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Thymidine Incorporation by Free-Living and Particle-Bound Bacteria in a Eutrophic Dimictic Lake

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The percentage of [*methyl*-^{3}H]thymidine incorporated into samples from a dimictic eutrophic lake and retained on polycarbonate membranes of 3.0-, 1.0-, and 0.2-μm pore size was studied in a lake with filamentous cyanobacteria as the dominant phytoplankton type throughout the period of thermal stratification. Water samples were also examined by epifluorescence microscopy for evidence of algal senescence and bacterial colonization of intact and damaged cyanobacterial filaments. A small percentage (2 to 20%) of bacterial activity was retained by filters with pore sizes ≥ 1 μm in epilimnetic samples. Epilimnetic samples also had a small percentage of cyanobacterial filaments, either intact or damaged, which were visibly colonized by bacteria in summer and fall samples. A significant proportion (20 to 35%) of bacterial activity was retained by filters with pore sizes ≥ 1 μm in samples collected from the metalimnion and hypolimnion during late summer and fall. The proportion of damaged cyanobacterial filaments was higher in these samples than in those from the epilimnion or from those obtained early in the summer. Furthermore, the filaments in these samples were more heavily colonized by bacteria. Overall, particle-bound production accounted for only 2 to 19% of total bacterial production from April to August in all water layers. It appears that the supply of colonizable particles (damaged cyanobacterial filaments) is an important factor affecting the level of particle-bound bacterial activity in this lake.

The contribution of particle-bound bacteria to bacterial production and heterotrophic activity in aquatic ecosystems has received a great deal of attention. Bacterial attachment to suspended solids has been observed in marine waters (1, 8, 10, 13, 23), rivers and estuaries (2, 3, 5, 15–17), and freshwater lakes and ponds (3, 6, 14, 19, 21, 22).

Although data on the distribution of attached bacteria are still fairly sparse, it is widely accepted that attached bacteria are responsible for only a small part of bacterial activity in pelagic marine waters (1, 17). The degree of bacterial attachment varies widely among the other aquatic systems studied. Attached bacteria dominate the microheterotrophic population of some rivers and estuaries, whereas other systems nearby have a low proportion of particle-bound bacteria (16, 17). A wide range in the degree of bacterial attachment was found in ponds and marshes (21) and eutrophic lakes in Sweden (22). Unfortunately, very few seasonal studies on the degree of bacterial attachment in aquatic systems have appeared in the literature (5, 7), and the importance of particle-bound organisms within a system is commonly assessed from a few samples taken within a short period of time.

We measured bacterial activity in a eutrophic dimictic lake using the [^{3}H]thymidine incorporation method of Fuhrman and Azam (11). Size fractionation of this activity was undertaken to determine the amount of thymidine incorporation because of attached bacteria in the lake water column on a seasonal basis.

MATERIALS AND METHODS

This study was conducted on Little Crooked Lake (Noble County, Ind.). Temperature and oxygen measurements were made with a YSI model 54 oxygen meter (Yellow Springs Instruments, Kettering, Ohio). Water samples were collected with an acid-washed Van Dorn bottle (Wildco Supply Co., Saginaw, Mich.). The location of the phytoplankton population maximum was determined from chlorophyll *a* measurements of discrete samples from the water column. Chlorophyll *a* concentrations were determined from the absorbance at 663 nm of dimethyl sulfoxide-acetone extracts (40:60) of organisms filtered onto glass fiber filters (25). Experiments were initiated 10 min after sample collection in the laboratory of the Crooked Lake Biological Station.

The measurement of bacterial activity was performed by the thymidine incorporation procedure of Fuhrman and Azam (11). Water samples were taken with an acid-washed Van Dorn bottle and transported at in situ temperature to the Crooked Lake Biological Station in acid-washed polypropylene bottles in an ice chest. Subsamples (15 ml) were dispensed into each of four sterile 25-ml screw-cap tubes. One tube of each set received Formalin (1% final concentration), and each tube received [*methyl*-^{3}H]thymidine (Schwarz-Mann; specific activity 60 to 71 Ci mmol^{-1}) to a final concentration of 2.5 nM. We found that this concentration supports maximum rates of thymidine incorporation in this lake (21a). The tubes were then inverted for mixing and incubated in the dark at in situ temperature for 20 min. A linear increase in the amount of label incorporated was observed for 1 h. After incubation, the tubes were fixed with Formalin (2.5% final concentration) and filtered through a graded series of polycarbonate membranes (Nucleopore Corp., Pleasanton, Calif.) with pore sizes of 5.0 μm or 3.0, 1.0, and 0.2 μm.

These filters were placed into 20-ml glass scintillation vials, and 5 ml of ice-cold 5% trichloroacetic acid (TCA) was added to each. The vials were incubated on ice for 20 min, after which the filters were replaced on the manifold, and the TCA was poured through. Comparison of total counts on all filters to those obtained from unfractionated samples showed no significant loss of labeled cells during the fractionation

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TABLE 1. Percentages of bacterial activity retained by polycarbonate membranes for Little Crooked Lake metalimnetic samples in 1982[a]

Date (1982)	Percent bacterial activity at the following pore sizes (μm):			
	5.0	3.0	1.0	<1.0
18 May	2.0 ± 0.2	0.9 ± 0.3	4.0 ± 0.4	93.1 ± 8.9
22 July		1.9 ± 0.1	9.0 ± 0.4	89.1 ± 4.8
5 August	8.1 ± 2.3		7.7 ± 2.1	84.2 ± 30.7
25 August	7.7 ± 0.4		30.9 ± 2.8	61.4 ± 7.2
26 October		39.7 ± 1.7	18.1 ± 2.1	42.2 ± 5.7

[a] Data are given as the mean of five subsamples from the same Van Dorn sample plus or minus the standard error of the mean.

procedure. Two rinsings with 3 ml of ice-cold TCA followed, and the filters were removed and placed in plastic minivials and dissolved with a drop of phenethylamine (New England Nuclear, Boston, Mass.). Three milliliters of ACS (Amersham, Arlington Heights, Ill.) liquid scintillant was added, and radioactivity was determined with a Tracor Delta 300 (Tracor Analytic, Austin, Tex.) liquid scintillation counter. Formalin-fixed controls had very low radioactivity, generally 100 to 150 dpm. Counting efficiencies were determined by the channels ratio method, and a quench curve was constructed from a series of quenched standards.

In previous studies (21a, 21b), we have used the thymidine incorporation procedure to study vertical and seasonal distribution of secondary production in this lake and have tested several factors influencing the calculation of rates of bacterial production from rates of thymidine incorporation. Our results have been comparable to those obtained by other workers in several systems (4, 11, 12, 20).

The condition and bacterial colonization of cyanobacterial filaments was determined by examining Formalin-fixed (2.5% final concentration) samples by epifluorescence microscopy. A 5-ml portion of sample was stained with Primulin (0.01% final concentration; Polysciences Corp., Warrington, Pa.) for 30 s and filtered through 0.45-μm nominal pore size black cellulose nitrate filters (Sartorius Filters, Inc., Hayward, Calif.). The cyanobacterial filaments were too large to be entrapped in the fibrous surface of the filters, so no difficulty was encountered in the use of cellulose nitrate filters. Cyanobacterial filaments were then examined, and the percentages of intact and damaged filaments bearing six or more attached bacteria were determined. For the purposes of this study, damaged filaments were defined as those having visible filament breaks and disrupted cells.

RESULTS

The size distribution of heterotophic activity in metalimnetic samples changed significantly from May to October 1982 (Table 1). In metalimnetic samples, the percentage of bacterial activity retained by 1-μm filters increased throughout the summer of 1982. The retention of incorporated label on filters with pore sizes greater than 1 μm remained about the same through August. In the October 1982, samples from both the 3- and 1-μm pore size filters retained a significant percentage of incorporated label. On this date, 3- and 1-μm-filterable bacterial activity accounted for 57.8% of the total.

In contrast to the metalimnion, epilimnetic bacteria were only associated with particles 1 to 3 μm in size during spring 1983. The importance of this size class diminished during the summer (Table 2). This pattern may have been due to suspended solids washed in by spring rains. Metalimnetic samples from 1983 showed a trend similar to that observed in 1982, but the importance of the larger size classes was not as great as in 1982. In these samples, 1-μm pore size filters retained increasing percentages of bacterial production from June through October. An increase in 3-μm filterable production analogous to that observed in the October 1982, samples was seen in September 1983. In fall, 1983, bacterial activity associated with particles ≥ 1 μm accounted for 33% of the total. In hypolimnetic samples collected in late summer and fall, bacterial production associated with particles ≥ 1 μm ranged from 31 to 34% of the total. Although there were increases in particle-bound activity in the fall, it is important to note that in most samples from Little Crooked Lake more than 80% of thymidine incorporation was associated with particles capable of passing through a 1-μm pore size filter. The free-living bacteria in these samples were primarily rods with lengths from 0.75 to 1.0 μm and widths from 0.25 to 0.5 μm that should pass through 1.0-μm pore size filters.

Filamentous cyanobacteria, such as those from the genera *Aphanizamenon*, *Oscillatoria*, and *Anabaena*, dominated the Little Crooked Lake phytoplankton in 1982 and 1983. We examined Formalin-fixed samples from epilimnetic, metalimnetic, and hypolimnetic waters from 1983 for damaged cyanobacterial filaments and for intact and damaged filaments colonized by significant numbers of bacterial cells. Samples from July, August, September, and October were examined.

In epilimnetic samples, 8 to 9% of intact filaments (of >200 filaments examined) carried six or more attached bacteria from July through September (Table 3). In the sample obtained in October, only about 2% of intact filaments carried bacteria. The percentage of damaged filaments carrying attached bacteria increased in epilimnetic samples from July through October. The increase in both the percentage of filaments damaged and the percentage of damaged filaments colonized by bacteria probably explains the observed increase in the 3.0-μm-filterable fraction of secondary production.

TABLE 2. Percentage of total bacterial activity retained by polycarbonate membranes for Little Crooked Lake in 1983

Zone	Date (1983)	Percent total bacterial activity at the following pore sizes (μm):		
		3.0	1.0	<1.0
Epilimnion	6 April	3.3 ± 0.2	13.5 ± 1.0	83.2 ± 5.0
	24 May	4.7 ± 0.3	15.4 ± 1.1	79.9 ± 8.0
	7 June	3.0 ± 0.3	4.3 ± 0.7	92.7 ± 17.2
	7 July	0.5 ± 0.0	5.1 ± 0.5	94.4 ± 22.9
	16 August	0.3 ± 0.0	1.7 ± 0.1	98.0 ± 2.4
	22 September	2.7 ± 0.3	1.9 ± 0.4	95.4 ± 26.1
	27 October	5.6 ± 2.0	6.7 ± 0.7	87.7 ± 4.2
Metalimnion	11 May	2.7 ± 0.4	10.7 ± 2.0	86.6 ± 30.1
	24 May	2.6 ± 0.9	2.7 ± 0.6	94.7 ± 20.1
	7 June	2.7 ± 0.2	1.0 ± 0.2	96.3 ± 32.9
	7 July	1.1 ± 0.2	3.4 ± 0.7	95.5 ± 6.7
	16 August	2.4 ± 0.4	2.7 ± 0.2	94.9 ± 15.2
	22 September	10.2 ± 1.6	7.5 ± 1.0	82.3 ± 2.1
	27 October	5.6 ± 1.5	26.8 ± 15.5	67.6 ± 3.0
Hypolimnion	11 May	6.5 ± 1.6	8.0 ± 1.0	85.4 ± 7.8
	7 July	2.0 ± 0.3	3.3 ± 0.2	94.7 ± 9.2
	16 August	11.7 ± 0.0	21.1 ± 0.4	67.2 ± 4.5
	22 September	7.3 ± 1.3	26.8 ± 2.9	65.9 ± 6.4
	27 October	2.4 ± 1.8	29.0 ± 5.0	68.6 ± 3.7

[a] Water column mixed.

Filaments colonized by six or more bacteria accounted for increasing percentages of intact filaments in metalimnetic samples from July through October (Table 3). The period of highest intact filament colonization, damaged filament occurrence, and damaged filament colonization in the metalimnion was October; this was also the period when the percentage of bacterial production retained by a 1-μm filter was highest. In the July through September samples, more than 200 filaments were counted for each date. October samples had few filaments (only 100 counted), and the damaged filaments were extensively disrupted. We observed many small fragments of filaments and other particles extensively colonized by bacteria.

In July and October, hypolimnetic samples contained very few filaments (less than 50 counted). Numbers of filaments were higher in August and September samples (more than 200 counted). All filaments seen in the October samples were damaged and heavily colonized (Table 3). The low percentage of bacterial production retained by 1.0-μm filters in July samples is probably more a function of low filament numbers than degree of colonization. The frequency of damaged filaments and degree of colonization in August and September samples probably account for the high retention of bacterial production on 1.0- or 3.0-μm filters observed for these months. The near absence of filaments and the large quantity of heavily colonized small debris observed in October samples account for the high percentage of bacterial production retained on 1.0-μm and the low amount retained by 3.0-μm filters for this month. In all samples examined, particles were primarily cyanobacterial filaments or fragments. Very few particles of abiotic origin were observed.

TABLE 3. Colonization of intact and damaged cyanobacterial filaments by heterotrophic bacteria in Little Crooked Lake from July to October 1983[a]

Zone	Date (1983)	Percent damaged[b]	Percent Colonized[c]	
			Intact	Damaged
Epilimnion	7 July	6	9	75
	14 August	9	8	80
	21 September	10	9	90
	27 October	16	2	91
Metalimnion	7 July	12	2	70
	14 August	5	13	100
	21 September	10	10	95
	27 October	36	38	100
Hypolimnion	7 July	20	31	88
	14 August	16	12	100
	21 September	20	4	90
	27 October	100		100

[a] Primulin-stained filaments were examined by epifluorescence microscopy, and intact, damaged, and colonized filaments were counted.
[b] Filaments with visible breaks and disrupted cells.
[c] More than six bacterial cells per filament.

DISCUSSION

The use of filter separation techniques has greatly improved understanding of the importance of bacterial attachment in aquatic ecosystems. The finding by Sheldon (24) that Nucleopore filters can be used to separate particles into size classes has led to widespread use of this method in many different aquatic ecosystems.

Although results of field studies of pelagic marine waters have indicated that bacterial attachment to particles plays a small role in the biological activity of these systems (1, 17), work in other aquatic ecosystems has demonstrated great variability in the importance of attached bacteria (2, 3, 5, 7, 8, 13, 15, 17, 19, 21, 22).

One difficulty in interpreting the accumulated data is that different substrates were used by these investigators. Most commonly, incorporation or uptake of glucose or amino acids in various size fractions has been measured. Use of different labeled substrates in the same system can give very different percentages of label retained by filters (21). Microautoradiographic results (12) have indicated that practically all bacteria capable of assimilating either glucose or amino acids can also be labeled with [*methyl*-^{3}H]thymidine. Consequently, use of this label should not select for particular subpopulations. Another major advantage of the use of the thymidine incorporation method is the specificity of labeling of the heterotrophic bacteria in mixed algal and bacterial populations (12). This is particularly important in view of the finding of significant incorporation of tracer-level glycine by natural phytoplankton (26).

In our study of the contribution of particle-bound bacteria to bacterial production in a dimictic eutrophic lake, we found a clear seasonal trend in the importance of attached bacteria. The increase in bacterial production retained on 1- or 3-μm pore size filter observed in metalimnetic and hypolimnetic samples in late summer and fall corresponds to increases in the frequency of damaged cyanobacterial filaments and the degree of colonization of intact and damaged filaments. We did not determine whether the cyanobacterial filaments colonized by bacteria were actively photosynthesizing, nor did we measure the cell specific rate of thymidine incorporation by attached or free bacteria. It is possible that the intact filaments we observed to be colonized by bacteria were moribund but had not been extensively damaged by the time our sample was taken. We can make no statement on the relative rates of growth of attached versus free-living bacteria. Although our method of determining the frequency of damaged filaments and degree of bacterial colonization of filaments is only semiquantitative, these microscopic observations explain our data on thymidine incorporation associated with particles. Samples in which greater than 20% of thymidine incorporation is particle associated tend to have a higher degree of filament colonization by bacteria. It is particularly interesting that samples containing very few intact filaments but a great many small particles, such as the October 1983 samples, have a high degree of retention of thymidine incorporation on 1.0-μm filters but low retention on 3.0-μm filters. Also, the low filament numbers observed in hypolimnetic samples and the high degree of disruption seen in damaged filaments is in agreement with the rapid rates of cyanobacterial decomposition observed by Fallon and Brock (9) for Lake Mendota, Wis.

Riemann (22) has observed a similar trend of increase in 1-μm-filterable ^{14}C-glucose-labeled particles from August to September 1977, in eutrophic Lake Mosso, Sweden. The data of Berman and Stiller (6) are more fragmentary but also seem to show seasonal variation in 3-μm or larger ^{14}C-glucose-labeled particles in Lake Kinneret, Israel, from 1975 to 1976. Numbers of attached bacteria in a brackish lake rapidly increased over a 6-day period during the degradation of a phytoplankton bloom (14). These observations, as well as our own data, can be explained by the attachment of bacteria to dead and moribund phytoplankton cells, filaments, and fragments during periods of algal senescence. The observation by Seki (23) of "many" bacteria attached to senescent phytoplankton, particularly at the intercellular connections of colonial phytoplankton, corroborates this view.

Several workers have found strong correlations between the percentage of attached bacteria and the concentration of suspended particles (5, 16). A linear relationship between the concentration of suspended solids and numbers of attached bacteria was observed in the Humber Estuary, England (15). Significant correlations between the proportion of bacteria attached and the concentration of particles have been recorded for the Bay of Fundy, Nova Scotia (7), Tokyo and Segami Bays, Japan (13), as well as ponds and marshes (19, 21). Our data indicate that cyanobacterial filaments in Little Crooked Lake may serve as particles for bacterial attachment but appear to only do so if they are moribund or dead. Therefore, during senescence the quantity of colonizable particles is greatly increased, as is the percentage of thymidine incorporation activity bound to the particles. However, this increase in the colonization of particles by active bacteria occurred at a time in 1983 when bacterial production in the lake was relatively low. The highest rates of bacterial production (19.8 g of C m^{-2}) occurred in August 1983, in Little Crooked Lake (21b). In September and October, when relatively large percentages of thymidine incorporation were associated with particles, secondary production values were two- to four-fold lower. Thus, in 1983, bacterial production associated with particles was not a major fraction of the seasonal total. In fall 1982, the large fraction of thymidine incorporation associated with particles in metalimnetic samples coincided with a period of high bacterial production. Consequently, particle-associated bacterial production was quantitively more important during this period. However, as in 1983, particle-associated bacterial production accounted for only a fraction of total bacterial production throughout most of the period studied.

The attachment of bacteria to particles has another important implication for lake bacterial production. Free-living bacteria have negligible rates of sinking (ca. 1 to 2 mm day^{-1}) (18), but settling rates of particle-bound bacteria can be much greater. Ducklow et al. (8) have measured settling rates of 0.1 to 1.0 m day^{-1} for attached bacteria in the Hudson River Plume and have estimated that 3 to 67% of the total daily bacterial production settled out of the water column. Although particle-bound bacteria seem to be a minor component of the Little Crooked Lake bacterial population throughout most of the productive period, the increase in the proportion of attached bacteria during the senescence of the phytoplankton bloom has important implications for lake productivity, owing to the potential loss of a significant fraction of the total bacterial production with sinking particles in the fall.

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