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Seasonal patterns of substrate utilization by bacterioplankton: case studies in four temperate lakes of different latitudes

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ABSTRACT: Utilization of 95 carbon sources by bacteria in 2 Canadian Shield lakes and 2 Texan reservoirs was determined using Biolog-GN microtiter plates. Triplicate plates were inoculated and incubated for 5 d, during which color development was monitored twice daily by optical density (OD_{595}). Optical densities in plate wells containing carbon substrates were corrected for blank absorbance at each measurement time. Then, data on optical densities of all substrate wells were selected from a single measurement time, to construct a community-level physiological profile. This measurement time was chosen so that plates would be compared at similar levels of color development. This data selection also generally maximized correlations of substrate utilization patterns determined from replicate plates. Multivariate analyses (ordinations) of the community-level physiological profiles identified several amino acids, carboxylic acids, and carbohydrates associated with seasonal patterns of substrate utilization. Multivariate analyses further revealed patterns in substrate utilization common to all 4 lakes: strong relative responses to amino and carboxylic acids in cool seasons and strong relative responses to carbohydrates in warm seasons. These patterns may be driven by seasonal events among phytoplankton that influence carbohydrate supply, and appear to be dampened in an oligotrophic lake, which does not support high algal abundance.

KEY WORDS: Bacterioplankton · Biolog-GN · Substrate utilization pattern · Community-level physiological profile

INTRODUCTION

Limnologists have a well-developed concept of 'seasonal succession' for phyto- and metazoan plankton (Sommer et al. 1986). This concept describes annual cycles of demographic and ecological events in populations and communities of these organisms in pelagic zones of lakes. Variations of successional cycles associated with lake trophic status, climate, and morphometry are at least partially understood. By contrast, seasonal succession of bacterioplankton is poorly understood. Although periods of high and low abundance and high and low growth rate and activity have been identified for bacterioplankton as a whole, there is little knowledge of seasonal events within the bacterioplankton assemblage to complement the detailed species-level knowledge of aquatic eukaryotes (Pedrós-Alió 1989, but see Höfle et al. 1999).

In part, this deficit stems from the traditional study of microbial assemblages as 'black boxes'—in which abundance and total activity have been quantified through a remarkable variety of analytical approaches. Techniques offering greater resolution for the study of microbial plankton assemblages are now emerging (Øvreås 2000). These include chemotaxonomic analyses (White et al. 1997) and molecular techniques ranging from DNA hybridization (Torsvik et al. 1994) to characterization of genetic variation (e.g. Muyzer et al. 1993, Øvreås et al. 1997, 1998, Øvreås & Torsvik 1998)

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to sequencing and phylogenetic analysis of specific genes (e.g. Giovannoni et al. 1990, Fuhrman et al. 1993). These techniques have great promise, but much work remains to relate measured genetic variation to ecologically important phenotypic variation.

In this paper we take a purely phenotypic approach to seasonal succession in bacterioplankton, characterizing patterns of potential carbon substrate use with Biolog-GN microplates (Garland 1997). Our interest in this technique lies in its potential to detect variations in the 'guild' structure of bacterioplankton assemblages (Pedrós-Alió 1989). Microbial guilds use similar energy sources, and electron donors and acceptors. In this context, bacterioplankton are typically aerobic heterotrophs, differentiated *inter alia* by their abilities to use particular substrates for growth. In the Biolog technique, such capabilities are tested using a battery of substrates (see 'Materials and methods' and 'Discussion' for further information). It is reasonable to hypothesize that seasonal differences in such capabilities occur. Seasonal changes in bacterioplankton genotypes have recently been documented (Höfle et al. 1999). Moreover, several studies have documented seasonal variations in bacterial uptake and turnover of carbohydrates (Hanisch et al. 1996, Bunte & Simon 1999) and amino acids (Rosenstock & Simon 1993, Simon 1998). Such variations in substrate turnover appear to be driven by variations in availability of substrates supplied by phytoplankton exudation and zooplankton grazing.

We hypothesized that the Biolog technique would detect seasonal patterns in substrate utilization, and that these patterns would have common features among lakes of different trophic status and climate. To address these propositions we studied 2 lakes in the cold temperate climate of western Ontario, Canada, and 2 lakes in the warm temperate climate of north Texas, USA. Also, the 2 Canadian lakes differed greatly in trophic status due to whole lake experiments on eutrophication.

MATERIALS AND METHODS

Field sites. Bacterioplankton were studied from 4 lakes located in 2 regions with differing climate and geology, and differing in trophic status, morphometry, and thermal stratification (Table 1). Two lakes (L227 and L239) were on the granitic Canadian Shield in the Experimental Lakes Area (ELA), Ontario, Canada, and 2 (Joe Pool Lake and Eagle Mountain Lake) are reservoirs on Mesozoic sedimentary rock in the Dallas-Fort Worth area, Texas, USA.

L239 is an oligotrophic glacial lake that is the subject of long-term monitoring (Schindler et al. 1990, 1996) and has been used as an unmanipulated reference lake for experiments in nearby lakes. L239 was stratified during the period of sampling, and its seston C:P and C:N ratios (Table 1) suggested severe nitrogen and phosphorus deficiency of phytoplankton throughout the growing season (Healey & Hendzel 1980). If stoichiometric ratios in the seston also characterize bacterioplankton, then phosphorus limitation is likely (Vadstein & Olsen 1989, Chrzanowski & Kyle 1996).

L227 is smaller and shallower than L239, and is the subject of 30 yr of experimental phosphorus fertilization (until 1990 nitrogen was also added), which has rendered it highly eutrophic (Schindler 1971, 1990). Cyanobacterial blooms often occur, and in the year studied here, a large population of *Aphanizomenon* spp. developed during the first half of the growing season, and collapsed in mid-summer. L227 was stratified during the period of sampling, and its seston stoichiometry suggested moderate nitrogen and phosphorus deficiency of phytoplankton, and phosphorus limitation of bacteria (Table 1). These stoichiometric indices also suggested that nutrient deficiency became more severe as the growing season progressed.

Joe Pool Lake is a reservoir in north Texas, and was impounded in 1986. It was only occasionally and weakly stratified during the period of sampling. The lake is mesotrophic, and peak algal abundance occurred in mid-summer (mixed-species bloom of the diatom *Aulacoseira granulata* and several cyanobacteria). Seston stoichiometry suggests severe nitrogen deficiency and moderate phosphorus deficiency of phytoplankton (Table 1), with nitrogen deficiency strongest in late spring and phosphorus deficiency strongest in late summer. Seston stoichiometry suggests phosphorus limitation of bacteria.

Eagle Mountain Lake is also a reservoir in north Texas, and was impounded in 1932. Like Joe Pool Lake, it was only occasionally and weakly stratified during the period of sampling. The lake is eutrophic and peak algal abundance occurred in late summer with abundant filamentous cyanobacteria. Seston stoichiometry suggests moderate nitrogen and phosphorus deficiency of phytoplankton (Table 1), with phosphorus deficiency severe during summer, and nitrogen deficiency severe during spring and autumn. Seston stoichiometry suggests phosphorus limitation of bacteria.

Sampling and laboratory analysis. Each lake was sampled about every 2 wk at a single station near the deepest point. Sampling was conducted from May to August in Canada, and from March to December in Texas (Table 1). Depth profiles of temperature and dissolved oxygen were taken using YSI instruments, and from these, the depth of the surface mixed layer was determined. When lakes were not stratified, the bottom was taken as the limit of mixing. Samples were

Table 1. Characteristics of the lakes studied: L239, L227, Joe Pool Lake (JPL) and Eagle Mountain Lake (EML). For total phosphorus, total nitrogen, seston composition ratios, and Secchi depth, the mean and range of measurements during the study period are reported

Parameter	Lake			
	L239	L227	JPL	EML
Location	49°40′ N, 93°43′ W	49°41′ N, 93°42′ W	32°38′ N, 97°0′ W	32°53′ N, 97°30′ W
Period studied (Julian days)	146–229	142–233	71–321	83–342
Surface area (ha)	56.1	5.0	3620	3653
Mean depth (m)	10.5	4.4	7.2	6.1
Water residence time (yr)	20	4.2	2.5	0.5
Total dissolved solids (mg l^{-1})	20	60	270	270
Total phosphorus (µM)	0.50	1.66	0.80	1.66
	(0.23–0.77)	(0.98–2.36)	(0.41–1.44)	(1.06–2.35)
Total nitrogen (µM)	23.8	43.4	35.3	35.3
	(21.0–26.0)	(36.1–54.8)	(26.9–63.0)	(23.5–52.6)
Seston C:P (molar)	428	317	321	188
	(187–752)	(204–404)	(79.3–826)	(50.6–301)
Seston C:N (molar)	22.6	14.0	17.2	12.7
	(18.4–28.2)	(9.0–20.2)	(11.1–23.0)	(10.2–16.6)
Seston N:P (molar)	17.8	23.5	18.9	14.9
	(8.5–22.9)	(14.6–33.7)	(7.1–49.5)	(4.2–67.8)
Secchi depth (m)	4.1	1.0	1.1	1.1
	(3.0–4.6)	(0.5–1.7)	(0.1–2.1)	(0.7–1.7)
Average thermocline depth (m)	4.1	1.9	None	None

taken with a Van Dorn bottle at discrete depths near the top, bottom, and middle of the mixed layer and were combined to create a pooled mixed-layer sample (PML). Three such pooled samples were taken during each sampling trip, thereby creating true triplicate samples.

Upon return to the laboratory each PML was subsampled for determination of a number of chemical and biological properties. Sub-samples were removed from each PML and preserved in formaldehyde (2% final concentration) for enumeration of bacteria. Aliquots were filtered (Whatman GF/F), immersed in saturated $MgCO_3$ (1 ml) and frozen for later determination of chlorophyll *a* (chl *a*) concentration, and additional filters were prepared for later determinations of particulate carbon, nitrogen, and phosphorus. Bacteria were enumerated by epifluorescence microscopy using DAPI as the fluorochrome (Porter & Feig 1980). Chl *a* was determined by fluorometry (Turner model 10-AU) following an overnight freeze-thaw extraction (without grinding) of pigments in 90% acetone (Marker et al. 1980). The fluorometer was configured as in Welschmeyer (1994) to measure chl *a* directly, without acidification to correct for pheopigments. Particulate carbon and nitrogen were determined with a Perkin-Elmer CHN analyzer, and particulate phosphorus was determined after wet digestion in persulfate (Menzel & Corwin 1965).

Assessment of substrate utilization potential. Community-level physiological profiles (CLPPs) of potential substrate use by bacteria were determined with Biolog-GN™ (original type) 96-well microtiter plates (Garland 1997). One CLPP was taken from each PML, giving triplicates for each sampling date on each lake. Biolog plates contain 95 carbon substrates, with the 96th well serving as a blank. Each well also contains a mineral medium, phosphate buffer, and tetrazolium dye, which is reduced to a colored compound if respiratory metabolism occurs in the well. Each well on a plate was inoculated with 150 µl of lake water, and the plates were incubated for at least 4 d. Incubations were conducted at room temperature in Texas (ca 22°C). Temperature-regulated rooms were unavailable in Canada, so a 9°C incubator was used for the first 3 samples from L227, and the first 2 from L239. All other plates were incubated at 19°C. We have assumed that incubation temperature affects only the rate at which substrates are used, and not patterns of relative substrate use. We recognize that this assumption is largely untested. Optical density (OD) was determined twice daily at 595 nm (OD_{595}) and data were electronically recorded. For the reasons noted below (see 'Discussion'), we equate increasing absorbance (respiration) with growth.

Constructing the CLPP. Data processing for each Biolog plate began by subtracting the OD of the blank well from all others, and then calculating the average (blank-corrected) well color development (AWCD) as the average OD of all wells on a single plate at a single reading time. Previous studies emphasize comparisons of CLPP among plates at comparable levels of AWCD (Garland 1997). Typically, AWCD started low (<0.1) for

all replicate plates from a sample, increased exponentially up to an OD of about 0.8, and approached a plateau above OD 1 after several days' incubation. Average pair-wise correlations between replicate plates for color development in the different wells usually reached a peak or a plateau at about the time that AWCD reached 0.4. For each plate, we selected data to construct the CLPP from the first reading time at which AWCD for all 3 replicate plates in a sample exceeded an OD of 0.4. Thus, data for the CLPP were taken at different times for different samples, but were taken at the same time for the replicates within a sample. The CLPP thus obtained consists of 95 readings of blank-corrected OD, corresponding to wells with different carbon substrates. In one sample only, we accepted data from a single plate even though its AWCD never reached 0.4 during incubation. Plate data selected by these procedures had an overall average AWCD of 0.60 (standard deviation 0.16), with a range of 0.2 to 1.25.

Multivariate statistical analysis. Prior to multivariate analysis, we subtracted both the mean color development for each plate, averaged over wells within a plate, and mean color development for each well, averaged over all plates in the analysis. This data 'centering' reduces the influence of wells (i.e individual substrates) that consistently produced a high color development, and plates that produced several highly colored wells (e.g. due to a high inoculum density). Thus, multivariate analyses focused on deviations of color development associated with growth on a particular substrate, from the mean color development for that plate and that substrate.

Given our current, poor knowledge of succession in bacterioplankton assemblages, the goal of this study was primarily exploratory, and thus indirect gradient analysis was used to identify major patterns of variation in the data. (Effective use of alternative, direct gradient analysis in relation to specific environmental variables generally requires prior hypotheses to structure the analysis [Ter Braak & Prentice 1988].) Ordination analyses were conducted separately for data from Canadian and Texan lakes to identify time trends and differences between lakes within each region. Preliminary ordinations using detrended correspondence analysis showed that gradient lengths of the extracted axes of variation were all <1.4 units of pooled standard deviation. This is far less than the threshold of 4 units at which nonlinear ordination methods are recommended (Ter Braak & Smilauer 1998), so the linear method of principal components analysis (PCA) was used. Although data were centered, they were not standardized, and thus the PCA was based on the covariance matrix. Multivariate analyses were computed with CANOCO 4 (Ter Braak & Smilauer 1998), and checked with PC-ORD (McCune & Mefford 1997).

Previous studies using Biolog assays suggested aggregating responses to substrates within functional classes (such as carbohydrates, amino acids, etc.) to better highlight general patterns of substrate utilization (Zak et al. 1994, Lehman et al. 1995). Therefore, the above analyses were repeated after summing responses to all substrates grouped within 11 functional classes (Garland & Mills 1991, Lehman et al. 1995): carbohydrates, carboxylic acids, amino acids, esters, alcohols, aromatics, amides, amines, polymers, brominated compounds, and phosphorylated compounds. A complete list of the substrates within each class may be found in Garland & Mills (1991), and all substrates are listed in Table 1.

RESULTS

Canadian lakes

Dynamics of chl *a*, bacterioplankton density, and temperature in all lakes are shown in Fig. 1. L239 is oligotrophic, with chl *a* reaching about 5 µg l^{-1} in late spring and falling to below 2 µg l^{-1} during summer. Bacterioplankton abundance increased about 2-fold from spring through mid-summer, reaching about 1.3×10^9 cells l^{-1}. In eutrophic L227 chl *a* reached about 70 µg l^{-1} by mid-summer. This peak was associated with a bloom of *Aphanizomenon* spp., which subsequently collapsed. Following collapse of the bloom, chl *a* remained below 20 µg l^{-1}. Bacterioplankton abundance reached its highest level, at about 1.0×10^{10} cells l^{-1}, shortly after the algal bloom collapsed. L239 and L227 were both stratified throughout the sampling period, with thermoclines near 5 m. Water temperatures in the mixed layer rose from about 16°C in late spring to 20–22°C in late summer. Water temperatures in the hypolimnion averaged 5.0°C in L239 (standard deviation 0.3°C) and 4.3°C (standard deviation 0.8°C) in L227.

Texan lakes

In Joe Pool Lake, chl *a* peaked in mid-year, attaining a level near 25 µg l^{-1}. Following this peak, chlorophyll remained below 10 µg l^{-1}. Bacterioplankton abundance was variable with several peaks. In Eagle Mountain Lake, chl *a* increased throughout spring and developed a broad, late summer maximum of about 30 µg l^{-1}. Bacterioplankton abundance attained its maximum in late spring at about 8.0×10^9 cells l^{-1}. Eagle Mountain and Joe Pool Lakes were never strongly stratified during sampling. Temperatures in the water column ranged between 11°C (spring) and about 30°C (summer).

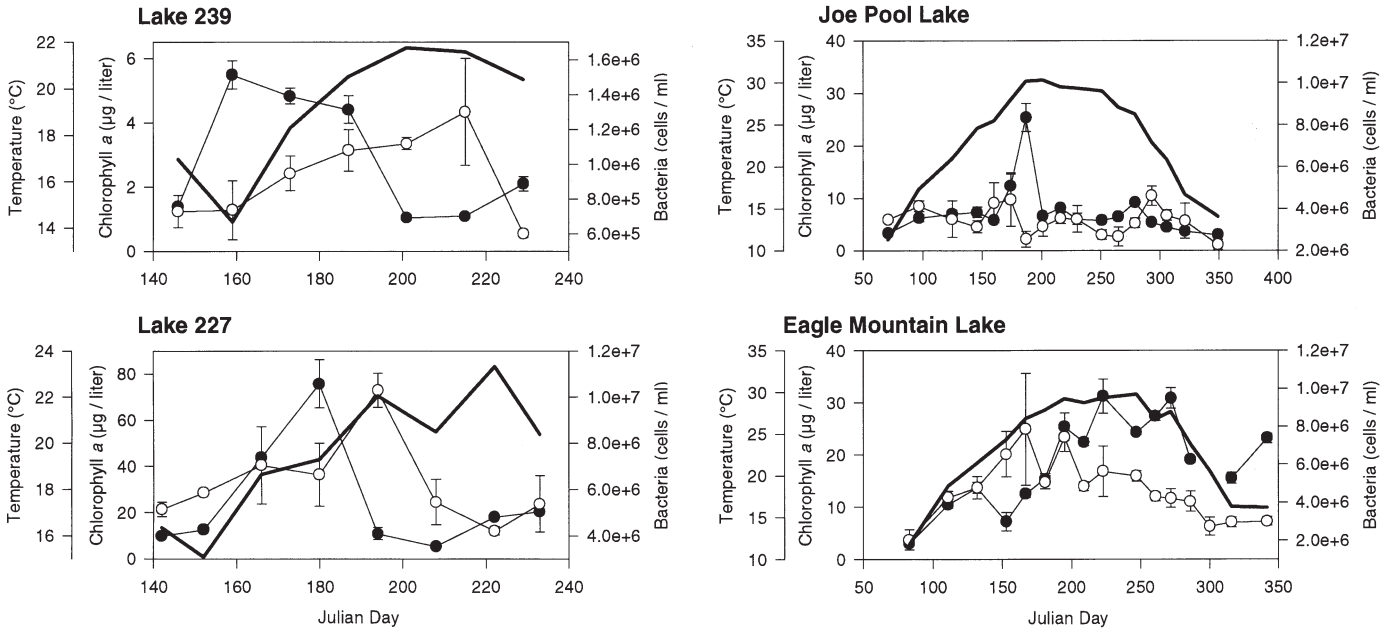

Fig. 1. Seasonal dynamics of phytoplankton (chlorophyll *a*), bacterioplankton, and temperature. Heavy solid line: temperature in the upper 5 m (L239 and L227), or in the whole water column (Joe Pool and Eagle Mountain Lakes); (●) chlorophyll *a*; (○), bacterial abundance

Community level physiological profiles

Properties of the CLPP

Example profiles from 2 dates in each lake (1) showed that measured utilization typically differed among substrates, (2) showed similarities among replicates within a sample, and (3) revealed differences and similarities among lakes and sampling times (Figs. 2 & 3). In displaying Figs. 2 & 3, relative responses for each well on each plate were calculated by subtracting the AWCD for that plate. Substrates were then grouped into 4 functional classes, and arrayed within each class in descending order of average utilization over all samples in this study (Table 2). This arrangement accounts for the general decreasing trend of

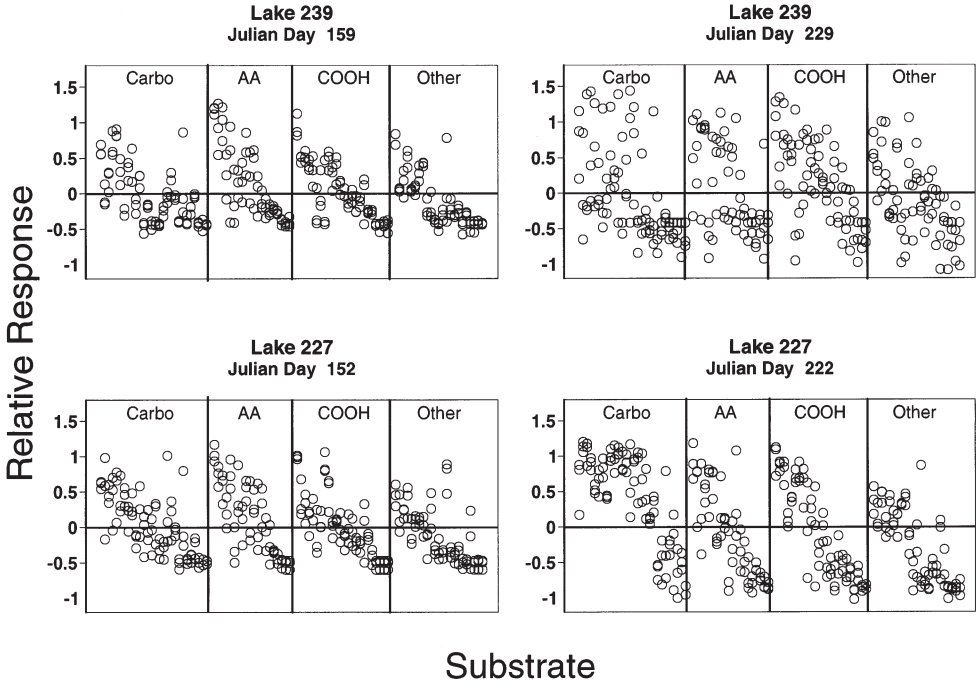

Fig. 2. Example of the community-level physiological profiles (CLPPs) from the Canadian lakes. Relative responses from the 3 replicate plates for selected sample dates are displayed: these are (blank-corrected) optical densities for the 95 wells on a plate, centered by subtracting the average optical density for all wells on the plate (AWCD). Substrates are arranged in 4 functional classes, and within each class they are arrayed in order of decreasing average utilization over the entire study (Table 2). Carbo: carbohydrates; AA: amino acids; COOH: carboxylic acids

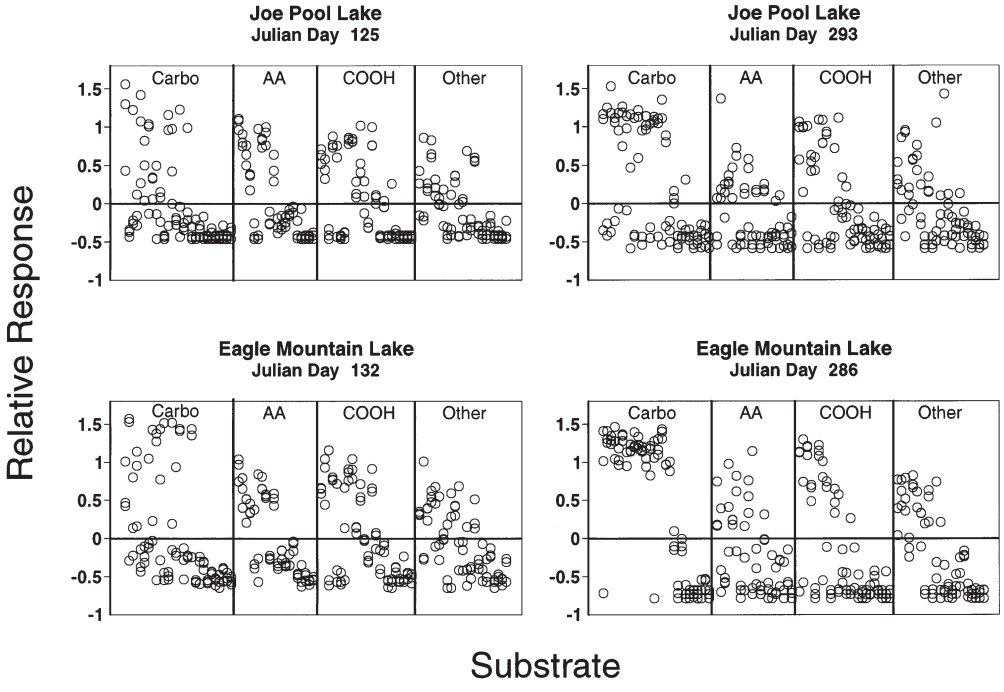

Fig. 3. Example CLPPs from the Texan lakes. Relative responses from 3 replicate plates for selected samples are displayed. Data are calculated and arranged as in Fig. 2

Table 2. Substrates on the Biolog-GN plate, arranged in functional classes, and ranked in decreasing order of average utilization over all samples in this study. In example profiles (Figs. 2 & 3) substrates within a class are arrayed in this order

Carbohydrates		Amino acids		Carboxylic acids		Other	
Well	Substrate	Well	Substrate	Well	Substrate	Well	Substrate
B6	α-D-glucose	F8	L-asparagine	D2	Cis-acotinic acid	C11	Methyl pyruvate
C8	D-trehalose	F10	L-glutamic acid	D7	D-gluconic acid	A3	Dextrin
C7	Sucrose	F9	L-aspartic acid	D6	D-galacturonic acid	H1	Urocanic acid
B10	Maltose	G1	L-histidine	D3	Citric acid	A5	Tween 40
A10	L-arabinose	G9	L-serine	E10	D-saccharic acid	H2	Inosine
B2	D-fructose	G8	D-serine	D5	D-galactonic acid lactone	H9	Glycerol
A8	N-acetyl-D-glucosamine	G12	γ-aminobutyric acid	D9	D-glucuronic acid	A6	Tween 80
B11	D-mannitol	G6	L-proline	E1	p-hydroxyphenyl-acetic acid	H12	Glucose-6-PO_4
B4	D-galactose	F6	L-alanine	E9	Quinic acid	H11	Glucose-1-PO_4
B12	D-mannose	G7	L-pyroglutamic acid	E6	D,L-lactic acid	H6	Putrescine
C6	D-sorbitol	F5	D-alanine	E4	α-keto-glutaric acid	A4	Glycogen
C2	β-methyl-D-glucoside	G2	Hydroxy-L-proline	D11	β-hydroxybutyric acid	A2	α-cyclodextrin
A12	Cellobiose	F7	L-alanyl-glycine	E12	Succinic acid	H3	Uridine
B5	Gentobiose	G4	L-ornithine	E2	Itaconic acid	F2	Succinamic acid
C1	D-mellibiose	G11	D,L-carnitine	E8	Propionic acid	H7	2-aminoethanol
C4	D-raffinose	F12	Glycyl-L-glutamic acid	E11	Sebacic acid	F1	Bromosuccinic acid
A7	N-acetyl-D-galactosamine	F11	Glycyl-L-aspartic acid	E7	Malonic acid	H10	D,L-α-glycerol PO_4
C5	L-rhamnose	G3	L-leucine	D12	γ-hydroxybutyric acid	H4	Thymidine
B7	m-inositol	G10	L-threonine	D8	D-glucosaminic acid	C12	Monomethyl succinate
A11	D-arabitol	G5	L-phenylalanine	D1	Acetic acid	H5	Phenylethyl-amine
C9	Turanose			E3	α-keto-butyric acid	F4	Alaninamide
B3	L-fucose			D4	Formic acid	H8	2,3-butanediol
B8	α-D-lactose			D10	α-hydroxybutyric acid	F3	Glucuronamide
A9	Adonitol			E5	α-keto-valeric acid		
C10	Xylitol						
B9	Lactulose						
C3	D-psicose						
B1	i-erythritol						

relative response seen within each class on each graph. Substrates with positive relative responses were utilized to a greater extent than the average substrate, and those with negative relative responses were utilized to a lesser extent.

Those substrates that consistently produced low relative responses are probably poorly degraded by the bacterioplankton studied here. However, most substrates apparently supported at least some bacterial activity at some times, as the overall occurrence of negative responses (wells with OD less than the blank) was only 9.6%.

Inspection of the example profiles suggests some differences among lakes and sampling times. Both Canadian lakes apparently show higher carbohydrate utilization at the later dates illustrated (Julian Day [JD] 229, August) than at the earlier dates (JD 159, June), and L227 shows greater carbohydrate utilization than L239 at both times (Fig. 2, compare relative response heights). Both Texan lakes show greater carbohydrate utilization at the later dates illustrated (JD 293, October) than at the earlier dates (JD 125, May) (Fig. 3). However, it is difficult to detect general patterns from simple examination of the many profiles obtained in this study, and therefore a multivariate technique (PCA) was applied.

Multivariate analysis

Canadian lakes: Relative eigenvalues of the first 4 principal components are 0.239, 0.092, 0.076, and 0.055. Jackson (1993) suggested that principal components be considered significant if their eigenvalues exceed expectations based on the broken-stick distribution, which applies to a null model of random data with no true correlations. CLPPs have 95 variables, yielding broken-stick expectations of 0.054, 0.044, 0.038 and 0.035. Thus the first 4 axes could be considered significant. To be conservative, however, we plotted and interpreted only the first 2 PCA axes (Fig. 4). The first 2 principal components accounted for 33% of the variance in CLPP data.

Eight substrates had >50% fit to the first 2 principal components, i.e. a regression of relative color development for these wells (after data centering) onto the first 2 PCA axes had $R^2 > 0.5$. For conventional statistics, a correlation of this magnitude would have $p < 0.01$, although here we merely use this as a heuristic criterion to identify substrates associated with overall patterns in the data. Fig. 4 shows their loadings (i.e. correlations with the first 2 axes) as arrows. Arrows strongly aligned to the positive direction of an axis indicate substrates positively correlated with the pattern summarized by that axis. Arrows aligned to the opposite,

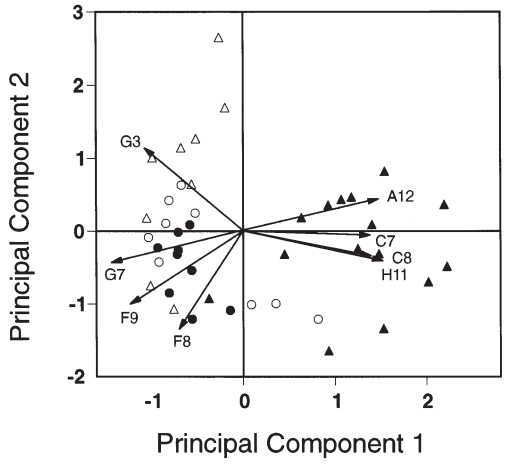

Fig. 4. Ordination diagram of CLPPs from the Canadian lakes, showing scores of the first 2 PCA axes. Filled symbols: L227; open symbols: L239; circles: samples taken prior to Julian Day 180; triangles: samples taken at or after Julian Day 180. Arrows show twice the loadings (for ease of display) for the substrates with greater than 50% fit to the first 2 PCA axes: A12, cellobiose; C7, sucrose; C8, trehalose; F8, asparagine; F9, aspartic acid; G3, leucine; G7, pyroglutamic acid; H11, glucose-1-phosphate

negative direction indicate substrates negatively correlated with the pattern summarized by that axis. The first PCA axis is thus positively associated with high color development for several carbohydrates — cellobiose, sucrose, trehalose, and glucose-1-phosphate — and negatively associated with high response to several amino acids — asparagine, aspartic acid, pyroglutamic acid, and leucine. Associations with the second PCA axis are less strong, but are positive for leucine and negative for asparagine and aspartic acid.

In the ordination plot, samples tend to separate between lakes for those samples taken at, or after, JD 180 (triangles in Fig. 4), which was the day the *Aphanizomenon* spp. bloom in L227 attained its maximum (Fig. 1). Prior to this event, patterns of color development were similar in the 2 lakes and associated with high respiration of the amino acids asparagine, aspartic acid, and pyroglutamic acid. Later in the season, patterns of substrate use diverged. Oligotrophic L239 was characterized by strong color development when leucine was the substrate, while eutrophied L227 was characterized by high color development in wells containing carbohydrates.

These broad multivariate patterns point to substrates that are important in developing a physiological profile but they do not imply that bacterioplankton in each lake responded similarly to each carbohydrate or amino acid, nor do they reveal time trends in the data. Time trends of color development were similar between lakes for some substrates (e.g. aspartic acid, cellobiose,

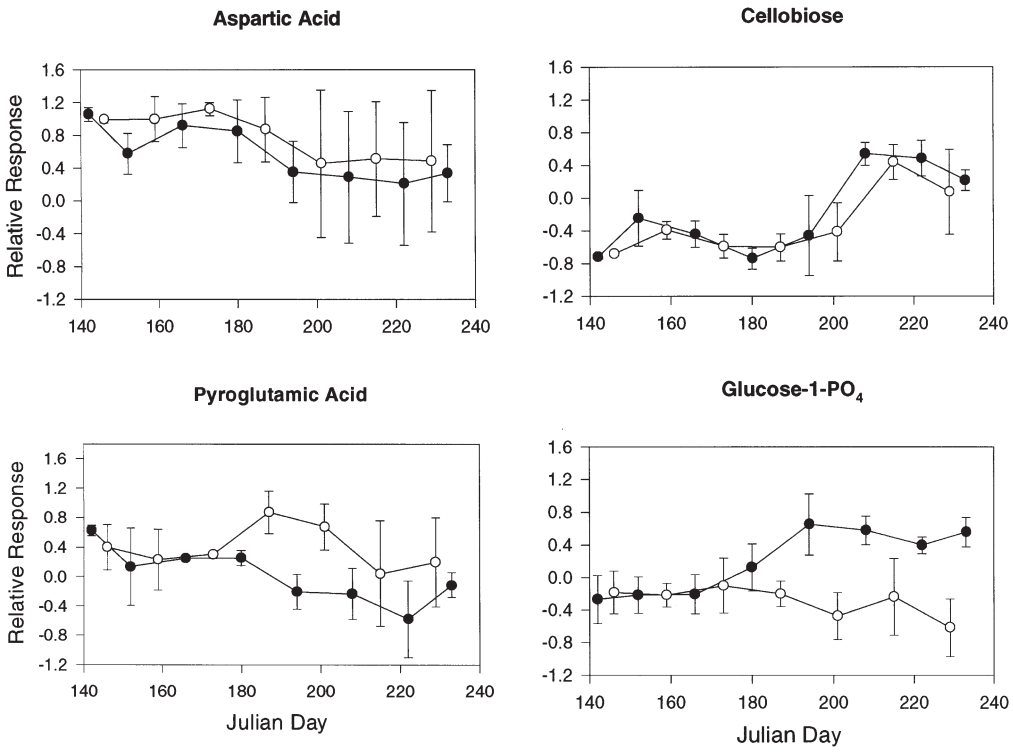

Fig. 5. Relative response for selected substrates, from CLPPs determined in the Canadian lakes. Relative response is the blank-corrected absorbance for the designated substrate, after subtracting the AWCD for the plate; data points show average of 3 replicate plates; bars show twice the standard error; (●) L227; (○) L239

Fig. 5), but quite different for others (e.g. pyroglutamic acid, glucose-1-phosphate). Color development was usually high in wells containing aspartic acid, indicating that it was among the better-metabolized substrates (Table 2), but the degree of response to this substrate declined seasonally in both lakes (Fig. 5). Color development in wells containing cellobiose was also similar in both lakes: low early in the growing season, but high at the end of the season (Fig. 5). Cellobiose was not among the substrates that were consistently well metabolized (Table 2), although it did produce a high response at times. The concordant changes in response to substrates such as aspartic acid and cellobiose in L239 and L227 suggests shared characteristics in the seasonal succession of bacterial phenotypes. For other substrates, temporal trends in color development diverge between these 2 lakes of very different trophic status. In L239 there was relatively high color development from pyroglutamic acid in mid- to late summer, whereas in L227 there was relatively low color development from pyroglutamic acid during the same period (Fig. 5). Also during mid- to late summer, bacterioplankton originating in L227 produced high color development from glucose-1-phosphate whereas bacterioplankton originating in L239 did not (Fig. 5).

The multivariate analysis identified color development patterns similar to those of pyroglutamic acid and glucose-1-phosphate. The amino acids asparagine and leucine produced higher responses in L239 than L227 in mid- and late summer, respectively. The carbohydrates sucrose and trehalose produced higher responses in L227 than L239 in late summer. Collectively, these data suggest a late season divergence in substrate use patterns in these lakes, with bacterioplankton in oligotrophic L239 producing strong color development from certain amino acids, and bacterioplankton in eutrophic L227 producing strong color development from certain carbohydrates. These relative differences developed against a general background of decreasing color development from amino acids, and increasing color development from carbohydrates in both lakes.

Texan lakes: Relative eigenvalues of the first 4 principal components are 0.176, 0.075, 0.057, and 0.047 and were considered significant according to the broken-stick criterion. As for the Canadian lakes, we plotted and interpreted only the first 2 PCA axes (Fig. 6), which accounted for 25% of the variance in potential substrate use.

Seven substrates had >50% fit to the first 2 principal components and Fig. 6 shows their loadings (as arrows) on the axes. A group of 6 amino compounds and organic acids were strongly related to the first axis — aspartic acid, pyroglutamic acid, 2-aminoethanol, α-ketoglutaric acid, quinic acid, and putrescine. An additional substrate, galactonic acid lactone, was negatively but weakly related to the first axis and more strongly related to the second axis. Of these compounds, only aspartic acid ranks high in Table 2, indicating consistently strong utilization overall in this study. The remaining substrates

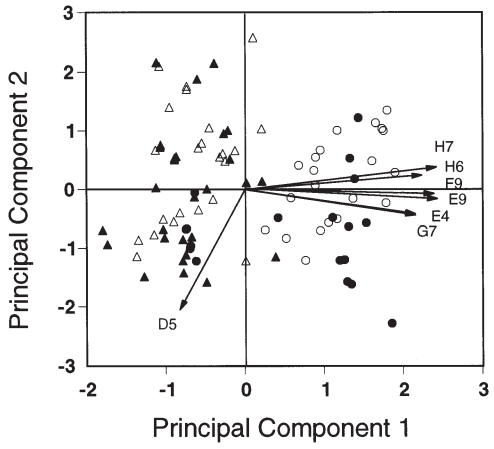

Fig. 6. Ordination diagram of CLPPs from the Texan lakes, showing scores of the first 2 PCA axes. Filled symbols: Eagle Mountain Lake; open symbols: Joe Pool Lake; circles: samples taken Julian Days 71 to 167 and 306 to 342; triangles: samples taken Julian Days 174 to 300. Arrows show 3 times the loadings for the substrates (for ease of display) with greater than 50% fit to the first 2 PCA axes: D5, galactonic acid lactone; E4, α-ketoglutaric acid; E9, quinic acid; F9, aspartic acid; G7, pyroglutamic acid; H6, putrescine; H7, 2-aminoethanol

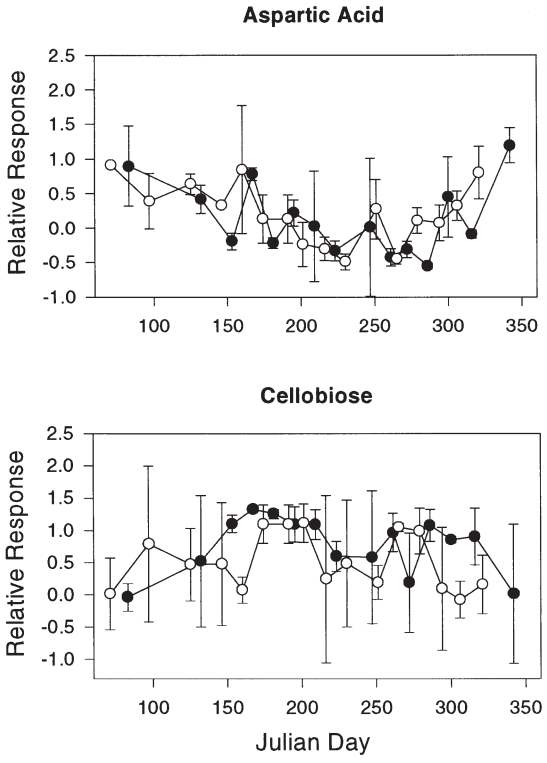

Fig. 7. Relative response for selected substrates, from CLPPs determined in the Texan lakes. Relative response is the blank-corrected absorbance for the designated substrate, after subtracting the AWCD for the plate; data points show average of 3 replicate plates; bars show twice the standard error; (●) Eagle Mountain Lake; (○) Joe Pool Lake

were generally mid-ranking in their overall use, and thus the multivariate pattern is strongly influenced by the variation in utilization of these substrates that were highly utilized only in particular samples.

The ordination plot shows no evident differences between lakes (Fig. 6). There was a distinct time trend, however, with samples shown as circles taken on JDs 71to 167 (March to June), and 306 to 342 (November to December) falling mostly on the positive end of PCA axis 1, and samples shown as triangles taken on JDs 174 to 300 (June to October) falling on the negative end. These sample dates do not correspond perfectly to the annual thermal cycles of these lakes (Fig. 1), although the period of poor color development from amino acids (negative scores on PCA axis 1, Fig. 6) does coincide with warm water temperature. During the period of positive scores on PCA axis 1, average water column temperatures in both lakes rose steadily from about 11 to 27°C. Negative scores were found while temperatures rose from about 27 to 30°C and then fell to about 20°C. A second period of positive scores on PCA axis 1 occurred while temperatures fell from about 20 to 16°C.

Examples of the time trends in color development from individual substrates (Fig. 7) illustrate the similar seasonal dynamics of the 2 Texan lakes. Aspartic acid was associated with high color development early and late in the growing season, but with a poor color development in mid- to late summer. Color development on cellobiose showed a less conspicuous, but generally opposite pattern.

Functional substrate classes

The aggregated analysis for Canadian lakes emphasizes the seasonal divergence of the 2 lakes, associated with color development from carbohydrates, amino and carboxylic acids (Fig. 8A). The first 2 PCA axes account for 94% of total variance and are significant (broken-stick criterion). All substrate classes have >70% fit to these 2 axes, with carbohydrates associated strongly with the first axis, and amino and carboxylic acids more strongly with the second. The other substrate classes correlate with the first 2 axes in a manner but roughly opposite to carbohydrates, amino and carboxylic acids (loadings not shown, for clarity).

Early in the growing season samples from L239 and L227 cluster in the upper left quadrant (circles), indicating that bacterioplankton produces poor color development from carbohydrates but strong color development from amino acids. Later, data from both lakes move to the right on the plot (triangles), reflecting higher color development from carbohydrates. Data from L239 plot high on the second axis, indicating strong relative responses to amino and carboxylic

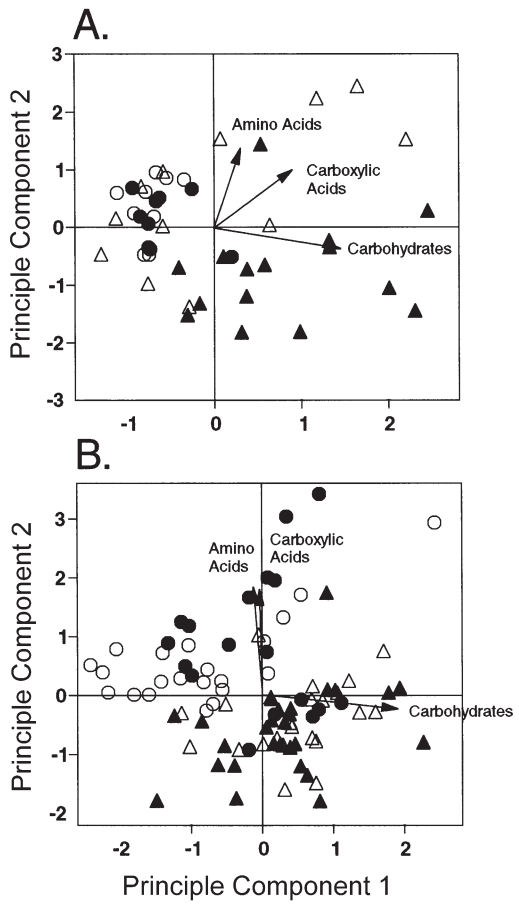

Fig. 8. Ordination diagrams based on the total responses to 11 substrate classes. Scores on the first 2 PCA axes are shown. (A) Canadian lakes: filled symbols, L227; open symbols, L239; circles, samples taken prior to Julian Day 180; triangles, samples taken at or after Julian Day 180. Arrows show 1.5 times the loadings (for ease of display) for all substrate classes. (B) Texan lakes: filled symbols, Eagle Mountain Lake; open symbols, Joe Pool Lake; circles, samples taken Julian Days 71 to 167 and 306 to 342; triangles, samples Julian Days 174 to 300. Arrows show twice the loadings for all substrate classes (for ease of display)

acids. Data from L227 plot on the negative end of the second axis, indicating relatively low color development from amino and carboxylic acids.

The aggregated analysis of data from Texan lakes also emphasizes seasonal patterns driven largely by carbohydrates and amino and carboxylic acids (Fig. 8B). The first 2 PCA axes account for 89% of total variance and are significant (broken-stick criterion). All substrate classes have >70% fit to these axes, except polymers (10%) and aromatics (45%). Carbohydrates are strongly associated with the first axis, and amino and carboxylic acids with the second. Other substrate classes correlate to the first 2 axes in a manner roughly opposite to carbohydrates, and amino and carboxylic acids (loadings not shown, for clarity). In spring and autumn, data from both lakes cluster primarily in the upper left quadrant (circles), indicating poor color development from carbohydrates and stronger color development from amino and carboxylic acids. In summer, data from both lakes plot lower and to the right (triangles), indicating strong color development from carbohydrates, and lower poor color development from amino and carboxylic acids.

The large amount of variance explained by the aggregated analyses and the high contributions of carbohydrates, amino acids, and carboxylic acids to the multivariate patterns suggest that measures of metabolism of these 3 functional classes should be informative. As simple indices of the metabolic responses to these functional classes, we added the relative responses to all substrates within a class on each replicate plate. For each sampling date, this gives 3 replicate estimates of the total response to carbohydrates, amino acids, and carboxylic acids.

In the Canadian lakes (Fig. 9A–C), total response to amino acids was high early in the growing season, when water temperature was about 16°C. Amino acid response then declined throughout the summer, to a greater extent in L227 than in L239. Total response to carboxylic acids peaked before mid-summer in both lakes, as water temperature rose to about 18°C. From then onwards, response to carboxylic acids was higher in L239 than in L227. Total response to carbohydrates peaked in late summer in both lakes, as water temperatures reached maxima of 22 to 24°C. Throughout mid- to late summer, carbohydrate response was higher in L227 than in L239.

In both of the Texan lakes (Fig. 9D–F), total response to amino acids was high, but variable, from winter through spring, becoming low during summer and most of autumn, and finally rising at the return to winter. Total response to carboxylic acids followed a similar pattern, while total response to carbohydrates followed an opposite pattern, with high response occurring throughout summer and most of autumn. In general, differences between lakes were small relative to variance between samples.

DISCUSSION

An ideal technique for assessing microbial communities would be free of methodological biases, identify a large number of phenotypic and phylogenetic variants, and distinguish between dominant and rare populations. No such technique currently exists. The one adopted here — substrate utilization patterns of natural samples inoculated in Biolog-GN microplates — has been widely applied in recent years, but has also been

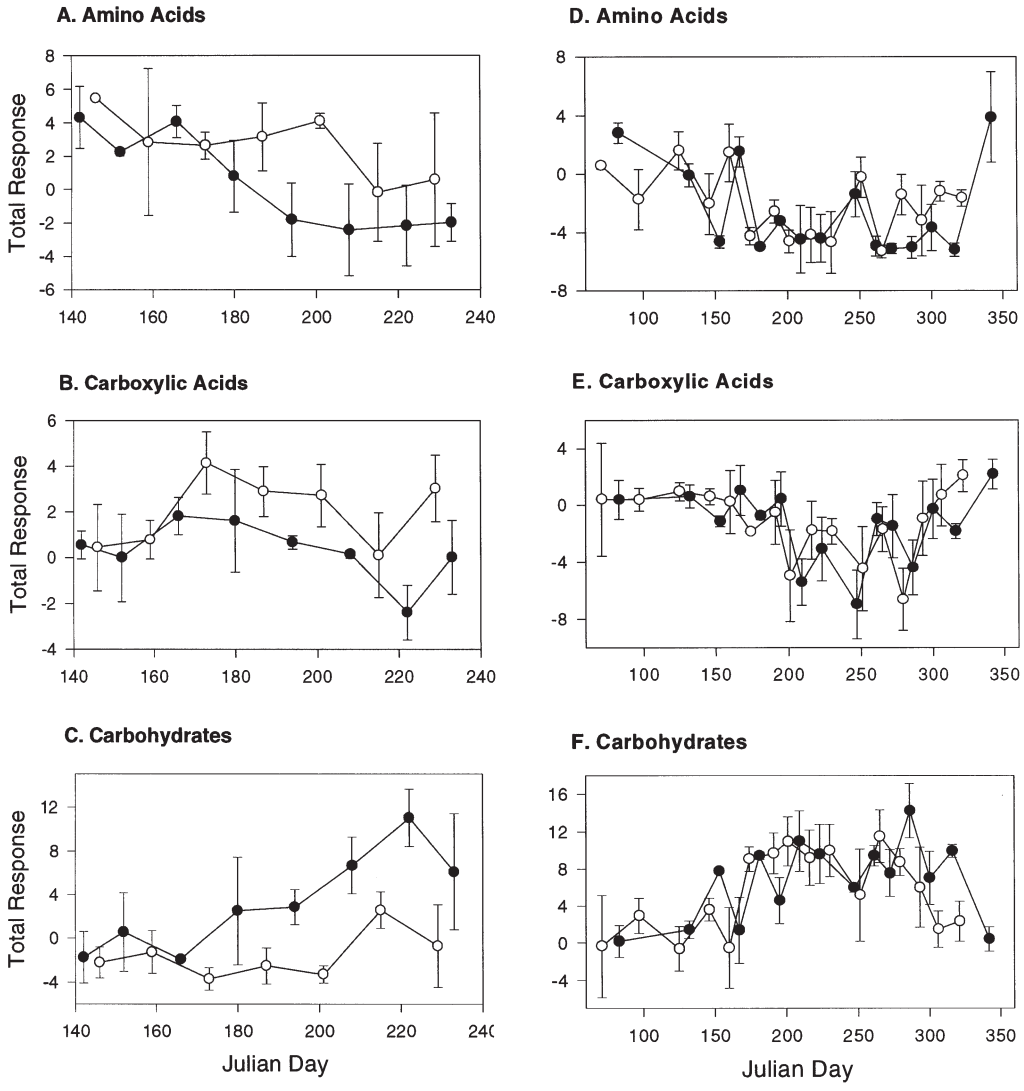

Fig. 9. Indices of total utilization of amino acids, carboxylic acids, and carbohydrates. (A to C) Canadian lakes. (D to F) Texan lakes

criticized (Konopka et al. 1998, Øvreås 2000). Certain aspects of this approach require commentary, as they influence the interpretation of its results.

Applied to dilute natural samples (such as lake water), the Biolog method involves culturing. A total population of about 10^8 cells appears necessary to produce measurable color development in a well (Haack et al. 1995). Here, 150 µl inocula of lake water contained 10^5 to 10^6 cells, and thus a period of growth in Biolog media was probably needed to produce color development, even if all inoculated cells used the substrate in the well. Moreover, variations in color development among different substrates are usually strongest when the AWCD of a plate is in its exponential phase, and the data used to construct a CLPP are often drawn from this phase (as was done here). Color development at this stage is associated with the most rapidly growing members of an assemblage (Verschuere et al. 1997), and thus the method is biased towards detection of such organisms. Other potential problems include the choice of substrates tested (originally chosen for use in identifying pathogenic bacteria), and the high representation of simple carbohydrates (28 compounds), amino acids (20 compounds), and carboxylic acids (24 compounds) among the 95 substrates.

Many of the Biolog substrates are known to occur in lake water, especially among the carbohydrates and amino acids used (Riemann et al. 1986, Jørgensen 1987, Chróst et al. 1989, Münster 1993, Jørgensen & Jensen 1994, Simon 1998, Bunte & Simon 1999). A large number of other substrates whose presence has not been documented (probably because it has not been sought) are common biochemicals with many potential sources in lakes. Despite the origins of the Biolog technique in medical microbiology, the sub-

strates involved may have relevance in the environments studied here.

The growth bias of the method is a more serious issue. It has been suggested that environmental samples are dominated by slow-growing, unculturable bacteria (Konopka et al. 1998). However, Biolog data are likely dominated by opportunists with rapid growth potential (given a suitable carbon substrate) and wide tolerance for factors such as temperature and ionic strength, and thus may not detect differences among samples resulting from other members of the assemblage. The opportunist populations whose activity likely dominates Biolog profiles need not be the majority of the members of the bacterioplankton community (Smalla et al. 1998). For the purposes of this study, aimed at detecting seasonal variations in bacterial communities, the worst case scenario would arise if the culturable organisms consisted of a single opportunist population. The method might then detect its variations in abundance but not any changes taking place among remaining, unculturable bacteria. Such a worst case scenario could be recognized in an ordination analysis. There would be a strong first axis and strong positive loadings for the group of substrates best for that population's growth. In graphs such as Figs. 4 & 6, there would be only 1 group of arrows closely aligned in the positive direction along the first axis of the PCA.

This pattern did not arise in this study. Instead, more complex substrate utilization patterns suggest that more than 1 population within the bacterioplankton in these lakes grows in Biolog assays, and that these populations differed in their abilities to grow upon carbon substrates. Moreover, consistent seasonal patterns were found in these lakes. A simple explanation of these patterns is that populations of opportunistic bacteria with different substrate utilization capabilities vary in abundance over the growing season. When abundant, such an opportunist population is a larger fraction of the inoculum, and contributes to greater color development in wells containing its favored substrates.

This scenario could perhaps also explain the large variance in response to individual substrates sometimes seen between replicate plates. For example, the time trend of aspartic acid in the Canadian lakes (Fig. 5) shows high variance late in the growing season. A low total abundance of bacterioplankton could yield high variance in total numbers of cells inoculated in each well, with attendant variation in measured responses. However, late season bacterioplankton densities are of the order of 10^6 cells ml^{-1}, yielding ca 10^5 cells in the inoculum of each well, making variation in total abundance an unlikely explanation for variation in measured responses. However, if specific opportunistic populations metabolizing a given substrate were becoming rare at this time, then their proportion in the inoculum would be highly variable, producing a more variable response. High variability among the same wells (substrates) on replicate plates could also indicate that high phenotypic diversity of bacterioplankton, with no single phenotype dominating, and thus high variability between replicate inocula in the representation of different types. Our subsequent interpretations are based on the presumption that populations of opportunistic bacteria will vary seasonally, producing patterns of substrate utilization potential.

The basic pattern found here is that high color development from carbohydrates occurs in mid- to late summer under relatively warm conditions, and that at other, cooler times of year color development from amino and carboxylic acids is higher. One possibility is that those opportunistic bacterioplankton capable of using a class of substrates increase in abundance when the relative supply of that substrate class is seasonally elevated. In all of the lakes studied here, stoichiometry indicates limitation of phytoplankton growth by minerals (Table 1). Phytoplankton with sufficient light for photosynthesis, but whose growth is otherwise limited, often release large proportions of fixed carbon as dissolved organic matter (Bratbak & Thingstad 1985, Lee & Rhee 1997), and much of this material can be carbohydrate (Biddanda & Benner 1997). In 2 of the lakes studied here — L227 and Joe Pool Lake — high relative responses to carbohydrates in Biolog assays commence after an algal bloom collapses. Autolysis and grazing both release algal carbohydrates to water (Münster 1993), and are plausibly associated with bloom collapse. In a third lake — Eagle Mountain Lake — high relative responses to carbohydrates in Biolog assays coincide with a broad, late summer peak of algal abundance, and it is again plausible that exudation, autolysis, or grazing transfer algal carbohydrates to lake water. Finally, oligotrophic L239 lacks a pronounced bloom of algae, and generally shows a weaker shift to carbohydrate utilization in late summer than the other lakes. We thus hypothesize that high utilization of carbohydrates is driven by seasonal variations in algal abundance, and transfer processes such as exudation, lysis and grazing.

Studies of substrate dynamics using chemical analyses and tracer kinetics support the notion that carbohydrate supply and bacterial utilization are associated with seasonal algal dynamics. Concentrations of total dissolved carbohydrates and several individual saccharides peaked as the spring bloom of algae in eutrophic Lake Plußsee (Germany) declined, and bacterial uptake of glucose increased steadily as the bloom developed and declined (Chróst et al. 1989). In Lake Constance (Germany) carbohydrate concen-

trations and bacterial utilization typically peak after the spring bloom and after blooms in late summer (Hanisch et al. 1996, Bunte & Simon 1999).

In Lake Plußsee, shifts in the dominant bacterioplankton genotypes occurred at the spring and autumn seasonal transitions (Höfle et al. 1999). Amino acid concentrations in the surface waters of this lake peak during these seasonal transitions (Münster 1993), and often coincide with maxima of zooplankton, leading Höfle et al. (1999) to suggest that enhanced amino acid concentrations produced by grazing on phytoplankton favored development of particular populations within the bacterioplankton. Similar dynamics of zooplankton and amino acids might occur in the lakes we studied here, and be related to the apparent patterns in substrate utilization that we detected. However, we have no data to address this possibility directly.

We have drawn a picture of seasonal succession in bacterioplankton that emphasizes relative capabilities to use different classes of carbon substrates. Biological interactions with phytoplankton and zooplankton may be major drivers of this seasonal succession in bacterioplankton. Biolog assays could be combined with *in situ* tracer studies of uptake kinetics and metabolism of selected substrates. These methods would provide independent pictures of microbial responses to substrates, and their seasonal or spatial concordance could help validate physiological profiling. Combined with genetic analyses of bacterial communities (Muyzer et al. 1993, Øvreås et al. 1997, 1998, Head et al. 1998, Øvreås & Torsvik 1998), a robust picture of structure and function of bacterial assemblages could emerge.

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