APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1997, p. 3111–3118 Vol. 63, No. 8
0099-2240/97/$04.00+0

Characterization of the Sediment Bacterial Community in Groundwater Discharge Zones of an Alkaline Fen: a Seasonal Study

TIMOTHY C. GSELL,[1]* WILLIAM E. HOLBEN,[2] AND ROY M. VENTULLO[1]†

Department of Biology, The University of Dayton, Dayton, Ohio 45469-2320,[1] and Division of Biological Sciences, The University of Montana, Missoula, Montana 59812[2]

Received 27 December 1996/Accepted 2 June 1997

The cell density, activity, and community structure of the bacterial community in wetland sediments were monitored over a 13-month period. The study was performed at Cedar Bog, an alkaline fen. The objective was to characterize the relationship between the sediment bacterial community in groundwater upwelling zones and the physical and chemical factors which might influence the community structure and activity. DNA, protein, and lipid synthesis were measured at three different upwelling zones by using [^{3}H]thymidine, [^{14}C]leucine, and [^{14}C]glucose incorporation, respectively. The physiological status (apparent stress) of the consortium was assessed by comparing [^{14}C]glucose incorporation into membrane and that into storage lipids. Bacterial cell density was determined by acridine orange direct counts, and gross bacterial community structure was determined by bisbenzimidazole-cesium chloride gradient analysis of total bacterial community DNA. Both seasonal and site-related covariation were observed in all estimates of bacterial biomass and activity. Growth rate estimates and cell density peaked in late July at 2.5×10^8 cells/g/day and 2.7×10^9 cells/g, respectively, and decreased in December to 2.0×10^7 cells/g/day and 1.5×10^9 cells/g, respectively. Across sites, membrane-to-storage-lipid ratios were generally highest in late spring and peaked in September for one site. Overall, the data indicate dynamic seasonal differences in sediment bacterial community activity and physiology, possibly in response to changing physical and chemical environmental factors which included the C/N/P ratios of the perfusing groundwater. By contrast, total cell numbers were rather constant, and community structure analysis indicated that the overall community structure was similar throughout the study.

Microbial biomass and activity are fundamental variables in determining the importance of microorganisms in a particular environment (4, 22, 28). By a variety of radiotracer methods, bacterial activity, or production, has been assessed in freshwater aquatic systems (21, 23, 24, 28), marine water (7, 11, 12), aquatic sediments (8, 10, 25), and soils (2, 3, 37). This work has provided evidence that bacteria are essential for the cycling of nutrients such as nitrogen, phosphorus, and sulfur and as food for other trophic levels in these environments (28). However, little is known about bacterial communities residing in sediments through which groundwater flows (37). Freshwater wetlands in general, and fens in particular, are not well studied with regard to their bacterial communities. Knowledge of factors regulating bacterial activity and production would help us to better understand nutrient flux and carbon flow in wetland systems.

The objective of this study was to characterize the relationships between the prevailing physical and chemical parameters and the bacterial community in groundwater upwelling zones in Cedar Bog, an alkaline wetland located near Urbana, Ohio. The specific objectives were to describe the seasonal patterns of activity, cell density, physiological status, and community structure of the heterotrophic bacterial communities near the surface of several groundwater discharge zones over a 1-year time span. These unique sites, where groundwater emerges from the subsurface to feed a primary stream in an alkaline fen, represent the interface between two vastly different environments. Key physical and chemical parameters were monitored for comparison and correlation with cell activity and numbers.

The activity of bacterial communities at these sites was determined by three different radiotracer methods, specifically, the incorporation of [^{3}H]thymidine into DNA and [^{14}C]leucine into protein in a dual-label experiment and incorporation of [^{14}C]glucose into membrane lipids. In addition, the physiological status, or apparent stress, of the microbial communities was assessed by analysis of ^{14}C allocation patterns between membrane and storage lipids. The cell density of the bacterial communities was determined by direct microscopic enumeration. The overall structure of bacterial communities was determined by analysis of DNA isolated and purified directly from the environmental samples. For this purpose, the approach, first described by Holben and Harris (18), which generates a profile of the bacterial community based on relative abundance of DNA versus percent G+C content was employed.

Statistical comparison of the data by Spearman's rank correlations indicated several apparent relationships between bacterial community status and the physical and chemical parameters measured. The data support the conclusion that, in this system, the overall bacterial community structure does not change significantly but the bacterial activity and physiological status are subject to large directional shifts seasonally.

MATERIALS AND METHODS

Site description and sampling. The study site was the Cedar Bog Nature Preserve located on Woodburn Road near Urbana, Ohio. The preserve covers 427 acres mapped out in quadrants. Contrary to its name, Cedar Bog is an alkaline fen and is located above several hundred feet of limestone gravel which was deposited over the last 2 million years by the three great glaciers. Ground-

* Corresponding author. Mailing address: Division of Biological Sciences, The University of Montana, Missoula, MT 59812. Phone: (406) 243-6365. Fax: (406) 243-4184. E-mail: tgsell@selway.umt.edu.

† Present address: Department of Biology, Wartburg College, Waverly, IA 50677.

water flowing from the hills to the east comes to the surface here in tiny springs termed discharge or upwelling zones. At these zones, there is a constant flow of cool, alkaline water that keeps the water table high. As the spring water moves to the surface of the bog meadow, the excess calcium carbonate dissolved from the limestone gravel below is deposited. This calcium carbonate deposit, called marl, forms the gray soil in the bog meadow, which is 0.5 to 1 m deep. In the bog meadow, several upwelling zones can be found in and adjacent to the east branch of Cedar Run, a primary stream located in the preserve which flows from north to south.

Groundwater and associated sediments were taken from three upwelling zones along the stream edge in quadrants D5 and D6 in the bog meadow. These sites are referred to here as (i) south pool, (ii) 60 N (60 m north of the south pool), and (iii) 100 N (100 m north of the south pool). Groundwater samples were collected by placing a hollow Plexiglas cylinder (30 cm in length by 15 cm in diameter) vertically over and into a groundwater discharge zone and allowing the artesian properties of the site to fill and overflow the tube (10 cm above the water surface) for at least 10 min. After this flushing period, groundwater was pumped aseptically through Teflon tubing into sterile, acid-washed 500-ml glass bottles (Corning Glass Works, Corning, N.Y.) with a Nalgene handheld pump (Nalge Corp., Rochester, N.Y.). Sediment samples were collected aseptically in sterile mason jars from the sediment surface (0 to 10 cm) of the discharge zone. All samples were transported to the laboratory at in situ temperature (~10°C).

Air and groundwater temperature were measured with a mineral spirit-filled thermometer following a 15-min equilibration period. The pH of the groundwater samples was determined with ColorpHast pH strips (EM Reagents, Gibbstown, N.J.) in the field and was confirmed with a pH meter (Orion Research Inc., Boston, Mass.) upon arrival in the laboratory. Water chemistry was determined on-site with Hach (Loveland, Colo.) and CHEMetrics (Calverton, Va.) chemical field kits. Phosphate levels were determined spectrophotometrically in the laboratory by an ammonium molybdate assay (36). The levels of inorganic nutrients in water from the discharge zone were determined by high-pressure ion chromatography (Dionex Corporation, Sunnyvale, Calif.). Nonpurgeable organic carbon was determined with a TOC 5000 analyzer (Shimadzu, Columbia, Md.). Annual precipitation data for North Springfield and Urbana, Ohio, which are adjacent to the study site, were obtained from the Miami Conservancy District (Dayton, Ohio).

Chemicals. All radioisotopes ([*methyl*-^{3}H]thymidine, 50 Ci/mmol; L-[U-^{14}C]leucine, 310 mCi/mmol; and D-[U-^{14}C]glucose, 280 mCi/mmol) were obtained from ICN Pharmaceuticals, Inc. (Irvine, Calif.). Chloroform, acetone, and methanol were gas chromatography–gas chromatography-mass spectrometry grade (Burdick & Jackson, Muskegon, Mich.). All other chemicals were reagent grade or better.

Enumeration of bacteria. Sediment samples (1 g [wet weight]) from each site were fixed with 2.5% (vol/vol) glutaraldehyde dissolved in 0.1% (wt/vol) sodium pyrophosphate and stored at 10°C prior to counting. Bacterial population densities were determined by the acridine orange direct count (AODC) method of Hobbie et al. (16) with modifications. Briefly, sediment slurries were homogenized and diluted (generally 1:10) into 10 mM phosphate buffer (pH 7.5). One-milliliter aliquots were subsequently stained with 4 drops (~0.2 ml) of 0.1% acridine orange for 4 min and then filtered onto 0.2-μm-pore-size filters (Poretics, Livermore, Calif.). The filters were placed onto microscope slides spotted with 1 to 2 drops of low-fluorescence immersion oil. At least 10 randomly selected fields with >10 cells per field were counted on each slide with an Olympus BHS microscope equipped with epifluorescence (Valencia, Pa.). With this microscope at ×1,000 magnification, there are 2.684×10^4 fields per stained area on each 25-mm filter. The average number of bacterial cells per gram of sediment was determined by the following calculation: number of cells per gram = (average number of cells per field) × (number of fields per filter [2.684×10^4]) × (dilution factor)/(sample volume). While direct microscopic counting of bacteria in soils and sediments can be problematic, the high bacterial biomass present and the modifications developed for these samples enabled its use.

Bacterial activity determinations: dual-label incorporation of [^{3}H]thymidine into DNA and [^{14}C]leucine into protein. The dual-label procedure employed involved incubation with 125 nM [^{3}H]thymidine and 160 nM [^{14}C]leucine for 1 h at in situ temperature. The incubation was followed by cold trichloroacetic acid (TCA) precipitation and extraction of macromolecules and fractionation following the type 3 procedure of Craven and Karl for carbonate sands (8) with the modifications described by Carman et al. (6). Briefly, ice-cold 10% TCA was thoroughly mixed with 2 g (wet weight) of sediment and put on ice for 45 min. This slurry was cleared by centrifugation at 7,500 × *g* for 10 min at 4°C, and the TCA was decanted. The TCA wash was repeated once, followed by two washes with ice-cold 100% ethanol, and the sediment was then air dried at room temperature. RNA and DNA were then solubilized in 1 N NaOH for 1 h, and the slurry was cleared by centrifugation at 7,500 × *g* for 10 min at 4°C. The resulting supernatant was treated with ice-cold 20% TCA–3.6 M HCl–0.1 g of diatomaceous earth for 20 min to acidify the mixture. This mixture was subjected to centrifugation at 7,500 × *g* for 10 min at 4°C, and the RNA was recovered in the supernatant. The pellet (containing the DNA) was washed three times with ice-cold 5% TCA, and the DNA was subsequently recovered from the supernatant following a hot 5% TCA treatment at 95°C for 30 min and centrifugation at 7,500 × *g* for 10 min at 4°C.

Protein was insoluble in the base solubilization treatment described above. To obtain protein, the pellet from that step was washed with 5% TCA, centrifuged at 7,500 × *g* for 10 min at 4°C, washed in 95% ethanol, and air dried. The dried sediment was subjected to alkaline hydrolysis by adding 10 ml of 1 N NaOH for 18 h at 37°C with gentle shaking to solubilize the protein. Protein was collected in the supernatant following centrifugation at 2,000 × *g* for 10 min at 4°C to clear the suspension.

Aliquots of the DNA, RNA, and protein fractions were added to scintillation vials containing 5 ml of Ready Safe cocktail (Beckman, Palo Alto, Calif.) and counted with a dual-label (^{3}H-^{14}C) program with an LS 3801 liquid scintillation counter (Beckman). Bacterial growth rates were calculated from the [^{3}H]thymidine activity in the DNA fraction by using a conversion factor of 10^{18} cells per mol of thymidine incorporated into DNA, an average freshwater value (31). Bacterial growth rates were estimated from the [^{14}C]leucine incorporation data by using an average freshwater conversion factor of 7.5×10^{16} cells per mol of [^{14}C]leucine incorporated into the protein fraction (31). This value was subsequently multiplied by 2 to compensate for the twofold isotope dilution that appears to exist in all environments (33).

Lipid analysis and physiological stress determination. Fresh sediment samples (5 g [wet weight], $n = 4$) were added to 50-ml Nalgene Oak Ridge tubes and then amended with 160 nM [U-^{14}C]glucose. Samples were incubated on an orbital shaker at 100 rpm at 10°C for 60 min. Lipids were extracted by the chloroform-methanol buffer procedure described by Guckert et al. (13). Total lipids in the chloroform layer were separated into three general lipid classes (neutral lipids, glycolipids, and polar lipids) by silicic acid column chromatography with columns containing 0.5 g of inert 100/200 mesh silica (Burdick & Jackson) as described elsewhere (14, 42). Briefly, lipids were loaded onto the column in 10 ml of chloroform and a series of mobile phases of increasing polarity were employed to selectively elute each class of lipids as follows: for neutral lipids, 10 ml of chloroform; for glycolipids, 10 ml of acetone; and for polar lipids, 10 ml of methanol. Following elution, the solvents in the respective samples were evaporated by nitrogen purging to a volume of 1 ml which was then added to 5 ml of Ready Safe cocktail (Beckman) and subjected to liquid scintillation counting. The ratio of label incorporated into membrane lipids (polar phospholipids) to that incorporated into storage lipids (glycolipids) was determined and used as an indicator of physiological status as described by Guckert et al. (14). Incorporation of label into the phospholipid fraction was also used to determine general microbial heterotrophic activity (42, 43). Physiological stress is defined as an indication of a change in the physiological status of the microbial community in general, based on changes in membrane-lipid-to-storage-lipid ratios. Higher ratios indicate increased stress.

Analysis of community structure. Bacterial community DNA from the south pool was isolated by direct lysis (17) and used to evaluate community structure from four seasonal samples by the bisbenzimidazole-CsCl gradient method described by Holben and Harris (18). The resulting profiles indicate the relative abundance of DNA of specific percent G+C content in the sample. Since percent G+C content of DNA is characteristic for bacterial populations at about the genus level, this analysis can be used to indicate the general structure of the bacterial community in an environmental DNA sample (18).

Statistical analysis. Significant correlations between data sets were tested with Spearman's rank correlation coefficient (r_s) (35). The nonparametric rank correlation was used because a linear relationship between any of the data was not assumed. All statistical analyses were performed with Statistix software, version 3.5 (Analytical Software, Tallahassee, Fla.). Significance levels were set for these two-tailed comparisons at $P = 0.05$ for r_s values ≥ 0.503, $P = 0.01$ for r_s values ≥ 0.635, $P = 0.002$ for r_s values ≥ 0.732, and $P = 0.001$ for r_s values ≥ 0.765 (45). n is 16 for all analyses unless otherwise specified.

RESULTS

Seasonal patterns of physicochemical parameters. Seasonal variations in groundwater temperature, groundwater pH, and air temperature at all three sites were determined. While air temperature varied seasonally as expected at this temperate latitude, the groundwater temperature was fairly constant throughout the year, ranging from 9.5°C in January to 13°C in late July (data not shown). The pH of the groundwater from the south pool ranged between 7.06 and 8.08 throughout the year and was similar at all three sites (data not shown).

Nutrient levels in south, 60 N, and 100 N pool groundwaters were measured (Fig. 1). Peak nutrient levels occurred in late summer and generally preceded increases in bacterial activity and cell number as discussed below. Nutrient ratios (C/N/P) from the south, 60 N, and 100 N pool groundwaters were calculated from these data. A C/N/P ratio of 100:10:1 is considered to be optimal for microbial activity (34). Generally, the closer the calculated groundwater nutrient ratio was to this optimal value, the greater the measured microbial activity dur-

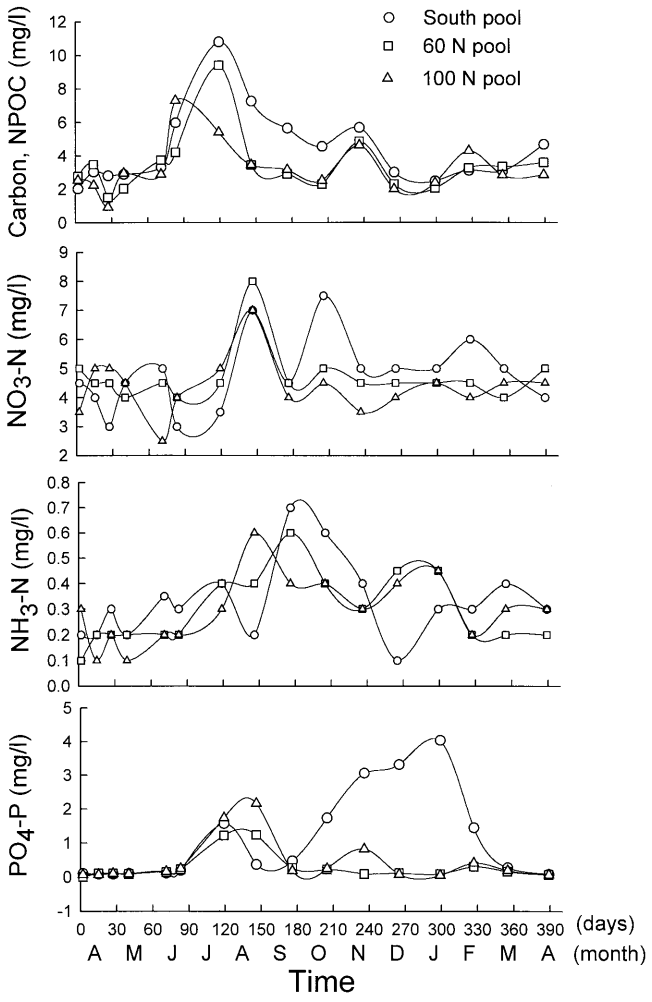

FIG. 1. Seasonal variation in carbon, nitrogen, and phosphorus levels in south, 60 N, and 100 N pool groundwater.

ing or immediately following that sampling time. The C/N/P ratios observed during June and July for the south pool (100:55:2 and 100:35:14.5, respectively) were, by far, the closest to the optimal levels for maximum growth rates of the seasonal sampling times. Closest-to-optimal ratios for the 60 N pool groundwater were also observed in June and July, being 100:100:5.5 and 100:52:12.8, respectively. Similarly, the most optimal ratios at the 100 N pool were 100:58:3.4 and 100:98:32 for June and July, respectively. By contrast, the least optimal nutrient ratios for the south pool and the 60 N and 100 N sites were observed in August and were 100:99:5.1, 100:247:36.5, and 100:217:62, respectively. It was at this sampling time that a dramatic drop in organic carbon and an increase in nitrate levels were observed (Fig. 1).

Seasonal patterns of bacterial activity. Time course experiments for substrate incorporation determinations were performed with sediment samples from all three study sites. For these experiments, 1-h incubation times were used based on observed linear uptake of the three substrates for more than 90 min under experimental conditions (data not shown). Growth rate determinations based on [^{3}H]thymidine and [^{14}C]leucine incorporation were always within the same order of magnitude and covaried temporally (Fig. 2). Activity values derived from DNA synthesis rates by the [^{3}H]thymidine incorporation method were generally higher than values derived from [^{14}C]leucine incorporation for each site but were within a factor of 2 for most of the study (Fig. 2). Bacterial activity was highest at the south pool site and peaked in July. Phospholipid synthesis rates (based on incorporation of ^{14}C from glucose into lipid), which can also be used as an estimate of heterotrophic bacterial activity, covaried with the dual-label activity measurements (Fig. 2 and 3A).

Comparative evaluation of the three sites. Overall, the physicochemical and biological parameters measured at the 60 N and 100 N sites were more similar than those measured for the south pool. Further, the observed carbon allocation patterns for membrane and storage lipids indicate that the north pool bacterial communities were more physiologically similar to each other and somewhat different from the community in the south pool (Fig. 3B). The south pool exhibited a large increase in apparent stress following the dramatic drop in activity in early fall (Fig. 2 and 3).

Bacterial growth rate determinations indicated that increases in bacterial activity (Fig. 2 and 3A) preceded increases in cell numbers (Fig. 4A) for all sites throughout the study. Population turnover estimates were derived by dividing the

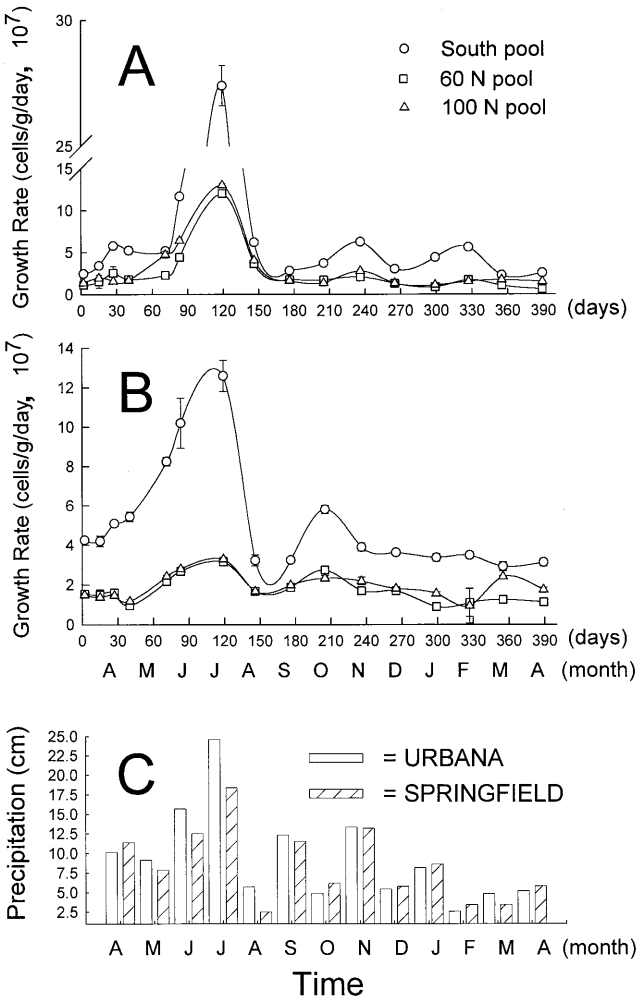

FIG. 2. Seasonal variation in incorporation of [^{3}H]thymidine into DNA (A) and [^{14}C]leucine into protein (B). Samples were incubated with both labels, 125 nM [^{3}H]Thy and 160 nM [^{14}C]leucine, for 1 h. Data represent the means ± standard errors of the means (n = 4). Local precipitation from Urbana and Springfield, Ohio, from April 1993 through April 1994 is also shown (C).

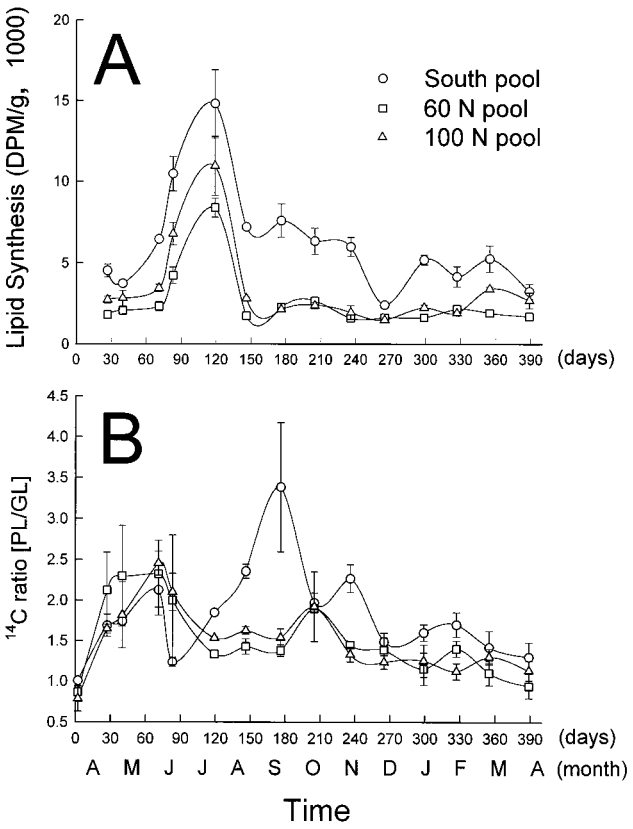

FIG. 3. (A) Lipid synthesis as measured by [^{14}C]glucose incorporation into phospholipid. (B) Ratio of [^{14}C]glucose incorporation into membrane to that into storage lipid for physiological status (stress) assessment. Data represent the means ± standard errors of the means (n = 4). DPM, disintegrations per minute; PL/GL, phospholipid-to-glycolipid ratio.

bacterial biomass values by the corresponding growth rate (determined by [^{3}H]thymidine incorporation) and are given in terms of the number of days required to replace the current bacterial biomass at that growth rate (Fig. 4B). Turnover values were highest in winter and early spring and lowest during the summer when bacterial activity and nutrient concentration peak and the C/N/P ratios are closest to 100:10:1. Despite the higher growth rates at the south pool (Fig. 3), the turnover times were similar to those obtained for the two north pools (Fig. 4B).

Spearman's rank correlations (r_s). Spearman's rank correlations were used to assess the relationships among the various physical, chemical, and biological parameters measured. With this approach, correlation values greater than the significance levels set for $n = 16$ for these two-tailed comparisons ($P = 0.05$ for r_s values ≥ 0.503, $P = 0.01$ for r_s values ≥ 0.635, $P = 0.002$ for r_s values ≥ 0.732, and $P = 0.001$ for r_s values > 0.765) were generally accepted as being significant (45). The results of these analyses are presented in Tables 1 and 2 and are highlighted below.

Turnover time values covary inversely with DNA synthesis rates from all sites and provided the strongest correlations in this study (-0.874, -0.948, and -0.877 for the south, 60 N, and 100 N pools, respectively [Table 1]). Less strong correlations were observed for turnover times and protein synthesis from all three sites and for phospholipid synthesis at the 100 N pool (Table 1). 100 N pool physiological status values also correlated with the turnover times (0.721) that were generated by using activity and biomass data from that site. Other significant correlations observed include south pool DNA and protein synthesis (0.538), south pool phospholipid synthesis and physiological status (0.512), 60 N pool DNA and protein synthesis (0.606), 60 N pool DNA synthesis and physiological status (0.677), and 100 N pool DNA synthesis and phospholipid synthesis (0.729).

Trends in thymidine incorporation values observed for the south pool during the 13-month study period correlated well with the values obtained for the 60 N (0.850) and 100 N (0.590) pools although the south pool was generally two to three times higher in activity (Fig. 2). DNA synthesis rates for the 60 N pool and the 100 N pool also correlated well (0.764). The protein synthesis rates were also correlated for the south pool and 60 N pool (0.564) and the 60 N pool and 100 N pool (0.765) but not between the south pool and 100 N pool (0.291). Phospholipid synthesis rates appeared to be correlated only between the 60 N and 100 N pools (0.592). Biomass measurements by AODC indicated a significant correlation only between the south pool and 60 N sites (0.594). In general, the data patterns yielded the strongest correlations between the two north pools, followed by the south and 60 N pools, and were weakest between the most spatially separated sites, the south and 100 N pools (some data not shown).

Good correlations between activity measurements and chemical and physical parameters were obtained for the [^{3}H]thymidine incorporation experiments (Table 2). For the south pool, DNA synthesis data correlated with C/N ratios (0.533). The south pool physiological status values showed

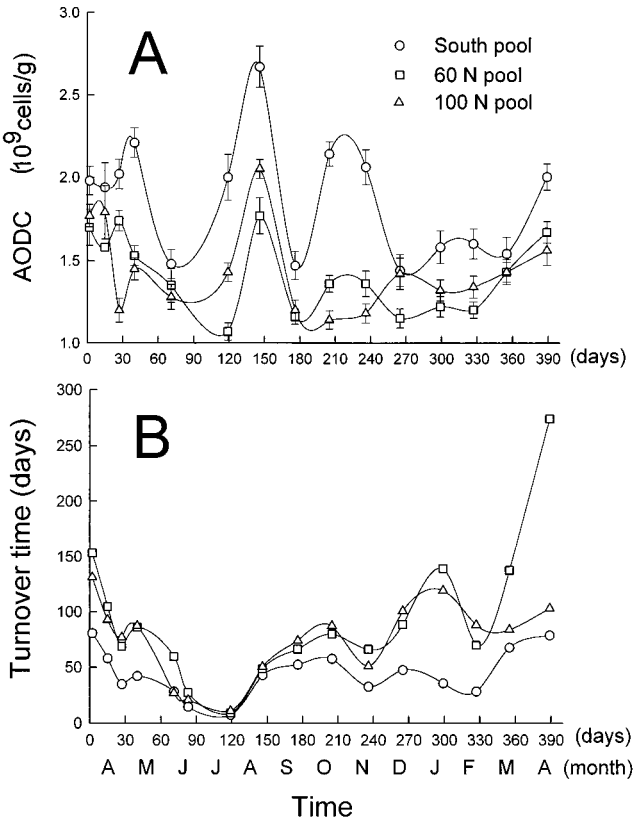

FIG. 4. (A) Seasonal variation in bacterial cell numbers as determined by the AODC method; (B) population turnover times based on AODC and thymidine incorporation data. Data represent the means ± standard errors of the means (n = 10 fields/sample).

TABLE 1. Correlations (r_s) among activity measurements, biomass, and metabolic state from the south, 60 N, and 100 N pools[a]

Activity, biomass, and metabolic state for pool	Cellular turnover time	Thy	Leu	Glu	AODC	PL/GL
South						
Thy	−0.874****	1				
Leu	−0.556*	0.538*	1			
Glu	−0.467	0.482	0.324	1		
AODC	−0.003	0.431	0.201	0.038	1	
PL/GL	−0.397	0.424	0.076	0.512*	0.202	1
60 N						
Thy	−0.948****	1				
Leu	−0.703**	0.606*	1			
Glu	0.318	0.355	0.454	1		
AODC	0.341	−0.139	−0.381	−0.181	1	
PL/GL	0.662*	0.677**	0.336	0.021	−0.055	1
100 N						
Thy	−0.877****	1				
Leu	−0.615*	0.478	1			
Glu	−0.544*	0.729**	0.409	1		
AODC	0.240	0.128	−0.314	−0.181	1	
PL/GL	0.721**	0.490	0.468	0.024	−0.055	1

[a] Activity measurements were determined by [^{3}H]thymidine, [^{14}C]leucine, and [^{14}C]glucose incorporation into DNA, protein, and phospholipids, respectively. AODC represents biomass estimates, and PL/GL represents the phospholipid-to-glycolipid ratio (stress). Cellular turnover time was based on thymidine incorporation activity estimates and AODC data. * to ****, weakest to strongest correlation, respectively.

similar trends compared to local precipitation (r_s of 0.520). Overall, bacterial activity values covaried most consistently with increasing organic carbon, water temperature, and precipitation (Table 2 and Fig. 2C). South pool phospholipid data also correlated well with the organic carbon (0.727), ammonia (0.601), and C/N ratios (0.545) obtained at that site, indicating that, as nutrient ratios at the site approached optimum, bacterial biomass and activity increased (Table 2).

Bacterial activity estimates at the 60 N and 100 N pool sites were also strongly correlated with groundwater chemistry and physical parameters measured at each site. For example, bacterial activities ([^{3}H]thymidine incorporation) throughout the year in the two north pools appear to be most strongly influenced by phosphate levels with r_s values of 0.558 and 0.524 for the 60 N and 100 N sites, respectively (Table 2). 100 N pool protein synthesis had an r_s value of 0.509 compared to the Urbana precipitation patterns. Groundwater temperature had an r_s of 0.529 for 60 N pool DNA synthesis and 0.567 for phospholipid synthesis (Table 2). Positive correlations of 0.567 and 0.767 were also obtained between groundwater temperature and 100 N pool DNA and phospholipid synthesis estimates, respectively. The pattern of 100 N [^{3}H]thymidine incorporation into DNA was also related to organic carbon levels (0.667).

Community structure. To further characterize the bacterial communities, DNA was isolated from south pool samples and the bacterial community structures were compared for spring, summer, fall, and winter samples. Although individual bacterial populations are not detected by this broad-resolution approach, the data indicate that no significant alterations in total bacterial community structure occurred in response to seasonal changes since nearly identical community structure profiles were obtained at each time point (Fig. 5). The lipid-based stress determinations were highest in the fall, but no comparable community shift was detected at that time by the percent G+C method (Fig. 3B and 5).

DISCUSSION

While seasonal fluctuations in air temperature were evident, due to the continuous upward flow of groundwater little change in groundwater temperature was observed. In fact, in January when the marl meadow was frozen, Cedar Run had algae growing in it and there was no indication of ice formation. Rainfall appears to initiate the primary peaks in activity

TABLE 2. Correlations (r_s) between measured community parameters and physical and chemical parameters from the groundwater[a]

Activity and parameter for pool	Organic carbon	NO_3-N	NH_4-N	PO_4-P	C/N	Groundwater temp	Precipitation for Urbana, Ohio
South							
Thy	0.497	−0.141	0.062	0.227	0.533*	0.334	0.413
Leu	0.488	−0.346	0.061	−0.047	0.212	0.467	0.214
Glu	0.727**	−0.044	0.601*	0.221	0.545*	0.475	0.477
AODC	0.202	−0.584	0.010	−0.208	0.231	0.022	0.052
PL/GL	0.468	0.423	0.442	0.422	0.309	0.090	0.520*
60 N							
Thy	0.356	−0.170	0.068	0.558*	0.215	0.529*	0.400
Leu	0.452	0.111	0.310	0.517*	0.309	0.358	0.358
Glu	0.302	−0.111	−0.308	0.313	0.386	0.567*	0.086
AODC	−0.209	0.382	0.420	−0.414	−0.419	−0.177	−0.445
PL/GL	−0.082	−0.274	0.240	0.240	−0.106	0.312	0.233
100 N							
Thy	0.667**	0.167	−0.363	0.524*	0.477	0.757***	0.339
Leu	0.385	−0.198	0.261	0.390	0.464	0.389	0.509*
Glu	0.253	0.338	−0.424	0.287	0.162	0.767***	0.146
AODC	−0.002	0.385	−0.116	−0.108	0.220	0.080	−0.235
PL/GL	0.235	0.044	−0.100	0.458	0.202	0.492	0.432

[a] Activity measurements were determined by [^{3}H]thymidine, [^{14}C]leucine, and [^{14}C]glucose incorporation into DNA, protein, and phospholipids, respectively. AODC represents biomass estimates, and PL/GL represents the phospholipid-to-glycolipid ratio (stress). * to ***, weakest to strongest correlation, respectively.

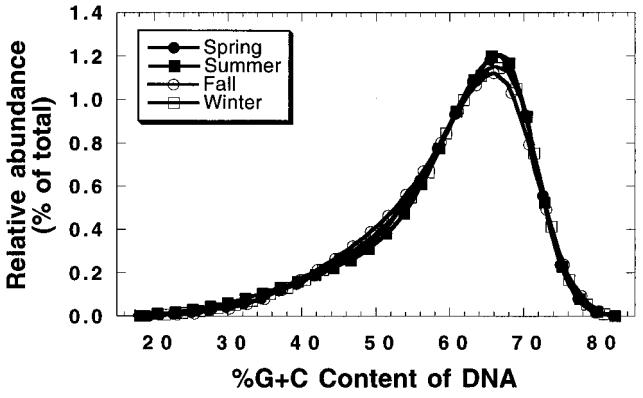

FIG. 5. Seasonal data for the percent G+C method of describing microbial community change. Dates for the south pool samples are as follows: spring, 22 April 1993; summer, 28 July 1993; fall, 22 October 1993; winter, 21 January 1994.

and nutrient levels in August and September. Secondary peaks in nitrogen and phosphorus in the south pool in the September-to-October time frame may have resulted from nutrient release due to disturbance of topsoil and cedar root mycorrhizal zone caused by cedar tree removal adjacent to this site. The groundwater was typically low in carbon and phosphate, particularly phosphate, with values generally less than 5 ppb. However, nitrate levels were much higher than expected for groundwaters, approaching 5 mg/liter, probably due to agricultural runoff and seepage. This is probably the case since the surrounding fields were fertilized twice a year, in both the spring and the fall. Nonpurgeable organic carbon levels varied but were much higher in summer (10.8 mg/liter) than during the other seasons (3 to 4 mg/liter). In groundwater-fed wetlands (fens), carbon levels generally range between 1 and 20 mg of C/liter (44).

It was expected that carbon, nitrogen, and phosphorus levels would affect the bacterial communities in the sediments from the three sites sampled. It is not only the absolute supply of nutrients that regulates growth but also the stoichiometry of the dissolved nutrient pool (15, 35). While the C/N/P ratio at these sites was never optimal, the N/P ratios do approach the optimal level. The C/N ratios are consistently low at all three sites, suggesting carbon limitation. Recent studies in freshwater lakes and streams have shown that N and P levels have profound effects on community growth rates and that the ratio of these important nutrients influences bacterial growth (19, 26, 27, 39–41). It has also been shown that low phosphate concentrations (below 5.0 μg/liter) and high N/P ratios can result in phosphorus limitation (1). Thus, it is possible that the C/N/P ratio in the waters perfusing Cedar Bog sediments is the key parameter regulating bacterial activity and growth.

Spearman's rank correlations (r_s) were made by using entire data sets from each site to compare the patterns of growth rates with measured chemical and physical parameters throughout the study. Overall, bacterial activity values correlated well between sites, with the sites that are closest in proximity having the best correlation. It is not clear whether this is due to differences in groundwater flow (hydrology), nutrients, or other parameters. Correlations between activity and physical and chemical parameters, although indicative of a regulatory role, did not consistently yield complementary results among activity measurements. For example, groundwater temperature alone displayed a significant correlation with both thymidine and glucose incorporation activity measurements at the 100 N pool. However, leucine incorporation activity data at this site does not show a correlation with groundwater temperature, and yet it does correlate with the thymidine incorporation pattern (Table 1). It should be noted that responses in the activity, cell density, and physiological status of the bacterial communities to patterns in physicochemical parameters may be lagging, which would affect the strength of the correlation because the patterns would be slightly offset.

The effect of phosphate concentration is more apparent for the two north pools, suggesting that in this system at least some sites may be regulated by this nutrient. However, for the south pool C/N ratio is suggested as a key regulator of growth based on the statistical comparisons between DNA and phospholipid synthesis and the nutrient ratios.

One aim was to compare three independent activity estimates since the combination of several methods to measure growth should give more reliable estimates (21, 31). Incorporation of [^{3}H]thymidine is specific for heterotrophic bacterial DNA, and [^{3}H]thymidine is reportedly not taken up by cyanobacteria, eucaryotic microalgae, autotrophic bacteria, or fungi (3, 12, 29, 37). While [^{3}H]thymidine labeling of DNA has been used extensively to measure bacterial activity, lack of specificity of incorporation and extraction variability have been observed in freshwater (7, 20, 32) and sediment (5, 9, 25) microbial communities with this approach. To overcome these potential limitations, data obtained by this approach and other bacterial activity estimates were compared. Uptake and incorporation of [^{14}C]leucine into total cellular protein (2, 7, 31) have provided an alternative technique for estimating bacterial growth. For each method, the amount of radiotracer incorporated into the corresponding macromolecules has been shown to be proportional to the growth rate of bacterial communities (10, 22, 30, 38, 44). Because growth determinations generally rely on cellular processes which are independent of one another but are all related to growth or new cell production, covariation during periods of balanced growth is anticipated. Growth rate estimates based on thymidine incorporation were only approximately twofold higher than those based on leucine incorporation, a small difference considering that the conversion factors employed differ by 2 orders of magnitude. Thus, the dual-label approach produced highly similar results based on DNA and protein synthesis rates, allowing greater confidence in the growth estimates obtained.

The rates of [^{3}H]thymidine, [^{14}C]leucine, and [^{14}C]glucose incorporation into DNA, protein, and phospholipids, respectively, by sediment heterotrophic bacteria were found to covary in the two north pools and the south pool. When label incorporation data were converted to growth rates (number of cells per gram per day), similar estimates of growth were obtained. The observed increases in growth rate and cell numbers in the south pool may be explained by higher nutrient levels, higher temperatures, or changing ratios of these nutrients. The different nutrient levels in the south, 60 N, and 100 N pool groundwaters may be due to differences in sediment composition, such as organic detritus or carbonate makeup common to these groundwater upwelling zones. Also, it appears that increases in precipitation influence availability of nutrients at the upwelling zones.

Few studies have addressed the effects of nutrients on activity and biomass of microbial communities in sediments (4). This study provides much-needed information concerning sediment bacterial communities from groundwater-fed ecosystems. The data indicate dynamic seasonal differences in some physical and chemical parameters. The data also indicate significant seasonal variation in growth and activity measurements of the bacterial community. The bacterial community profiles based on the percent G+C content of component

populations in the complex bacterial community provided a profile of the structure of the entire bacterial community at the time of sampling in terms of relative abundance of DNA versus percent G+C content. While limited in resolution for individual bacterial populations, this approach has been successfully employed to demonstrate gross changes in microbial community structure in response to anaerobiosis and carbon amendment (18). While we cannot preclude the possibility that the abundance of specific individual populations varies seasonally, it is apparent that there are no major shifts in overall community structure during the course of the year. Thus, the data support the interpretation that, while the activity and physiological status of the entire community are subject to large directional shifts seasonally, the overall bacterial community structure and biomass do not change significantly.

The data provide new information regarding seasonal variation in the bacterial community in an understudied wetland environment and implicate many factors that may influence activity and biomass of the bacterial consortium in the sediment compartment of groundwater discharge zones in Cedar Bog. Since no protozoa were evident in the bog sediments, it is likely that bacterial biomass and activity in this system are regulated by nutrients or other physicochemical parameters rather than by predation.

ACKNOWLEDGMENTS

This work was partially funded through the Research Institute at the University of Dayton.

We are grateful to Terry Jawarski, curator of the Cedar Bog Nature Preserve, for his assistance and knowledge concerning the site and to the Ohio Historical Society for permission to access and sample the three study sites on the East Fork of Cedar Run. We also thank Richard Lynch for technical assistance with bacterial community structure analysis.

REFERENCES

1. **Arvola, L.** 1991. Recent trends in the water chemistry of lake Paajarvi. Lammi Notes **18:**1–5.
2. **Baath, E.** 1994. Measurement of protein synthesis by soil bacterial assemblages with the leucine incorporation technique. Biol. Fertil. Soils **17:**147–153.
3. **Baath, E., and T. Johansson.** 1990. Measurement of bacterial growth rates on the rhizoplane using ^{3}H-thymidine incorporation into DNA. Plant Soil **126:**133–139.
4. **Bott, T. L., and L. A. Kaplan.** 1985. Bacterial biomass, metabolic state, and activity in stream sediments: relation to environmental variables and multiple assay comparisons. Appl. Environ. Microbiol. **50:**508–522.
5. **Brittain, A. M., and D. M. Karl.** 1990. Catabolism of tritiated thymidine by aquatic microbial communities and incorporation of tritium into RNA and protein. Appl. Environ. Microbiol. **56:**1245–1254.
6. **Carman, K. R., F. C. Dobbs, and J. B. Guckert.** 1988. Consequences of thymidine catabolism for estimates of bacterial production: an example from a coastal marine sediment. Limnol. Oceanogr. **33:**1595–1606.
7. **Chin-Leo, G., and D. L. Kirchman.** 1988. Estimating bacterial production in marine waters from the simultaneous incorporation of thymidine and leucine. Appl. Environ. Microbiol. **54:**1934–1939.
8. **Craven, D. B., and D. M. Karl.** 1984. Microbial RNA and DNA synthesis in marine sediments. Mar. Biol. **83:**129–139.
9. **Ducklow, H. W., S. M. Hill, and W. D. Gardner.** 1985. Bacterial growth and the decomposition of particulate organic carbon collected in sediment traps. Continental Shelf Res. **4:**445–464.
10. **Fallon, R. D., S. K. Newell, and C. S. Hopkinson.** 1983. Bacterial production in marine sediments: will cell-specific measures agree with whole system metabolism? Mar. Ecol. Prog. Ser. **11:**119–127.
11. **Fuhrman, J., and F. Azam.** 1980. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. Environ. Microbiol. **39:**1085–1095.
12. **Fuhrman, J., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation of field results. Mar. Biol. **66:**109–120.
13. **Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White.** 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microb. Ecol. **31:**147–158.
14. **Guckert, J. B., S. C. Nold, H. L. Boston, and D. C. White.** 1992. Periphyton response in an industrial receiving stream: lipid based physiological stress analysis and pattern recognition of microbial community structure. Can. J. Fish. Aquat. Sci. **49:**1–9.
15. **Hessen, D. O.** 1992. Dissolved organic carbon in a humic lake: effects on bacterial production and respiration. Hydrobiologia **229:**115–123.
16. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
17. **Holben, W. E.** 1994. Isolation and purification of bacterial DNA from soil, p. 727–751. *In* R. W. Weaver et al. (ed.), Methods in soil analysis, part 2. Microbiological and biochemical properties. SSSA Book Series no. 5. Soil Science Society of America, Madison, Wis.
18. **Holben, W. E., and D. Harris.** 1995. DNA-based monitoring of total bacterial community structure in environmental samples. Mol. Ecol. **4:**627–631.
19. **Horrigan, S. G., A. Hagstrom, I. Koike, and F. Azam.** 1988. Inorganic nitrogen utilization by assemblages of marine bacteria in seawater culture. Mar. Ecol. Prog. Ser. **50:**147–150.
20. **Karl, D. M.** 1980. Cellular nucleotide measurements and applications in microbial ecology. Microbiol. Rev. **44:**739–796.
21. **Kirchman, D. L., E. K'nees, and R. Hodson.** 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl. Environ. Microbiol. **49:**599–607.
22. **Kirchman, D. L., H. W. Ducklow, and R. Mitchell.** 1982. Estimates of bacterial production and growth rates based on changes in biomass and uptake rates. Appl. Environ. Microbiol. **44:**1296–1307.
23. **Kirchman, D. L., R. E. Murray, and R. E. Hodson.** 1986. Rates of DNA and protein synthesis by heterotrophic bacteria in aquatic environments: a comparison between the thymidine and leucine approaches, p. 631–637. *In* F. Megusar and M. Gantar (ed.), Perspectives in microbial ecology. Slovene Society for Microbiology, Ljubljana, Yugoslavia.
24. **Moriarty, J.** 1986. Measurement of bacterial growth rates in aquatic systems from rates of nucleic acid synthesis. Adv. Microb. Ecol. **9:**245–292.
25. **Moriarty, J., and P. C. Pollard.** 1981. Bacterial biomass and productivity in sediments. J. Microbiol. Methods **7:**91–101.
26. **Munster, V.** 1991. Extracellular enzyme activity in eutrophic and polyhumic lakes, p. 96–122. *In* R. J. Chrost (ed.), Microbial enzymes in aquatic environments. Springer-Verlag, New York, N.Y.
27. **Pace, M. L., and E. Funke.** 1991. Regulation of planktonic microbial communities by nutrients and herbivores. Ecology **72:**904–914.
28. **Riemann, B., and R. T. Bell.** 1990. Advances in estimating bacterial biomass and growth in aquatic systems. Arch. Hydrobiol. **118:**385–402.
29. **Riemann, B., P. K. Bjornsen, S. Newell, and R. Fallon.** 1987. Calculation of cell production of coastal marine bacteria based on measured incorporation of ^{3}H-thymidine. Limnol. Oceanogr. **32:**471–476.
30. **Riemann, B., J. Fuhrman, and F. Azam.** 1982. Bacterial secondary production in freshwater measured by ^{3}H-thymidine incorporation method. Microb. Ecol. **8:**101–114.
31. **Riemann, B., R. T. Bell, and N. O. G. Jorgensen.** 1990. Incorporation of thymidine, adenine and leucine into natural bacterial assemblages. Mar. Ecol. Prog. Ser. **65:**87–94.
32. **Robarts, R. D., R. J. Wicks, and L. M. Sephton.** 1986. Spatial and temporal variations in bacterial macromolecule labeling with [*methyl*-^{3}H]thymidine in a hypertrophic lake. Appl. Environ. Microbiol. **52:**1368–1373.
33. **Simon, M., and F. Azam.** 1989. Protein content and protein synthesis rates of planktonic marine bacteria. Mar. Prog. Ser. **51:**201–213.
34. **Sims, R. C., D. L. Sorensen, J. L. Sims, J. E. McLean, R. Mahmood, and R. R. DuPont.** 1984. Review of in place treatment techniques for contaminated surface soils, vol. 2. Background information for in situ treatment. EPA/520/2-84-003b. Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio.
35. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry. W. H. Freeman and Company, New York, N.Y.
36. **Strickland, J. D. H., and T. R. Parsons.** 1977. A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa, Ontario, Canada.
37. **Thorn, P. M., and R. M. Ventullo.** 1988. Measurement of bacterial growth rates in subsurface sediments using the incorporation of tritiated thymidine into DNA. Microb. Ecol. **16:**3–16.
38. **Tibbles, B. J., C. D. Davis, J. M. Harris, and M. I. Lucus.** 1992. Estimates of bacterial productivity in marine sediments and water from a temperate saltmarsh lagoon. Microb. Ecol. **23:**195–209.
39. **Toolan, T., J. C. Wehr, and S. Findlay.** 1991. Inorganic phosphorus stimulation of bacterioplankton production in a meso-eutrophic lake. Appl. Environ. Microbiol. **57:**2074–2078.
40. **Tulonen, T.** 1993. Bacterial production in a mesohumic lake estimated from [^{14}C]leucine incorporation rate. Microb. Ecol. **26:**201–217.
41. **Tulonen, T., K. Salonen, and L. Arvola.** 1992. Effects of different molecular weight fractions of dissolved organic matter on the growth of bacteria, algae, and protozoa from a highly humic lake. Hydrobiologia **229:**239–252.
42. **Vestal, J. R., and D. C. White.** 1989. Lipid analysis in microbial ecology:

quantitative approaches to the study of microbial communities. Bioscience **39:**535–541.

43. **White, D. C.** 1983. Analysis of microorganisms in terms of quantity and activity in natural environments, p. 37–66. *In* J. H. Slater, R. Whittenbury, and J. W. T. Wimpenny (ed.), Microbes in their natural environments. Cambridge University Press, Cambridge, United Kingdom.
44. **White, D. C., G. A. Smith, M. J. Gehron, J. H. Parker, R. H. Findlay, R. F. Martz, and H. L. Fredrickson.** 1982. The groundwater aquifer microbiota: biomass, community structure and nutritional status. Dev. Ind. Microbiol. **24:**201–211.
45. **Zar, J. H.** 1972. Significance testing of the Spearman rank correlation coefficient. J. Am. Stat. Assoc. **67:**578–580.