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Growth of Heterotrophic Bacteria and Algal Extracellular Products in Oligotrophic Waters

GORDON A. McFETERS,* SIDNEY A. STUART, AND SUSAN B. OLSON
Department of Microbiology, Montana State University, Bozeman, Montana 59715

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The unexpected observation of 200 to 400 coliform bacteria per 100 ml in an unpolluted pristine stream was studied within Grand Teton National Park, Wyo. The high numbers of waterborne bacteria occurred in mid- to late summer at a location where there was a coincidental bloom of an algal mat community. Periphyton samplers were used to measure the algal growth that coincided with the increase in number of bacteria. Laboratory studies followed the growth of various coliform bacteria in the supernatant obtained from a *Chlorella* culture isolated from the mat community. Mixed natural bacterial populations from the stream and pure cultures of water-isolated fecal and nonfecal coliforms increased by two to three orders of magnitude at 13°C when grown in the algal supernatant. Radioactive algal products were obtained by feeding an axenic *Chlorella* culture ^{14}C-labeled bicarbonate under laboratory cultivation at 13°C with illumination. Radioactive organic material from the algae became incorporated into the particulate fraction of pure cultures of coliform bacteria as they reproduced and was later released as they died.

The occurrence of certain indicator bacteria in water has been extensively used to detect water contamination of public health significance. Most notable among the microorganisms used in this manner is the group of enteric bacteria known as coliforms (1). Although these microorganisms have served us well as indicators of potential health hazards, this practice is not perfect. For example, it is known that indicator bacteria in water are subject to starvation and die-off phenomena that correlate reasonably well with the behavior of some common enteric pathogenic bacteria (20). As a result, the indicator bacteria may be considered as valid signals of hazard from pathogenic bacteria under many conditions, but, unfortunately, the same cannot always be said for the public health threats from water-borne viruses and toxic chemicals. Additionally, the source of indicator microorganisms may not be exclusively the gut of warm-blooded animals (12, 13).

Symbiotic communities composed of algae and bacteria have been described (2, 4), and it is thought by some authors (2, 3, 8, 9, 11, 14, 23, 38, 40) that such circumstances may represent an ecologically important source of organic nutrients for the growth of heterotrophic bacteria in natural aquatic ecosystems. However, no natural communities of that kind have ever been reported involving sanitary indicator bacteria, despite the observation that various bacteria are usually found in mass cultures of *Chlorella* and some are thought to grow using algal excretions (19, 23, 33). The metabolism and growth of enteric bacteria in pristine streams has, however, been suggested (16, 17, 22).

The present study was initiated to investigate the unexpected occurrence of high numbers of coliform bacteria in a pristine alpine stream within Grant Teton National Park, Wyo. The indicator bacteria were found in the water at times and locations that coincided with algal blooms, consisting of *Chlorella* and other species. Field and laboratory experiments were done to examine the premise that extracellular products excreted by an alga from the natural community (*Chlorella*) could support the growth of indicator bacteria. The implications of these findings are discussed from the standpoint of the basic ecological relationships of algal mats serving as sources of organic nutrients for the growth of "natural" coliform bacteria.

MATERIALS AND METHODS

Location of study sites. Field studies were carried out within Grand Teton National Park, Wyo. Sample sites were located along the stream that served as the outlet for Surprise Lake at 2,916 m (9,560 feet) above mean sea level. This alpine stream flowed a vertical distance of 234 m (767 feet) along a south-facing slope 0.5 mile (ca. 0.8 km) before entering Garnet Creek at 2,682 m (8,793 feet) above mean sea level. This small stream formed a thin film, 1 to 3 cm, in many places as it flowed over boulders in the stream bed. Although the watercourse was located slightly below timberline,

only a few trees were present along with other alpine vegetation. No formal trails were located in the vicinity of the upper 600 m of the watercourse. However, a main trail in Garnet Canyon intersected the lower end of the stream. The fauna was limited to marmots and picas.

Bacteriology. Water samples were collected at locations in the Surprise Lake outlet stream at regular intervals as a part of a surveillance program within Grand Teton National Park (30). The iced samples were transported by foot and then by car (in less than 4 h) to the field laboratory at Moran, Wyo. Tests were conducted for total coliform bacteria by membrane filtration according to standard methods (1). Representative colonies were picked from the coliform plates, put on enrichment media, and transported to Montana State University Water Microbiology Laboratory where they were further characterized.

Physical and chemical water analysis. Temperature, pH, and conductivity were taken at all sites to provide a general physical and chemical evaluation of the waters under investigation. Weather conditions were also noted before, during, and after sampling periods to correlate bacteriological results with rainfall and runoff.

Algal sampling in the field. The amount of chlorophyll *a* accrual within the stream was determined and correlated with the number of water-borne bacteria. The apparatus used to collect periphyton was an adaptation of an artificial substrate sampler used by Bahls (Ph.D. thesis, Montana State University, Bozeman, 1971). A single acrylic plastic plate with a collecting surface area of 100 cm^2 on each side was bolted to a 1-m concrete reinforcing rod that was secured by rocks so that it was positioned lengthwise across the stream. All plates were positioned uniformly so that the top surface of each plate was about 1 to 3 cm below the water surface and parallel to the direction of the current flow. Six plates were positioned in the Surprise Lake outlet stream. The first plate was analyzed after 2 weeks, and one plate was analyzed each following week for the next 5 weeks. Razor blades were used in the field to scrape the periphyton samples into wide-mouth sample bottles (250 ml) containing 200 ml of sterile water. The samples were shaken, iced, and transported to the laboratory where a 10-ml portion was taken from each bottle and tested for coliform bacteria. The remaining sample was filtered onto a membrane filter (Millipore HAWP04700, Millipore Corp., Bedford, Mass.), put into a dessicator that was sealed from light, and held at 4°C for phytopigment analysis by established methods (1). The amount of pigment per unit surface area of sample was calculated as follows: milligrams of chlorophyll/square meter = milligrams of chlorophyll/1 × volume of extract (liters)/area of substrate (square meter).

Algal growth in the laboratory. Samples of algae were collected in the field by scraping the periphyton samplers or rocks from the stream. These algal samples were placed in Gorham medium (18) and grown for several weeks under constant illumination with fluorescent lights (5,380 lux). The cultures were then streaked onto a solid Gorham medium containing 30 μg of tetracycline per ml to prevent bacterial growth and incubated for several more weeks in sealed plates under constant illumination at 10°C. Algal colonies were then picked from the plates into liquid Gorham medium and cultivated as before, and the growth was followed at regular intervals using a fluorometer (G. K. Turner Associates, Palo Alto, Calif.). Algal cultures were checked at regular intervals for bacterial contamination. Broth containing algae was diluted with sterile phosphate buffer (1) and 1-ml portions of undiluted algal cultures were added to 1, 0.5, and 0.1× Trypticase soy broth supplemented with 0.3% yeast extract and 0.5% glucose (TGE broth). These tubes were incubated at 10, 20, and 35°C and observed for bacterial growth after 1, 2, 7, and 14 days.

Algal products and bacterial growth. Batch cultures of an axenic alga isolated from the field, tentatively identified as *Chlorella*, were grown in 4 liters of Gorham medium in a fermenter with aeration, agitation, and constant illumination (ca. 500 lux) at 12°C. After a stationary population was achieved, usually within 10 to 14 days, the supernatant was separated from the algae by centrifugation followed by filter sterilization with 0.45-μm membrane filters. The sterile supernatant was stored at 4°C. Radioactive supernatant was obtained by growing *Chlorella* in Gorham liquid medium enriched with $NaH^{14}CO_3$. Batch cultures containing 1.5 liter of Gorham liquid medium were grown as described earlier for 10 to 12 days. At that time, 0.25 mCi (9.3 mCi per mmol) of $NaH^{14}CO_3$ (New England Nuclear Corp., Boston, Mass.) was dissolved in 5 ml of sterile phosphate buffer and added to the batch culture. The radioactive supernatant was harvested as described above. This supernatant was acidified with 1 N HCl to pH 3 and vigorously aerated for 10 min to purge unincorporated inorganic ^{14}C. This method has been shown effective for the purpose described while not affecting the concentration of volatile organic compounds (2). Sodium hydroxide (1 N) was added to readjust the pH to 8.0. This radioactive algal supernatant was filter sterilized and stored as described above. Both radioactive and unlabeled algal supernatant solutions were used as potential media for bacterial growth. Pure cultures of bacterial isolates from algal mats in the outlet of Surprise Lake and other waters were grown in TGE broth for 24 h. These cells were harvested by centrifugation (3,020 × *g*) for 10 min and washed twice with sterile buffer solution (1). After the final wash, the bacteria were suspended in sterile buffer and diluted to the desired population density. These bacteria were used as inocula for the growth studies in algal supernatant at final concentrations of less than 10^4 viable organisms per ml. The mixtures of bacteria plus sterile algal supernatant and diluent, as controls, were placed in sterile 500-ml flasks and held at 13°C. Portions were removed at timed intervals, and the bacteria enumerated by the pour and streak-plate methods using TGE agar were incubated for 24 h at 35°C.

In the studies where radioactive algal supernatants were used as potential bacterial growth media, the details of preparation and inoculation were done as previously described. At timed intervals, portions were removed for bacterial enumeration as described above and for determination of bacterial radioactivity. To

this end, 10-ml portions were removed and filtered using 25-mm diameter, 0.45-μm mean pore diameter membrane filters (Millipore Corp.). The filters were oven-dried at 105°C, treated with 4 ml of toluene, and added to 10 ml of Aquasol cocktail (New England Nuclear) in a scintillation vial. Radioactivity was determined using a Beckman LS-100C liquid scintillation system (Beckman Instruments, Fullerton, Calif.). Liquid samples such as the filtrate were counted by adding 1.0 ml or less of the filtrate to the scintillation cocktail as described previously. The adsorption of dissolved radioactive material, as suggested by Nalewajko and Lean (25), was checked by filtering 10 ml of the sterile radioactive supernatant and coating as described above. The values obtained were subtracted from the experimental values.

Chemical determinations on supernatants. The method of Calkins (5) was used to determine the glycolic acid concentration in the algal supernatant solutions. Standard curves of concentrations between 3 and 30 mg per liter indicated that good accuracy was attainable at these levels. A Beckman model 915 carbonaceous analyzer (Beckman Instruments) was used to determine the level of dissolved organic carbon in the filter-sterilized algal supernatants.

RESULTS

Populations of indicator bacteria were monitored in several alpine streams within Grand Teton National Park, Wyo., during the summer months of 1973 through 1975, as a part of a surveillance program conducted for the National Park Service. These studies (30) revealed low numbers of the classical sanitary indicator bacteria in most of the waters under investigation. However, a small stream that served as the outflow of Surprise Lake consistently contained total coliform counts of greater than 200 per 100 ml in midsummer. Coliform bacterial counts at the lower end of the Surprise Lake outlet stream (sample site G-5) were significantly greater than those observed in other streams within that area of the park (Fig. 1).

Samples were collected and analyzed for total coliform bacteria at six sites within the outlet stream of Surprise Lake to establish the source of the high bacterial numbers found at site G-5. The coliform counts were consistently low at the point where Surprise Lake emptied into the outlet stream (site 1) and increased as the stream progressed down the steep narrow canyon (Fig. 2). The coliform bacterial populations of other sample sites further upstream in the outlet of Surprise Lake were even greater (Fig. 2) because the lowest site (G-5) dried up each year before the occurrence of the maximal number of bacteria.

Macroscopic observations of this stream failed to reveal any obvious source of elevated bacterial counts. However, the emergence of a benthic

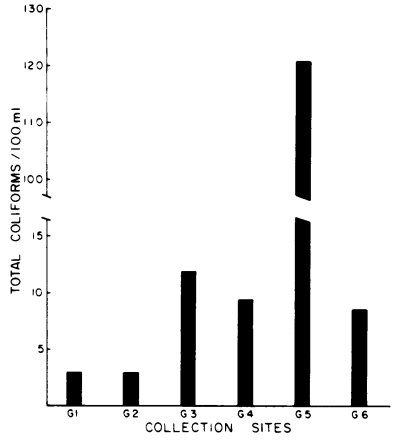

FIG. 1. *Bacteriological profile of Garnet Canyon showing geometric means of total coliform populations. The data were collected during the summers of 1973, 1974, and 1975. Sample site G-5 was located on the lower regions of the stream that served as the outlet of Surprise Lake. Sample sites G-1 through G-4 and G-6 were located along the main stream within Garnet Canyon.*

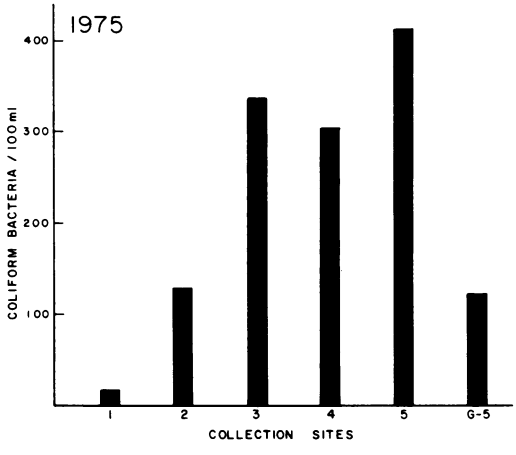

FIG. 2. *Geometric mean of coliform bacterial populations at six sites in the Surprise Lake outlet stream between the outlet of the lake and site G-5. The data were collected during the summer of 1975.*

algal community was noted on the rocks within the stream coincident with the high number of coliforms that typically occurred in late July through August. The benthos of the Surprise Lake outlet stream appeared as a thin, slippery, golden-green film that covered the rocks that were under 1 to 3 cm of water. Algae belonging to the genera *Gleocapsa, Stigonema,* and *Chlorella* were tentatively identified from representative samples scraped from rocks and colonized microscope slides. The time and extent of algal

growth, as chlorophyll a accrual, was compared with coliform increases observed in the stream water. The data obtained during 1975 are seen in Fig. 3. Since the algal community was measured as chlorophyll accrual at the end of 2-week growth intervals, each data point should be viewed as an average for that 2 weeks and can, therefore, be considered as being approximately 1 week late on the graph. With that in mind, the increase in water-borne coliform bacteria occurred at the same time as the algal community became established and began to proliferate. The lower sample sites (5 and G-5) dried up in early August precluding further bacterial sampling at those sites. This problem became even more severe in the summer of 1976 due to abnormally dry conditions when no contiguous data comparable to that found in Fig. 3 were obtained. The fragmentary evidence that was obtained in 1976 was consistent with that of 1975.

The algal samples that were scraped from the colonized Plexiglas plates for chlorophyll measurement were also tested for coliform bacteria. The mean value of data collected in 1975 was 8.4×10^4 coliform bacteria per m^2 (maximal value was 2×10^6 per m^2) of the algal communities that were at least 2 weeks old at the time of harvest.

In 1975 the water temperature of the Surprise Lake outlet stream was 0°C at the start of the summer, increased gradually to 10°C by July 22, and peaked at 13°C in the last week of August. The temperature then decreased until it was 7°C by September 2. This stream was also exposed to bright sunlight during the summer months since it was not shaded by trees and was on the steep south-facing slope. Specific conductance of the stream water was less than 10 μmho, and the pH values were 6.5 to 7.1.

Laboratory studies were performed with *Chlorella* to examine the growth dynamics of selected bacteria in the presence of algal extracellular products. These experiments were conducted using *Chlorella* that were obtained from the natural algal mats and subsequently treated with antibiotics to eliminate associated bacteria. The resultant axenic algal culture could then be used to produce supernatants without interference from bacterial contaminants. Bacteria were inoculated into filter-sterilized algal supernatants (Table 1). In the initial experiment, an inoculum of a mixed, natural bacterial population obtained by membrane filtration from the Surprise Lake outlet stream was added to algal supernatant and incubated for 5 days at 4 and 35°C. A 100-fold increase in the viable population was observed. This experiment was repeated using pure cultures of coliform isolates from the Surprise Lake outlet stream and the East Gallatin River, Mont. An incubation temperature of 13°C was used throughout the remaining experiments since that was the temperature observed in the field at the peak of the algal and bacterial blooms. In these experiments the bacterial growth response was between two and three orders of mganitude (Table 1, Fig. 4). No effort was made to correlate dissolved organic carbon concentrations and growth rate response of the bacteria in these experiments. Control suspensions of bacteria in diluent alone demonstrated die-off within 4 days.

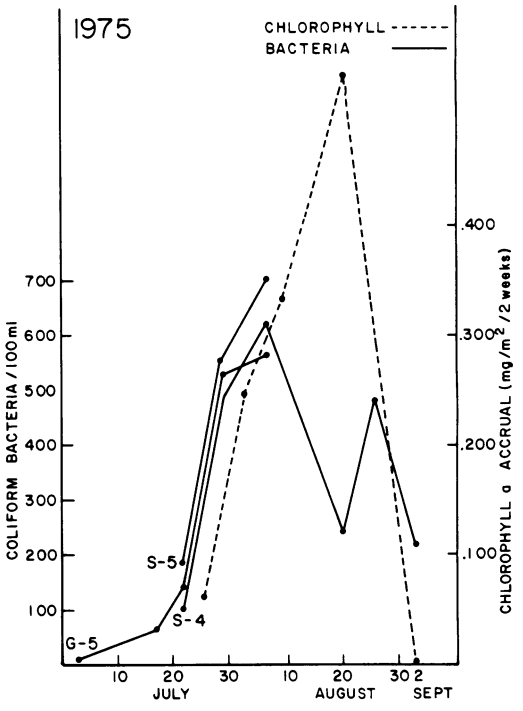

FIG. 3. *Chlorophyll a accrual and total coliform bacterial populations for three sites in Surprise Lake outlet with dates of sample collection during the summer of 1975.*

Further studies were done on the growth response of selected indicator bacteria in radioactive (^{14}C) algal supernatant. A fecal coliform (*Escherichia coli*) and a bacterium belonging to the genus *Klebsiella* were used in this experiment and yielded results that were virtually identical (Fig. 5). The radioactivity was incorporated into the particulate fraction with concomitant removal from the medium as the bacteria were actively growing. However, as the bacteria left stationary phase and their death became exponential, the process was reversed

TABLE 1. *Growth of coliform bacteria in filter-sterilized extracellular products of Chlorella*

Bacterium	Initial population (no./ml)	Final population (no./ml)	Days of incubation	DOC[a] (mg/liter)	Temp (°C)
E. coli	9.6×10^3	1.6×10^5	15	19.5	13
Enterobacter	1.0×10^4	2.6×10^5	15	19.5	13
Enterobacter	1.1×10^3	2.6×10^5	15	19.5	13
Enterobacter	9.1×10^3	1.0×10^5	15	19.5	13
E. coli	9.2×10^3	2.3×10^4	8	19.5	13
Enterobacter	8.5×10^3	2.3×10^4	8	19.5	13
Klebsiella	1.9×10^3	1.4×10^5	8	43.5	13
E. coli	1.3×10^3	1.09×10^5	8	43.5	13
E. coli[b]	2.4×10^3	1.09×10^5	8	43.5	13
Salmonella	4×10^1	9×10^1	23	33.6	13
Klebsiella	1.0×10^5	3.0×10^5	10	43.5	13
Enterobacter	1.2×10^5	2.8×10^5	10	43.5	13
E. coli[b]	6.2×10^4	8.0×10^6	4	43.5	13
SLO[c] mixed culture	1.1×10^4	1.0×10^6	5	NA[d]	4
SLO mixed culture	1.1×10^4	1.4×10^6	5	NA	35

[a] DOC, Dissolved organic carbon concentration of each algal supernatant that was used. The glycolic acid concentration of these solutions was between 2.0 and 4.3 mg/liter.

[b] Fecal coliform.

[c] SLO, Surprise Lake outlet.

[d] NA, Not applicable.

with increased radioactivity within the medium accompanied by a decrease in the particulate fraction.

DISCUSSION

With the exception of the Surprise Lake outlet stream, the occurrence of coliform bacteria in streams within the Garnet Canyon region of Grand Teton National Park in 1973 through 1975 was minimal, but gradually increased as the streams merged and flowed toward the valley (Fig. 1). This pattern was noted in a previous report (30) in which it was concluded that human recreational activities such as hiking, backpacking, and mountain climbing had little effect on the levels of aquatic sanitary indicator bacteria observed. Further, it was regarded that the density and complexity of biological communities surrounding the streams were the factors primarily responsible for the populations of water-borne indicator bacteria. That conclusion is shared in another paper describing the effect of multiple use on water quality within a timbered mountainous watershed (29). Also, the number of coliform bacteria found in the streams and lakes of Grand Teton National Park were not excessive when compared with other reports dealing with alpine waters (28, 29, 34).

The observations of relatively high numbers of coliform bacteria in the Surprise Lake outlet stream (Fig. 1 to 3) was unexpected in view of the light recreational activity within this pristine watercourse. The data in Fig. 1 to 3 represent circumstantial evidence that the number of coliform bacteria in the outlet stream of Surprise Lake may have increased because of a symbiotic relationship with the algal mat community. Further experiments were, therefore, carried out to examine the premise that extracellular products excreted by an alga from that benthic community (*Chlorella*) could support the growth of indicator bacteria. This finding could serve as a possible explanation for the relatively high numbers of coliform bacteria in the outlet stream of Surprise Lake found during July and August.

There have been numerous statements in the literature that algae may provide an ecologically important source of organic molecules for the growth or maintenance of heterotrophic bacteria (2, 8, 9, 11, 23, 40). It has also been demonstrated that organic compounds excreted by algae serve as a source of bacterial nutrients (2, 3, 14, 38). We have shown that different bacteria, including a mixed natural population from the Surprise Lake outlet stream and pure cultures of coliform bacteria, are capable of significant growth in the presence of *Chlorella* extracellular products (Fig. 4 and 5, Table 1). Further, it was observed that bacteria took up approximately 10% of the radioactive algal excretions coincident with active reproduction (Fig. 5). This finding was also reported by Bauld and Brock in a thermal mat community (2). It was further noted that much of the radioactivity incorporated within the bacteria was lost as they entered the death phase of their growth cycle.

Hellebust (14) reported that some phytoplankton are capable of excreting up to 25% of their photo-assimilated carbon during their log growth phase. From the amount of chlorophyll

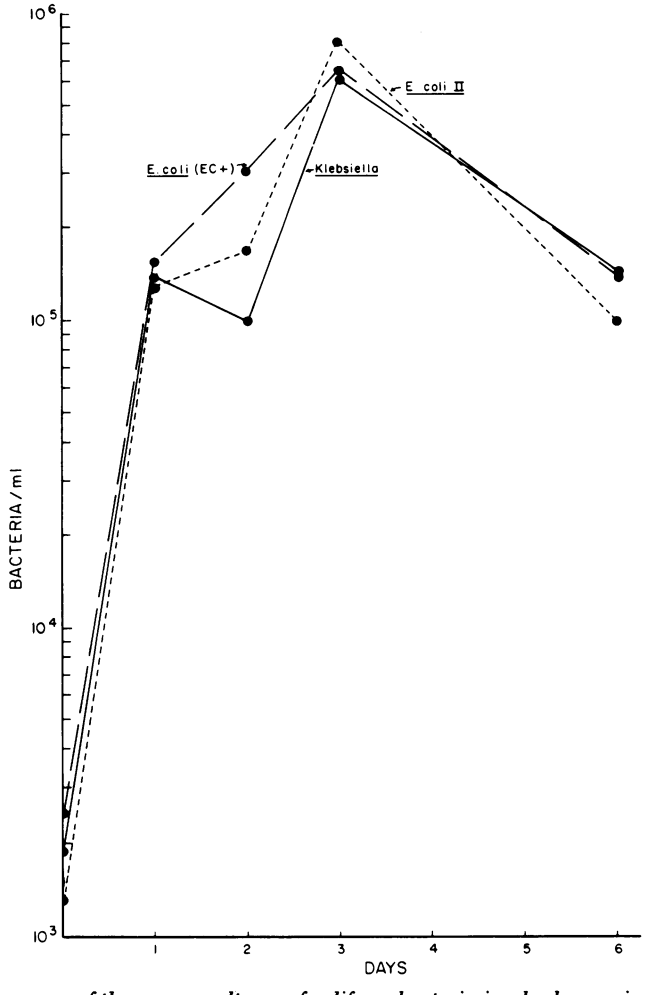

FIG. 4. *Growth response of three pure cultures of coliform bacteria in algal organic extracellular products at 13°C. The concentration of dissolved organic carbon was 43.5 mg/liter, and the concentration of glycolate was 2.3 mg/liter.*

a in Surprise Lake outlet (Fig. 3) and an assimilation number of 3.7 mg of carbon per mg of chlorophyll a per h as given by Ryther and Yentsch (27) and again by Wright (37), one should expect 1.98 mg of carbon being photoassimilated per m^2 of surface per h. Based on McGrew and Mallette's study (21), this level of organic solutes would be sufficient to maintain bacteria associated with the algae. On the other hand, the flow of the stream would most likely wash some of this excreted organic material downstream. The work of Hendricks and Morrison (16, 17) supports the plausability of coliform bacterial growth in pristine streams when they demonstrated that stream sediments may adsorb organic matter that is metabolized by those bacteria. In this way, the sediments may serve as a matrix on which dilute water-borne organic compounds from algal productivity and other sources are concentrated and made available for the metabolism and possible growth of associated heterotrophic bacteria. Other findings (22) also favor this model for bacterial reproduction in pristine waters. However, based on the high number of bacteria recovered from the algal community (as high as $2 \times 10^6/m^2$), we propose that bacteria were also closely associated with the algae under natural conditions; perhaps entrapped within the slime matrix of the photosynthetic cells. In that nutrient-rich microenvironment, the bacteria could have reproduced, and the organisms that were detected in the free-flowing stream water would have been fugitives of the mat community released by reproduction or other forces.

Under natural conditions, the interaction be-

tween algae and bacteria within mat communities is probably highly complex. Wright and Hobbie (39) suggested that the algae require one to three orders of magnitude greater concentration of organic compounds to metabolize heterotrophically than do bacteria. Therefore, symbiotic bacteria closely associated with the algae function as a "sink" for such solutes and prevent algal utilization of these solutes. As a consequence, such communities may reduce the level of dissolved organic compounds in natural waters (2, 23, 24, 26, 33, 39). However, there is evidence to suggest that such bacteria may be under nutrient limitation (24) and that glycolate is an important bacterial nutrient in algal excretions (15, 24, 31, 38). Also, there may be considerable variation in the bacterial populations within the natural, undisturbed mat community with time. The seemingly low coliform data point (Fig. 3) on August 20 might represent successional variation within the bacterial population accompanying the climax of the algal community. Further, from the demonstrated ability of some fecal coliform bacteria to grow, using algal secretions (Table 1, Fig. 4), the bacterial component of mat communities could contain fecal coliforms, and it is not inconceivable that the 200 fecal coliform per 100-ml limit for primary contact waters would be exceeded in such a situation (32).

The environmental factors responsible for the establishment of this and similar mat communities in nature are unknown. However, it was noted that algal colonization in the Surprise Lake outlet stream occurred when the water temperature rose above 10°C in midsummer and disappeared later when it fell to 7°C and below. It should also be pointed out that the Surprise Lake outlet stream was shallow and on a south-facing aspect and, therefore, received intense solar radiation during the time the mat community bloom was observed. Laboratory experiments have demonstrated the critical role of illumination in determining the quantity of or-

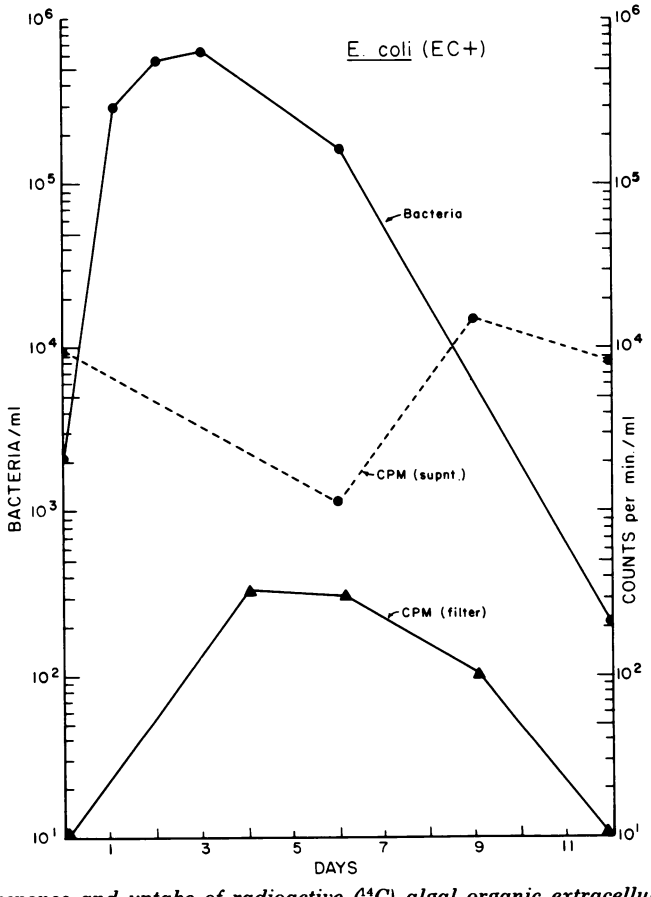

FIG. 5. *Growth response and uptake of radioactive (^{14}C) algal organic extracellular products by fecal coliform (E. coli) at 13°C. The CPM (filter) represents the amount of radioactivity retained on a 0.45-μm mean pore diameter membrane filter, and the CPM (supnt.) is the amount of activity passed through the filter.*

ganic products excreted by *Chlorella* (36). From these observations, once temperature and illumination cease to be limiting factors, algal growth occurs, the mat community becomes colonized and productive (4, 10, 36), giving rise to sufficient levels of excretory activity, and a symbiotic population of bacteria becomes established.

The relationship between *Chlorella* algae and various bacteria has been studied. Most authors agree that certain bacteria are capable of interacting with this alga in a mutualistic manner under conditions of mass culture (19, 23, 33), and some suggest that potential pathogens may be involved (35). Our findings suggest that some coliform bacteria may grow in a natural aquatic environment associated with algal mat communities. As a consequence, substantial numbers of coliform bacteria may be found in uncontaminated and pristine waters under certain environmental conditions exclusive of human presence or that of other warm-blooded animals. In such a circumstance, the classical interpretation of coliform data relative to sanitary significance could be in error. Furthermore, the origin in this manner of bacteria that are potentially pathogenic, such as enteropathogenic *E. coli* and *Klebsiella* species, should be considered a possibility from our data and in view of the suggestion by Cherry et al. (6) that salmonellae may grow on aquatic plant surfaces. In conclusion, our findings support the view expressed by others (7) that the probability of finding surface waters containing no pathogenic or indicator bacteria is very low.

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