels in the stream water of Pine Creek during 1980.

suggests that bacteria in the planktonic environment may associate themselves with free-floating phytoplankton. Such a phenomenon is well documented (26,30,40) and is believed to represent an important survival mechanism for bacteria existing in cold, nutrient-poor waters such as that of Pine Creek.

b. Epilithic bacteria.

The population patterns of the bacteria in the sessile mat community for the 1980 and 1981 seasons were characterized by mid-season (July and August) values of approximately 10^8 cells/cm^2 followed by a decrease of nearly one log by early autumn. As with the planktonic population, there was no correlation of bacterial numbers and in temperature. In fact, correlation of the sessile population with any single environmental parameter is difficult due to the complexity of the mat community structure. Microscopic examination of the mat has shown that the sessile bacteria tend to be intimately associated with phototrophic organisms, suggesting that their population patterns should correlate closely. For the Pine Creek system no such correlations were found to exist during either the 1980 or 1981 season, but while the sharp fluctuations characteristic of chlorophyll a were not apparent in the bacterial population patterns, their decrease in numbers in September and October appeared to follow that of the phototrophs. This may be explained by the fact that both the sessile bacteria and phototrophic organisms were deeply embedded within an

extracellular polymeric matrix that made up a large portion of the mat. As phototrophic activity declined in August a large portion of the mat was removed from the rock surfaces by the mechanical shear forces of the stream water. This sloughing process undoubedly transported bacteria away from the surfaces also.

The data on sessile bacterial populations in Pine Creek suggest that there were mechanisms in force which kept the magnitude of the bacterial population below a certain level. In fact, no reports exist in which bacterial numbers of an attached biofilm system exceed 10^9 cells/cm^2. Three mechanisms are considered in this discussion: 1. Nutrient limitations. 2. Fluid shear force. 3. Bacterial metabolism.

One limiting factor to the numerical magnitude of sessile bacterial populations may be the availability of, and competition for nutrients. It has been shown that for systems where the sessile population obtains its nutrients from the overlying bulk fluid, cell density is a function of nutrient diffusion into the film (Trulear, unpublished). This maximal thickness would likely impose an upper limit on the number of bacteria that could be present in a given area of film material. In a biofilm such as that in Pine Creek, organic nutrients were produced within the mat by phototrophic organisms in the form of excreted soluble organic products, and it is assumed that this characteristic virtually eliminated the need for the trans-

port of organic nutrients from the bulk fluid (see page 101). Further, results from carbon flux experiments indicated that bacteria were not nutrient-limited with respect to algal products throughout much of the season when the entire epilithic community was proliferating. Despite this, the numbers of bacteria were actually lower at this point than at times when carbon sources may have been scarce, suggesting that factors other than nutrient limitations determine maximum bacterial numbers in sessile communities.

The shear forces of the stream water, maintaining a film thickness compatible with the physical forces of the stream, could play a role in removal of bacteria from the mat community. For nearly all sampling dates in the 1980 season, total bacterial counts of stream water samples obtained downstream from the mat were higher than those taken upstream, suggesting that bacteria were being continually removed from the mat and transported downstream. This loss may have been compensated for by growth of the mat bacteria but because the temperature of the stream never exceeded approximately 13C, bacterial growth rates (see Table 4) were probably not high enough to overcome the losses from the mat brought about by mechanical shearing.

The metabolism of the bacteria themselves could also play a role in limiting their numbers within the mat. The low alkalinity levels of the stream water, 9-11 mg/l, indicate that there was a low capacity for buffering in the system. If the deeper layers of the mat

were anaerobic, organic acids produced by bacterial metabolism could lower the pH of their microenvironment to the point where growth was inhibited.

3. Coliform bacteria.

Studies examining the die-off rates of coliform and indicator enteric bacteria in both natural and laboratory settings has led to the general opinion that the cold, nutrient deficient stream waters of alpine regions represent an environment that is not conducive to the survival and growth of these organisms (53,70). Nevertheless, coliform and sometimes pathogenic bacteria are found in surprisingly high numbers in pristine mountain streams (20,54,65), suggesting that certain components of these streams are capable of supporting the growth of fastidious organisms. McFeters, et al. (54) found that large numbers of coliform bacteria were associated with an epilithic algal-bacterial film that exists in the mountain streams of an alpine region in Grand Teton National Park, Wyoming. It was further demonstrated that these bacteria incorporated labelled extracellular products of Chlorella, a representative alga of the film, indicating that the phototrophic population of the epilithic community provides a nutrient-rich microenvironment in which coliform bacteria are able to proliferate.

The general composition of the epilithic mat community at Pine Creek is not unlike that encountered in the Teton studies, and coli-

form bacteria were found to exist in Pine Creek during the summer months. However, the patterns seen are quite different; coliforms appeared in the stream water at only one point during the 1980 season in any appreciable numbers. This was in the form of a substantial peak in mid-July that was followed by a rapid disappearance to non-detectable levels. Two possibilities for this occurrence are examined in this discussion: 1. Increased transport of coliforms into the stream from the drainage. 2. Actual growth of coliforms within the mat.

Sudden peaks in aquatic bacterial populations are often associated with hydrologic events that significantly increase allochthonous input into the stream (52). Pine Creek differs from systems such as these in that its main feed is a large, deep reservoir; its level, and accordingly its flow, appears to be affected only by snowmelt in the spring. Further, the coliform peak occurred only in samples taken either from the mat itself or downstream; samples taken upstream from the mat remained low in coliform numbers.

It is thus hypothesized that coliform bacteria grew almost exclusively in the epilithic mat community of Pine Creek, and that conditions favorable for their proliferation existed at only one particular stage in the development of the mat. The high numbers seen in samples taken downstream from the mat in July were likely the result of mechanical shear forces exerted by the flowing stream

water which transport organisms into the water column from the mat.

The precise nature of the conditions that stimulate growth of the coliforms is not known. However, it should be pointed out that although the number of colonies exhibiting a green metallic sheen on m-Endo broth did exhibit a major fluctuation, the total numbers of colonies seen on these plates did not vary greatly between sampling dates. This may imply that coliform populations did not have the patterns just described, but rather that conditions within the mat were responsible for changes in their physiology which were not manifested on m-Endo as a typical coliform phenotypic reaction.

Phototrophic growth and activity.

The growth and metabolism of the phototrophic community, as monitored by chlorophyll a and primary productivity measurements, peaked at the same time of year as did stream water temperatures during the 1980 season. These data agree with the results of other workers (1,18,62) who also note that algal growth follows temperature patterns. However, both of these parameters exhibited sharp declines in August while water temperatures were near the highest levels recorded for the season. This phenomenon was repeated during 1981 and suggested that factors other than temperature were affecting algal growth, including the following: 1. Nitrogen and phosphorous. 2. Interactions of chemical and physical parameters. 3. Light and CO_2 limitation. 4. Silica concentrations.

Nitrogen and phosphorous levels have been implicated in governing the growth and physiological states of aquatic phototrophs (29,37,60). Although a rigorous and systematic examination of these parameters was not performed for logistical reasons, the samples that were collected and analyzed showed nitrites, nitrates and phosphates consistently present at very low concentrations (0.1 mg/l). Fluctuations that may have occurred were too small to be resolved by the analytical methods used, and as a result, algal population correlations with these parameters were not possible.

Another method used recently to detect nutrient limitations of phototrophic organisms involves analyzing the chemical composition of the cells (21,62). Perry (60) found that the chlorophyll a:ATP ratios of P-limited and N-limited cultures differ by a factor of at least five in chemostat studies. The extreme changes observed in the phototrophic population of the Pine Creek mat suggested that the use of chlorophyll a:ATP ratios would provide better insight into inorganic limitations than would chemical analysis of the extremely oligotrophic stream water. The results of these investigations (data not shown) were also inconclusive. Chlorophyll a:ATP ratios for the mat phototrophs were approximately midway between those reported by Perry for N- and P-limitations, and showed no significant deviations that would justify the changes observed in the phototrophic population of the mat in August.

Several researchers have concluded that in natural aquatic environments there may be an interaction of two or more chemical or physical parameters that affect algal growth (37,73,81). Albright, et al. (1) note in their studies on sub-Arctic Canadian rivers that reductions in temperature and photoperiod were responsible for the decline of the phototrophic communities. Maddux and Jones (45) found that nitrogen, phosphates, and light all act in concert to influence algal growth, and postulate that the interactions are probably not limited to these three parameters exclusively.

As the phototrophs proliferated during the summer months, the mat increased to a thickness that may have restricted penetration of light and carbon dioxide into its deeper layers. This phenomenon was most noticeable during the 1981 season when the mat approached a thickness of approximately 2 mm. Several investigators have demonstrated that phototrophic carbon fixation rates are influenced by CO_2 concentrations (39,42,57), and its loss from the deeper layers of the mat may have been reflected in the low primary productivity values seen after mid-August. The only other source of CO_2 for the sessile phototrophs was that produced by bacterial respiration, but Jones (39) reports that this accounts for only 10% of algal consumption.

The fact that >85% of the mat phototrophs were diatoms suggests that silica (SiO_2) was an essential inorganic nutrient. Table 1 shows

that silica levels in the water began a decline in early August that continued until concentrations had dropped by nearly an order of magnitude to levels which were considerably lower than values reported to be the minimum concentrations required for the support of diatom growth (37). Coinciding with the reduction in silica concentrations was a sharp decline in both chlorophyll a levels and primary productivity rates in the mat community. It is likely then 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 were not able to survive in the Pine Creek system.

Algal Excretion

Several researchers have demonstrated the release of soluble organic compounds by phototrophic organisms (23,24,51,68). Most of these studies outlined the physiological bases behind this phenomenon, and demonstrated the effects of numerous environmental parameters on the excretion rates of pure cultures. But in the complex environment of a sessile stream community, even one as relatively simple as Pine Creek, the behavior of algal cells with respect to this process is largely unknown. Previous publications (3,24) 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 as the physiological state of the organisms is varied.

Since the attached phototrophic population of Pine Creek exhibits such dramatic seasonal fluctuations, it provided an ideal natural system for the study of excretion processes.

The carbon flow experiments made it possible to monitor this important component of algal metabolism. Table 8 lists the fraction of total phototrophic fixation that was excreted by the algal cells as soluble organic products. Because the bacterial respiration component could not be quantified, it did not seem valid to compare 3 and 24 hour fractions. These values were always above 50% (for 3 hour samples) in the period from 6-25 to 8-11, during which total production rates were also comparatively high. But after August 11, transformation rates of $^{14}CO_2$ by phototrophs declined appreciably, and only 10% of the primary production metabolites were excreted. From an examination of Figure 12 it is evident that this was precisely when primary production rates per unit of algal biomass decreased abruptly from the high rates observed in July. Since the radiant energy of the sunlight during incubations did not vary significantly throughout the season, the data suggest that the phototrophs became severely stressed as these changes occurred. A comparative analysis of these two parameters makes it apparent that the algal population excreted the greatest amount of metabolic intermediates when growth and production rates were highest. As cells became stressed, their metabolism underwent a conservative shift

Table 8. Percentage of total $^{14}CO_2$ photoassimilation excreted by algal cells in the Pine Creek mat during 1981.

Date	Total $^{14}CO_2$ assimilation by phototrophs (dpm)	% excreted as soluble ^{14}C compounds	Silica concentration in stream water (mg/l)
6/25	-	-	1.25
7/8	64142	85.3	0.95
7/21	98554	54.6	0.99
7/28	81712	69.7	1.44
8/11	81742	67.5	0.24
8/21	6248	10.3	0.09
9/2	0	-	0.15

that resulted in a majority of transformation products being incorporated into cellular material. This is in agreement with the results of pure culture studies by other investigators (24,68,75). The factors cited most often as influencing excretory rates in these and other studies (57,73) were light intensity and CO_2 availability. The possible loss of these two parameters from the mat as its thickness increased during the season has already been discussed.

Cell density is also mentioned as a factor governing extracellular release of organic products in both pure cultures and natural phytoplankton populations (3,24,75). There appears to be an inverse relationship between the two parameters, which is explained in terms of an equilibrium between intra- and extracellular concentrations of the compounds in question. The design of the carbon flow system diluted the mat community substantially, and may have been responsible for the relatively high excretion values observed for the phototrophs in Pine Creek. It must also be kept in mind that the populations studied here were exposed to a flowing environment. Diffusional interactions between the mat community and the overlying water (see page) may have rendered levels of extracellular organic products within the mat independent of cell concentration.

Bacterial activity by studies of heterotrophic potential.

Results of studies in 1980 illustrate the seasonal trends of bacterial catabolic capability in the Pine Creek mat community. All

aspects of heterotrophic activity increased dramatically with seasonal progression. Vmax, indicative of the maximal uptake rate of a given substrate by the population, increased to values in September and October which were up to three orders of magnitude higher than the lowest levels seen in August. This is in contrast to results obtained by Ladd, et al. (43) in their studies on a sub-alpine stream system in Alberta. Although they too note seasonal patterns with respect to heterotrophic activity, the changes they observed were considerably less than those seen in the present study. This can be best explained by the fact that their study was conducted in a stream that experienced much higher allochthonous carbon loading. As a result, the adherent bacterial population was probably exposed to elevated levels or organic nutrients from the stream water, making their immediate microenvironment less variable with respect to seasonal nutrient patterns.

The low rates of glutamate uptake by the epilithic bacteria of Pine Creek were characteristic of bacterial populations that are largely in a low metabolic state and perhaps dormant. This phenomenon has been postulated as a means for bacteria to withstand various forms of environmental stress, including starvation (64). The cold, nutrient deficient waters of Pine Creek may not have been able to support the maintenance of the sessile bacterial population at certain times of the year, in which case dormancy would provide a mechanism

for survival until conditions became more favorable for growth.

In the past, Vmax has been considered useful for estimation of biomass (80), a concept that still is considered largely valid for laboratory cultures. Our findings agree with later reports by Wright and Burnison (79) which indicate that in a natural environment Vmax is not a proper estimator of biomass. Results from this work and those published by Wright (77) reveal that maintenance energies of aquatic bacteria are relatively high, with more than 65% of added labelled substrate being mineralized to $^{14}CO_2$. As a result, the activity of bacterial populations can vary without a concomitant change in their numerical magnitude.

Limitations of 1980 data.

The data obtained from studies performed in 1980 were helpful in determining population patterns and trends of metabolic activity among the microorganisms existing within the sessile community of Pine Creek. However, they were limited in the sense that they provided little insight into the ecological interactions occurring among the microorganisms which led to the formation of an epilithic mat community and determined its seasonal evolution. First, the measurements performed on each population were independent of the other, which had the effect of negating any interactive processes that may have occurred as a result of the close physical associations between the algae and bacteria. The labelled substrate used

to determine heterotrophic activity was not necessarily representative of the substrates normally present in the mat, and may have given misleading results with respect to bacterial degradative activities. Finally, all activity experiments of 1980 were performed in the laboratory under artificial lighting conditions that were considerably different from those at the sampling location. All of these factors had the effect of creating an assay environment that was radically artificial relative to natural conditions, and raised the possibility that the results were not entirely representative of the processes that actually took place among the microorganisms in the sessile community of Pine Creek.

A more detailed analysis of the ongoing ecological processes in Pine Creek was obtained with the carbon flux experiments during the 1981 season. The addition of labelled carbon in an inorganic form to the mat community circumvented any interruptions of carbon flow between the various trophic levels. In situ incubations under natural lighting conditions resulted in a system that more accurately defined phototrophic production parameters. Moreover, the labelled nutrients seen by the bacterial population were the substrates normally present in their environment, and were present at concentrations that closely approximated natural conditions. Finally, isolation of the phototrophic and heterotrophic microorganisms by size-differential filtration, as well as retention of the resulting filtrate, made

it possible to partition the labelled carbon into pools for a more accurate description of carbon flow within the mat.

The method also has limitations, foremost of which is the inability to detect $^{14}CO_2$ produced by bacterial mineralization. This leaves the assay of degradative activity dependent on incorporation values. Because the studies involve the use of isotopes, the samples must be removed from rock surfaces and incubated in closed containers which are different than the flowing environment typically seen by the microorganisms. Incubation times and volumes of the system were designed to minimize any changes brought about by microbial activities during the assay period, but the integrity of the mat community structure was altered nonetheless.

The separation of algal and bacterial populations employing filters of various pore sizes is admittedly an imperfect one, due to 1) size overlaps of the two populations of interest, 2) the inability to completely separate algal and bacterial cells that were closely enjoined within the slime matrix of the mat, and 3) breakage of algal filaments into fragments small enough to pass through the larger pore size filter. However, the data obtained in these experiments and results of other researchers (7,19) suggest that the errors can be adequately compensated for, and that the corrected values are an accurate estimate of absolute levels.

In spite of the limitations discussed here, the carbon flux

system provided a means by which natural communities could be observed with a minimum of disruption, and allowed for a more detailed and precise analysis of the ecological processes occurring between phototrophic and heterotrophic populations.

Bacterial uptake of phototrophic metabolites.

Early investigations of aquatic environments demonstrated the fact that bacterial cells are often closely associated with or attached to phytoplankton (59). This apparently symbiotic relationship is even more pronounced in an algal-bacterial mat community, and several studies have been aimed at determining whether algal associated bacteria are able to utilize the products that phototrophs have been shown to excrete. One report demonstrated that bacteria cocultured with algae possess higher growth rates than cultures without algae (46). This and other studies also showed that non-axenic algal cultures had lower concentrations of organic compounds in the growth medium than their axenic counterparts (46,55). Later it was shown that bacteria incorporated radioactively labelled compounds from an algal culture incubated under $^{14}CO_2$ (3,54).

The results of algal population and bacterial activity measurements for the Pine Creek mat in 1980 are summarized in Figure 14, and suggest that the heterotrophic bacterial population responded to a decline in the phototrophic community with higher catabolic rates.

Two mechanisms are considered here to explain this trend. 1. A change in algal excretion of photoassimilated compounds. 2. An increase in algal lysis products.

The catabolic activities of the bacterial population may have increased in response to quantitative or compositional changes in the soluble organic compounds excreted by the phototrophs as their activity was waning (23). However, an examination of Table 5 (Columns 2 and 4) reveals that bacterial consumption rates were highest when primary production was at a seasonal minimum. The values would indicate that bacteria were substrate limited at this time of year with respect to algal extracellular products, a situation which may have induced a high-affinity transport system capable of scavenging these materials at low concentrations. However, it is doubtful that the induction of such a system would have a demonstrable effect on Vmax rates; this change would more likely be reflected in a decrease of the transport constant K_t.

Based on the preceding arguments, it is more probable that bacterial catabolic activity was triggered in response to an accumulation of algal lysis products in the mat. As the phototrophic population declined in August of 1980, one would expect to see a release of lysis products from the cells which could be utilized by the heterotrophic bacteria. Geldreich, et al. (28) noted sharp increases in standard plate count densities of a man-made reservoir

after copper sulfate treatments were used to attenuate a severe algal bloom. These lysis products could have been easily trapped within the slime matrix of the mat community, creating an environment extremely rich in bacterial nutrients.

The carbon flux experiments performed in 1981 were the first to directly demonstrate bacterial utilization of extracellular algal products in a sessile community without previous separation of the two major components of interest. During light incubation of the experimental systems there was a concomitant rise in both primary production metabolites and bacterial incorporation of ^{14}C-compounds. In the dark, radioactivity in the bacterial fraction increased with time as filtrate radioactivity declined (Figure 16). The seasonal patterns observed for these experiments were noticeably different from those obtained by heterotrophic potential methods. Over the course of the 1981 season, the activity of the heterotrophic bacteria was greatest when primary production rates were at their highest points of the season (Figure 15). The reductions in activity in the two populations also appeared to coincide, at least up to the point where algal products became limiting to the bacteria (August 21). 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, and others (9,39,69) have shown that bacteria respond positively to the release of extra-

cellular metabolites by the phototrophs.

These results support the theory that bacteria in close association with algae act as carbon sinks for the extracellular intermediates of phototrophic production (3), and respond positively to the release of these compounds with higher metabolic activity. The patterns observed also serve to illustrate the dependence of the sessile bacteria on these excreted compounds for their survival in the nutrient deficient waters of Pine Creek.

Influence of heterotrophic substrate on results.

The epilithic bacteria of Pine Creek exhibited very different patterns of heterotrophic activity in 1981 as opposed to the results obtained by heterotrophic potential methods. In 1980 uptake rates of glutamate peaked in September long after the seasonal maximum for primary production had occurred. Using the carbon flux system, maximum bacterial utilization rates of algal extracellular products were highest when photoassimilation rates of CO_2 by the algal population reached a maximum for the year.

The marked differences in the results obtained over the two seasons raises important questions concerning the validity of data obtained when using substrates not entirely representative of the composition and concentrations produced by the natural phototrophic population to measure bacterial metabolic activity. Several substrates have been used in the past for measurements of heterotrophic

activity. Among these, glucose (13,30,33,77,80), acetate (56,80), and glutamate (43,79) have been the most commonly employed. Unfortunately, many of these studies were either performed independently of each other or were not designed to compare results obtained with two or more substrates. Wright and Hobbie (80) found that glucose transport constants were three times higher than those for acetate, with the disparity observed attributed to the complexation of acetate in the pH range of their system. Bacterial uptake kinetics for both glucose and alanine in a benthic environment were obtained by Chocair and Albright (13), but these values were not found to be significantly different.

All of the studies just mentioned were performed in either lake or stream environments where bacteria are likely to be associated with phytoplankton or sessile (attached) algal cells. Since it appears that these bacterial populations possess metabolic systems with a high degree of specificity for the extracellular products of the phototrophic cells (3, see page 95) it would seem that studies of bacterial metabolic activity using substrates representative of these extracellular metabolites would yield the most accurate data.

The most abundant extracellular algal product appears to be glycollate (68,73,74); other compounds are excreted in small amounts or not at all, depending on the species being examined (75).

To test whether the glutamate kinetics of 1980 were an accurate

reflection of the metabolic status of the sessile bacteria, the experiments were repeated in 1981 using both ^{14}C-glutamate and ^{14}C-glycollate as the test substrates. Vmax values for glycollate on that day (August 21) were ten times higher than those for glutamate (Table 8), with appropriately lower turnover times.

These results, together with the patterns observed for bacterial utilization of in situ algal extracellular products, cast some doubt on the ability of kinetic studies using substrates not typical of those found in natural systems to accurately assess the metabolic activities of the bacterial populations. As mentioned previously, glycollate appears to be the compound most representative of soluble algal products in aquatic systems, and should logically be the substrate of choice in many environments. But to ensure that an investigation produces data that accurately reflects the actual processes occuring in situ, a more careful analysis of the soluble organic regime is required.

Nutrient flux within the mat.

1. Direct flux from phototrophs to bacteria.

Based on population and heterotrophic activity measurements of both sessile and planktonic bacterial populations, it has been suggested that the former group plays a much more active role in stream purification processes by removal of organics from the overlying water column (27,33,43). This concept has been successfully

employed for several years for the treatment of wastewater using trickle filters, rotating biological contactors, and the like. But the Pine Creek environment is unique in that there is little organic matter (<4 mg/l) in the stream water. This 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 bacteria within the mat community. This appeared to be the case when the heterotrophic potential data of 1980 was analyzed. The mat bacteria did not utilize glutamate added to the system at any appreciable rates until primary production had virtually ceased (Figure 14). Two lines of evidence obtained in 1981 were used to confirm this assumption

a. SEM observations.

During visual examinations of the mat community using SEM, bacterial cells were primarily observed either on the surface of phototrophic cells or enclosed within the slime matrix. Given these two physical orientations, algal excretory products would appear to be a major nutrient source for the bacteria, either through direct flux from cell to cell, or by an accumulation within the polymeric slime matrix.

b. Carbon flux experiments.

Bacterial uptake of soluble algal excretory products occurred during both the light and dark incubation periods. The

uptake rates were extremely high for the first three hours incubation, during which time light-driven photosynthesis was occurring. When light was removed from the system, primary production was virtually halted. Bacterial uptake of algal products still occurred, but at much reduced rates relative to the first three hours in which light was entering. The time-course results of these experiments are illustrated in Figure 16. During light incubation (0-3 hrs.) the phototrophs appeared to be actively excreting organic products, and bacterial uptake rates of these compounds were relatively fast. As the experimental samples were switched to dark conditions, this release of organic material 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, as evidenced by the decline in radioactivity of the filtrate during dark incubations. 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 were not only dependent on algal production for their nutrition, but preferentially used algal products directly as they were excreted from the cells. The efficiency with which this removal occurred suggests that the bacteria possessed an extremely high affinity for algal extracellular products, a phenomenon that has been alluded to previously (55,80).

2. Flux of organics from the stream water.

Ladd, et al. (43) have suggested that the slime matrix represents a diffusional barrier that impedes transport of nutrients from the overlying bulk fluid to the sessile organisms. Unfortunately, the flow regime of the stream channel, crucial in calculations of transport rates of organic compounds to benthic surfaces, were not adequately characterized in this study. Thus it was impossible to ascertain the contributions, if any, of the soluble organics of the stream water to the total substrate pool of the sessile bacteria. But whereas the concentration of organic material in Pine Creek was extremely low (<4 mg/l TOC) even before considerations of diffusional barriers, it is concluded that organics from the stream water were an insignificant nutrient source for the sessile bacteria. Indeed, under these circumstances it is possible that there was a diffusional transport of soluble compounds out of the mat into the overlying stream water, a process that would contradict the accepted models of biofilm communities in flowing environments.

It should be stressed that the low levels of organic material in Pine Creek make it and the processes occurring within somewhat unique. But despite this, the data obtained from investigations of Pine Creek can easily be applied to more complex environments. The discussions of the last two sections have led to the conclusions that sessile bacteria exhibit a high specificity for algal extra-

cellular products and are in a position to use them directly as they are released. This implies that whenever phototrophs are an integral part of a sessile community, the efficienty of removal of organic material from aquatic systems by bacteria will necessarily be reduced as these bacteria preferentially utilize the soluble metabolites of primary production.

Nutrient limitations of heterotrophs.

It was mentioned in a previous section that competition for available substrate by the sessile bacteria may have been a crucial factor in limiting their population. Such a conclusion is warranted in light of the fact that total organic carbon levels of the stream water never exceeded 4 mg/l.

The carbon flux experiments of 1981 did make it possible to measure the algal contribution to this substrate pool. It was assumed that radioactivity in the filtrate fluid resulted from the excretion of soluble organic compounds by phototrophs during primary production. Despite the observation that bacteria were utilizing these compounds during the incubation (as evidenced by radioactivity on the 0.2 µm filters), production was far greater than consumption for a better part of the sampling season, as indicated by an excess of activity in the filtrate fraction. In the latter part of August, primary production rates decreased significantly, and the bacterial population removed all extracellular products from the system almost

immediately. The low levels of activity in the bacterial fraction, relative to previous points in the season, suggest that the contributions of soluble algal excretory products to the substrate pool of the mat environment was far less than the potential for removal by the bacterial population. The patterns observed in 1980 were essentially the same; Vmax rates in September were much higher than primary production rates by the phototrophs (Table 5). If algal extracellular products were a primary source of nutrients for the bacteria (see page 96), their loss would indicate that the bacteria were indeed substrate limited at this point in the season.

Disappearance of the mat community

Shortly after the decline in phototrophic activity was detected in late August, a large portion of the mat was uniformly removed from rocks lying on the streambed, leaving a tenacious community observed by SEM to be at the most only two cell layers thick. Evidence obtained by Jorgenson, et al. (41) and Revsbech, et al. (61) suggests that changes in oxygen levels within the mat may have been responsible for the abrupt disappearance of the mat. Using microprobe techniques, these researchers were able to demonstrate that biofilm communities which contain a phototrophic zone are saturated with oxygen in that zone when illuminated. Upon removal, oxygen rapidly disappears in the deeper layers, the rate of depletion being dependent on diffusional transport into the mat and the heterotrophic

activities of the sessile bacteria.

In the Pine Creek mat, the drop in primary production after mid-August represented a major loss of oxygen to the sessile bacteria, whose catabolic activity (as indicated by heterotrophic potential data) had not diminished. Due to the thickness of the mat at this time (ca. 2mm), diffusional transport from the stream water was probably unable to compensate for the depletion of oxygen under these circumstances (12,63). The resulting anaerobiosis may have resulted in the formation of bacterial metabolic products that weakened the overall mat structure (34) and facilitated its removal from the substratum by the shear forces of the stream water.

SUMMARY

The immobilization of microorganisms on rock surfaces represents a mechanism which enables them to survive and proliferate within the extremely oligotrophic waters of Pine Creek.

The mat community is a very dynamic system, experiencing a striking evolution as both phototrophs and heterotrophs respond to rapidly changing environmental pressures. The phototrophs appear to be healthy and actively growing as the water temperature of the stream increases through July, with high rates of primary production per unit biomass and excretion of a large percentage of soluble organic photoassimilation products. These activities peak briefly in August before dropping rapidly, indicating a stressed population at this point in the season.

The heterotrophic bacteria appear to be dependent on the phototrophs both as an attachment substrate and as a source of organic nutrients. Bacterial cells are characteristically observed either directly attached to algal cells or within the dense polymeric slime matrix of the mat. The utilization rates of algal extracellular products by bacteria are greatest at a point in the season when primary production and excretion rates are also at a maximum and the declines of these activities in August are concomitant.

There is also an apparent preference for these excreted algal products by the heterotrophic bacteria in the Pine Creek mat. Up-

take rates for glutamate in late August are significantly lower than those for glycollate, an abundant component of algal release material. Further, during the period in which the phototrophs are actively metabolizing, there is little uptake of any glutamate added to the experimental system. But when the decline of algal production in August results in a scarcity of excreted compounds, bacterial uptake of glutamate increases dramatically.

Short time-course sutdies of carbon flow experiments revealed a direct flux of nutrients from the phototrophs to the heterotrophs. Bacterial incorporation rates of soluble extracellular products are highest in the light when rates of release from algal cells are also at a maximum.

The decline of activity in the phototrophic population appears to be triggered by a sudden drop in the silica concentrations in the stream water in August, combined with the possible loss of light and CO_2 from the deeper layers of the mat. At this point the bacteria are substrate-limited with respect to excreted organic products of the algae, and probably turn to algal lysis products for their maintenance and growth. But with the loss of algal oxygen production from within the mat, the heterotrophic activities of the bacteria may create anaerobic conditions, resulting in the formation of products that weaken the overall structure of the mat and facilitate its removal from the substratum by the stream water.

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