Changes in bacterioplankton metabolic capabilities along a salinity gradient in the York River estuary, Virginia, USA

Gary E. Schultz Jr[1], Hugh Ducklow[2],*

[1]University of Houston, Dept of Biology and Biochemistry, 3201 Cullen Blvd., Houston, Texas 77204, USA
[2]College of William and Mary, School of Marine Science, PO Box 1346, Gloucester Point, Virginia 23062, USA

ABSTRACT: Changes in metabolic capabilities of bacterial communities along the estuarine salinity gradient may affect the extent of organic matter processing and bacterial growth and accumulation during transit through the system. As part of a larger study of estuarine microbial processes, we attempted to quantify differences in bacterial community structure using Biolog plates. Biolog GN plates (Biolog, Inc., Hayward, CA, USA) were used to determine differences in bacterioplankton community metabolic potential. Biolog GN microplates are 96-well microtiter plates in which each well contains an individual carbon source as well the redox dye tetrazolium violet. As bacteria grow and oxidize each substrate, a purple color is formed that can be quantified spectrophotometrically. The resultant patterns are a function of the original community inoculated into the sample wells. Samples were taken weekly from May 1997 through May 1998 at a fixed location. Samples were also collected bi-monthly from July 1997 through May 1998 at 6 stations located along the salinity gradient. Principal component analysis shows clear differences in the patterns of community metabolic capabilities along the salinity gradient. Bacterial communities were separated by both temperature and salinity. Rates of color development mimicked the pattern of a strong landward gradient in specific growth rates. Biolog analysis is shown to be a powerful tool for identifying shifts in bacterial community composition in space and time, and provides a useful guide for deeper analysis of bulk property data.

KEY WORDS: Bacterioplankton · Biolog · Community · Estuary

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INTRODUCTION

Estuaries are typically regions of steep physical, biological and chemical gradients. Salinity may range from 0‰ to near marine salinities over short distances. Depending upon latitude, estuary temperatures may vary considerably on an annual basis. Estuaries are also greatly influenced by human activities. These activities may significantly change the organic matter loadings of an estuary over a short distance (Day et al. 1989). Physical factors such as wind and circulation are capable of changing estuarine conditions over a much smaller temporal scale than occurs in the open ocean.

These gradients may have a large effect on the composition of the bacterial community as it is carried from the upper reaches of the estuary out to the sea (Ketchum et al. 1952, Painchaud et al. 1996).

Due to these considerations and others, it is difficult to identify the factors that control bacterioplankton abundance and production in an estuary. There have been a number of studies that have attempted to determine these controls (e.g. Ducklow & Kirchman 1983, Wright & Coffin 1983, Coffin & Sharp 1987, Wright et al. 1987, Findlay et al. 1991, Hoch & Kirchman 1993, Shiah & Ducklow 1994, 1995, Goosen et al. 1997). Some of these studies focused on a fixed location within the estuary; others collected data along a transect of the salinity gradient and lumped all the data

*Corresponding author. E-mail: duck@vims.edu

together to perform data analyses. A few of the studies separated data by position or salinity, but these separations were made without regard to possible differences in microbial community structure. Not surprisingly, strong relationships, such as those seen between bacterial and primary production in the open ocean and in lakes (Fuhrman & Azam 1980, Bird & Kalff 1984, Lancelot & Billen 1984, Cole et al. 1988, Currie 1990, White et al. 1991), are not seen in estuarine systems (Ducklow & Kirchman 1983, Wright & Coffin 1983, 1984, Painchaud & Therriault 1989, Findlay et al. 1991, Ducklow & Shiah 1993; but see Goosen et al. 1997). This lack of strong relationships could be due to differences in the metabolic capabilities of different bacterial communities encountered within the estuary in space and time. If one could distinguish between different microbial assemblages, data could possibly be stratified for analysis in a more insightful and informative manner.

Determining the types of bacteria that exist in any water sample is complicated by several factors. Marine bacteria are difficult to culture (Ferguson et al. 1984). Nucleic acid hybridization, or molecular probing, has been used to detect genes of specific bacterial metabolic potential in marine bacterial consortia without the need for cultivation (Barkay et al. 1989). However, this technique provides no information on whether the activity in question is actually occurring (Walia et al. 1990). Another molecular method that is useful in detecting organisms that are not culturable is polymerase chain reaction (PCR). PCR is used to amplify 16s rRNA sequences and then clone those sequences into vectors (DeLong et al. 1989, Giovannoni et al. 1990, Ward et al. 1990, Schmidt et al. 1991, Moyer et al. 1994, 1995). These methods provide a great deal of information on the phylogenetic diversity of a sample, but no information concerning the metabolic capabilities of the cells present. Lee & Fuhrman (1990, 1991) used purified microbial community DNA to probe for similar sequences in the same and different environments. This gave them a quantitative measure of the relative similarity between populations. However, this method, like the other molecular methods discussed above, is very time consuming. The effort and cost required to process samples limit the appeal of these methods.

In the late 1980s, Biolog, Inc. (Hayward, CA, USA) developed a technique to test bacterial isolates for strain identification using sole carbon source utilization. Biolog chose 95 different carbon substrates, based on their ability to distinguish between clinically important species (Bochner 1989). Although the Biolog technique was developed for use in clinical microbiology, Garland & Mills (1991) used Biolog plates to differentiate between bacterial communities in a marsh creek

and in an ocean inlet. Since that time, several other investigators have used Biolog plates to characterize environmental communities. Studies have been performed on soil communities (Garland & Mills 1991, Bossio & Scow 1995, England et al. 1995, Haack et al. 1995, Vahjen et al. 1995, Wunsche et al. 1995, Knight et al. 1997), plant leaves (Harvey & Miller 1996), tree and crop rhizospheres (Garland & Mills 1991, Garland 1996), compost (Insam et al. 1996), wastewater (Guckert et al. 1996, Victorio et al. 1996) as well as marine bacterioplankton (Jellet et al. 1996). These studies have generated a great deal of controversy over the use of these plates to provide answers to fundamental questions of microbial diversity (Konopka et al. 1998).

Konopka et al. (1998) raised several valid points. Color development in the wells does not occur until a density of approximately 10^8 cells ml^{-1} is reached. Since cell density in natural samples is typically much less than this, this technique must be regarded as a culture-based method. In addition, the substrates in the wells may not be the substrates found in the environment from which the sample was taken. Thus, the results cannot be used to determine the makeup of the original community, or the metabolic 'fingerprint' of the original community. The pattern that results from inoculation of the wells, however, must be a function of the original community. Thus, even critics of Biolog plates concede that the plates can be used to characterize environmental samples that differ from each other in their response patterns (Konopka et al. 1998). We believe that prudent use of the information obtained from analysis of Biolog plates used with samples from the natural environment may be valuable in determining the factors that separate microbial communities. This information may be especially valuable to stratify data in meaningful ways such that patterns and relationships in large data sets may be discovered.

The purpose of our study was to use Biolog plates to distinguish between the metabolic potential of different bacteria communities in an estuary in space and time and to determine the environmental factors that control the separation of communities. Data were then stratified by community to focus further data analysis.

MATERIALS AND METHODS

Sampling. *York River transect samples:* Samples were collected bi-monthly from July 1997 to May 1998 at 6 stations along the salinity gradient of the York River estuary (Fig. 1). Stations were approximately 9 km apart with Stn 1 located at the mouth of the York River and Stn 6 located in freshwater in the Pamunkey River approximately 60 km from the mouth. Samples
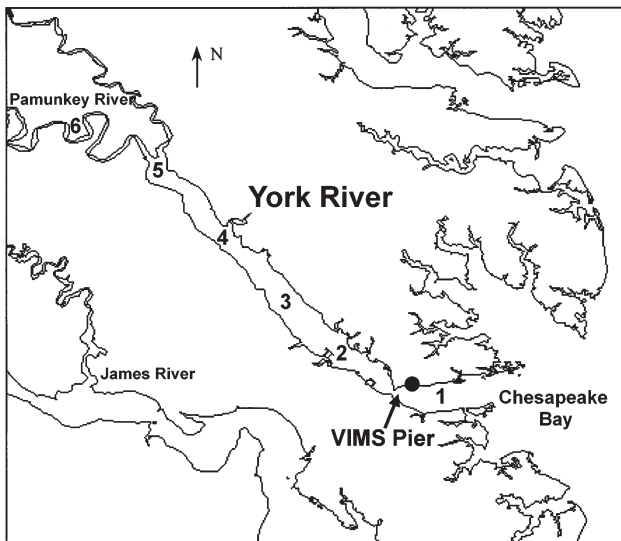
Fig. 1. Map of site locations (Stns 1 to 6) and the Virginia Institute of Marine Science (VIMS) pier. York River estuary, Virginia, USA

were collected 1 m below the surface at each station using a clean 2.5 l Niskin bottle. Approximately 750 ml were transferred to a clean 1 l polycarbonate bottle. Samples were kept in the dark and were brought back to the lab at *in situ* temperatures. These samples were collected as part of a larger study on bacterioplankton dynamics (Schultz & Ducklow unpubl.). Water temperature and salinity were measured. Bacterial abundance and biovolume were measured by epifluorescent microscopy and image analysis of cells stained with acridine orange (Shiah & Ducklow 1995). Bacterial production was estimated from [^{3}H-methyl]-thymidine (^{3}H-TdR) incorporation and average cell biovolume for each sample using appropriate conversion factors (Ducklow & Carlson 1992, Shiah & Ducklow 1995). A measure of specific growth rate was obtained by dividing production by biomass. Chlorophyll *a* (chl *a*) was determined fluorometrically by DMSO/acetone extraction according to Burnison (1980). Water was also collected for determination of dissolved organic carbon (DOC), dissolved organic nitrogen (DON), dissolved oxygen (DO) and nutrients (NH_4, TDN [total dissolved nitrogen], NO_2 and NO_3).

Pier samples: Samples were collected from the York River weekly from May 1997 to May 1998 from the Virginia Institute of Marine Science (VIMS) ferry pier located on the VIMS campus (Fig. 1). Samples were collected 1 m below the surface with a clean 1 l polycarbonate bottle. Samples were analyzed for bacterial cell abundance, bacterial activity and chl *a*. Biolog plates were immediately inoculated with sample in the lab.

Biolog plates. Biolog GN plates were used to determine differences in bacterioplankton community metabolic potential. Biolog GN microplates (Biolog, Inc.) are 96-well microtiter plates containing 95 carbon sources. These sources are supplied in each well in a dried-film form (Bochner 1989). Each well also contains the redox dye tetrazolium violet. As bacteria grow and oxidize each substrate, reduced nicotinamide adenine dinucleotide (NADH) is formed. The tetrazolium violet dye functions as a substitute electron acceptor during this biochemical oxidation (Siedler 1991). The reduced product is a colored, insoluble formazan seen as a purple color that is quantified spectrophotometrically. The 96th well is a control that contains tetrazolium dye only. It is assumed that the resultant pattern occurring on the Biolog plate is a function of the original community inoculated into the sample wells.

All samples were transferred from the 1 l sample bottles to the Biolog plates using an 8-channel multi-pipette. Care was taken not to touch the interior of the wells to prevent cross-contaminating substrates, and tips were discarded between plates to prevent cross-contamination of plates. Due to a problem with condensation forming inside the plate tops, 125 µl of sample were transferred to each well rather than the manufacturers' recommended 150 µl. Immediately upon inoculation, the zero time-point absorbance of each plate was read before incubating the samples at their *in situ* temperature.

Changes in color development were measured using a single-channel automated spectrophotometric microplate reader (EL×800; BIO-TEK Instruments, Inc., Winooski, Vermont, USA) at a wavelength of 590 nm. Plates were measured approximately every 6 to 24 h for changes in color development until maximum color development was reached. Maximum color development took from 72 h post-inoculation in warm water months to as long as 240 h post-inoculation in cold water months.

Overall color development for each plate was expressed as average well color development (AWCD) after Garland & Mills (1991). AWCD = $\sum[(R - C)/95]$, where R is the absorbance of each response well, and C is the absorbance of the control well. Garland & Mills (1991) suggested using a standard AWCD to eliminate biases caused by differences in initial cell abundance per well. Since color development follows a sigmoidal curve with incubation time, an AWCD value in the middle of the linear phase would give the greatest difference in substrate utilization patterns. Therefore, the measured sample with AWCD closest to 0.5 (±0.2) was used in all cases.

To reduce the influence of rate of color development among the plates, the absorbance value for each well was transformed according to Garland & Mills (1991)

by subtracting the absorbance of the control well from the absorbance of each well and dividing by the AWCD. Negative responses were set to zero before analysis.

Hierarchical cluster analysis. Hierarchical cluster analysis (HCA) was used to determine differences between patterns of substrate utilization with the Ein*Sight v 4.0 software package (InfoMetrix Corp.). The data were assembled into a large matrix. Each sample was placed into one of n rows with p columns of variables, in this case the absorbance measurements. The samples were treated as n points in a p-dimensional space. HCA groups data into clusters with similar attributes by determining the relative distances between pairs of samples in data space (Lebart et al. 1984). Euclidian distance and centroidal clustering were used in this analysis. When distances were relatively small, the samples were assumed to be similar with respect to the measured variables. Data from HCA were used to construct a dendrogram (Fig. 2). Each of the branches on the far left is called a leaf (see Fig. 2). The shorter the leaf, the greater the similarity and, therefore, the smaller the distance between the samples. Similarity was measured from 0.0 to 1.0, with 1.0 corresponding to an exact duplicate and 0.0 indicating maximum *relative* dissimilarity within the data set.

Principal components analysis. Principal components analysis (PCA) was used to determine differences between patterns of the substrate utilization profiles. Again, the software package Ein*Sight v 4.0 was used to perform PCA. To compare Biolog plates, the samples must be compared with the 95 variables. PCA is an exploratory tool, useful for reducing the number of variables in a given data matrix by collapsing the dimensionality of the data. This is accomplished by projecting the original data onto new axes, or principal components (PCs) (Lebart et al. 1984). These PCs are ranked according to the amount of the variance of the original sample that is accounted for by each PC. PC1 accounts for most of the variance, PC2 accounts for the next greatest amount of variance and so on. Basically, the farther apart 2 samples are in their original (95-dimensional) space, the farther apart they will be in 2-dimensional space. This allows the use of PCA to observe relationships between samples by plotting the scores of their PCs in 2 or more dimensions. Since the first 2 PCs account for the greatest amount of variance in the samples, they are typically the scores that are plotted.

RESULTS

Patterns of sole carbon source utilization

VIMS pier

Hierarchical cluster analysis: HCA was performed on the transformed Biolog data collected from the ferry pier to group the data by similarity. The data
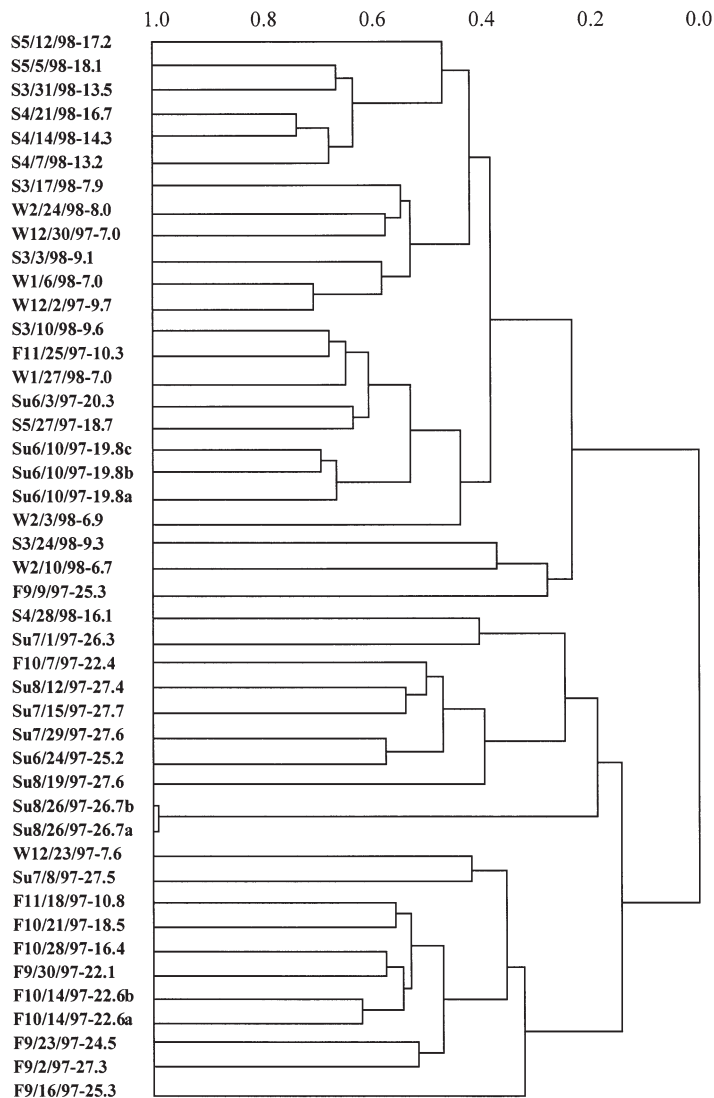

Fig. 2. Results of hierarchical cluster analysis (HCA) on Biolog results obtained for samples collected at the VIMS pier. Each leaf (left of figure) is labeled by season, date (mo/d/yr) and temperature. Season is indicated by S = spring (March, April, May), Su = summer (June, July, August), F = fall (September, October, November) and W = winter (December, January, February). Numbers at the top of the figure indicate percent similarity between paired samples. Samples labeled Su8/26/97-26.7a and b are machine replicates. Samples labeled Su6/10/97-19.8a, b and c, and F10/14/97-22.6a and b are sampling replicates

separated into 2 main clusters (Fig. 2). Temperature was the most obvious difference between the 2 clusters. The upper leaves were all samples with water temperature less than 21°C (except for 1 sample on September 9, 1997) while the lower leaves were mostly (14 of 19) samples from water temperatures greater than 21°C. Similarly, 2 clusters were also separated by season. Spring and winter samples (March, April, May and December, January, February respectively) were concentrated in the upper group, while summer and fall samples (June, July, August and September, October, November respectively) were concentrated in the lower group.

Sample variability was tested in 3 ways. The same samples were run twice throughout the incubation to test for variability in the spectrophotometer's reading. The similarity between these samples was very close to 1.0 (Fig. 2; August 26, 1997). On June 10, 1997, 3 samples were taken from the same 1 l bottle and incubated together. These samples were grouped by HCA with a similarity >0.6 (Fig. 2). Finally, 2 samples were collected in 2 separate 1 l bottles on October 14, 1997. These samples were also grouped together at a similarity of ~0.6 (Fig. 2). Together, these results suggest that focus should be concentrated on samples with less than 60% similarity.

Principal components analysis: PCA was performed on the 46 transformed Biolog data sets from the VIMS pier near the mouth of the York River. The samples showed a distinct separation by temperature along the first PC (Fig. 3). PC1 was positive for samples taken when the temperature was >21°C and negative for samples taken when the temperature was <21°C. Temperature was positively related to PC1 scores ($r^2 = 0.58$;
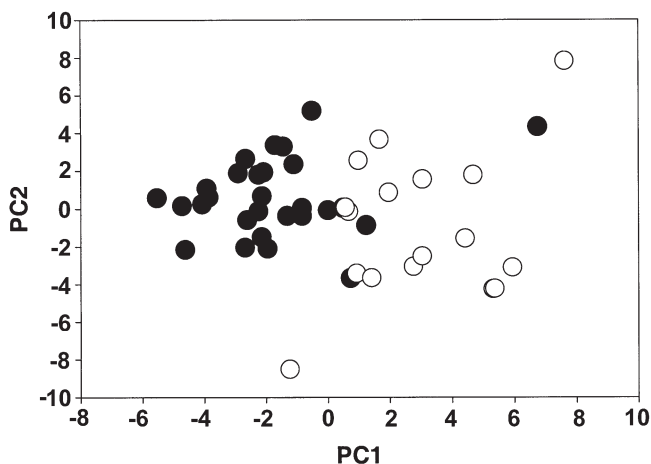

Fig. 3. Score plot for principal components PC1 and PC2 from principal components analysis of Biolog data obtained from samples collected at the VIMS ferry pier. Samples are labeled according to temperature. (O) Samples collected when temperature >21°C. (●) Samples collected when temperature <21°C

$p < 0.0001$) but was not significantly related to PC2, and the spread along PC2 was about the same for both temperature groups. Nearly 60% of the variance was accounted for in the first 10 PCs, with PC1 accounting for 11.2% and PC2 accounting for 8.9%. The small amount of variation accounted for by each individual PC was due to the high dimensionality (95) of the original data set.

Salinity also affected the separation of samples. From May 1997 to May 1998, the salinity at the VIMS pier ranged from 10 to 23.1‰ (VIMS Scientific Data Archive at http://www.vims.edu/data_archive/pier/). Salinity was not related to PC1, but was negatively related to the scores of PC2 ($r^2 = 0.33$; $p < 0.0001$). Thus, samples with high salinity values (>12‰) were relatively lower along the PC2 axis than samples with lower salinity values.

York River transect

Hierarchical cluster analysis: HCA separated the data from the York River transect into 5 distinct clusters (1 of the 5 is a lone station) (Fig. 4). Of the 17 members of the first cluster (A), only 3 had salinities >10‰. These 3 included 2 anomalous samples from Stn 5, which has a salinity ~5‰ over most of the year. The other sample (Stn 1 with salinity ~12‰) was also anomalous since Stn 1 typically has a salinity >18‰. The second cluster (B) in Fig. 4 comprised only Stn 6 from September 1997. This was a time of low freshwater flow when the salinity at the normally freshwater Stn 6 was >3‰. The third cluster (C) was composed of Stns 1, 2, 3 and 4 during 1 sampling period (July 1997). The fourth cluster (D) had 9 samples from November and January and 1 sample from March, and all but 1 sample had salinities greater than 10‰. The last cluster (E) was made up of samples from Stns 1, 2, 3, and 4 during September 1997.

The 2 clusters that were most dissimilar (similarity ≈ 0.0), were also split by salinity. In the upper cluster, 15 of the 22 members had salinity <10‰. The lower cluster had 14 members with only 1 having a salinity <10‰.

Principal components analysis: PCA was performed on the bi-monthly samples from the 6 stations along the salinity gradient of the York River (Fig. 5). The first 12 PCs accounted for more than 70% of the variance in the data, with PC1 accounting for 16.2% and PC2 accounting for 10.6%. Although there was much overlap, distinct patterns were present in the PCA of the station data (Fig. 5). The spread of the data seemed to indicate a general progression from Stn 1 scores in the upper left quadrant to Stn 6 scores in the lower right quadrant.
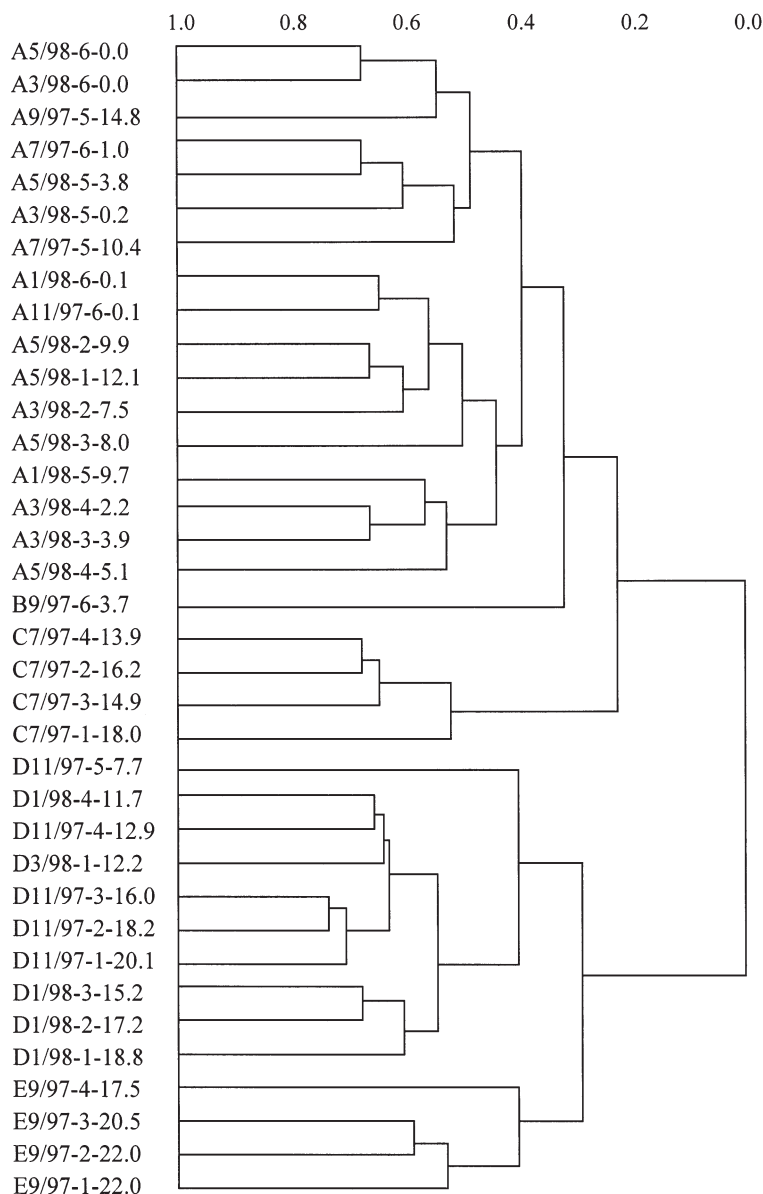
Fig. 4. HCA results for Biolog data obtained for York River transect samples. Leaves are labeled by date, station number, and then salinity. The initial letter identifies the 5 groupings discussed in the 'Results'

To determine the factors with the greatest influence on pattern development, multiple factor linear regressions were performed between all parameters measured (temperature, salinity, bacterial cell abundance, bacterial activity, chl *a*, DOC, DON, DO, NH$_4$, TDN, NO$_2$ and NO$_3$) and the scores of the PCs. Temperature and salinity were the only 2 parameters with a significant relationship with the scores of the PCs. Temperature and salinity together accounted for approximately 63% of the variance in PC1 (p < 0.0001) and approximately 68% of the variance in PC2 (p < 0.0001). Re-

examination of Fig. 5 with this information showed a distinct separation of the data points by temperature (Fig. 6). Points from samples with temperatures above 21°C were generally located above and to the right of those with temperatures below 21°C. Samples were also separated by salinity (Fig. 7). Points from samples with salinity greater than 12‰ were generally above and to the left of those with salinity less than 12‰.

To relate the utilization of individual carbon substrates to the differences in the sole carbon source utilization patterns, the correlation between the substrate variables and the PCs (Table 1) was examined. The higher the correlation, the more important that substrate was for differentiating between samples. A high correlation does not imply that the carbon source was well utilized. A high correlation simply means that the *difference* in the utilization of that carbon source was highest between samples. Samples with higher PC1 scores showed a higher response to those carbon sources positively correlated to PC1 than samples with lower PC1 scores. For these data, the substrates that were most important in differentiating between sole carbon source utilization were those which explained >25% of the variance in the data (−0.5 > r > 0.5, using Pearson's product moment regression). This r is not very large, but no single variable explained more than 43% of the variance in this set of data.

By examining Fig. 5 and Table 1, carbon substrates that were most important in differentiating between bacterial communities in the York River can be determined. In general, Stn 1 had the lowest PC1 values. This indicates that the bacterial community at Stn 1 utilized the substrates that were positively correlated to PC1 relatively *less* than did the bacterial community at Stn 6. These substrates were comprised of several classes of substrates including carboxylic acids (γ-hydroxybutyric acid, D-galacturonic acid, β-hydroxybutyric acid, and D-saccharic acid), an aromatic acid (urocanic acid), carbohydrates (m-inositol, D-psicose, and D-melibiose), 2 amines (2-amino ethanol, and putrescine), and amino acids (D,L-carnitine, L-pyroglutamic acid, L-phenylalanine, L-leucine, and hydroxy L-proline). Lower PC1 values at Stn 1 also indicate that the bacterial community there uses relatively *more* of the substrates that were negatively correlated to PC1 with respect to
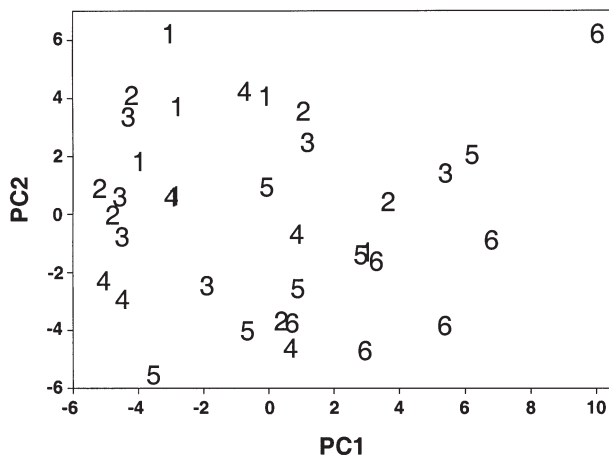
Fig. 5. PC1 plotted against PC2 for the Biolog data collected from the sites along the length of the York River, July 1997 to May 1998. Numbers indicate the station number for each of the 6 mo sampled

Fig. 6. Score plot of PC1 against PC2 of Biolog data obtained from water samples collected bi-monthly along the length of the York River, July 1997 to May 1998. Samples are labeled by temperature. (○) Samples collected when temperature >21°C. (●) Samples collected when temperature <21°C. The line was drawn by eye to suggest a general separation of data

Stn 6. These substrates included 2 carboxylic acids (D-gluconic acid, and D,L-lactic acid), 2 carbohydrates (cellobiose, and D-mannitol), 3 amino acids (glycyl-L-glutamic acid, and L-alanyl-glycine) and 2 polymers (glycogen and α-cyclodextrin).

Stn 1 had generally higher PC2 values than did Stn 6. For the substrates that were positively correlated to PC2, this indicates that the bacterial community at Stn 1 used relatively *more* of these substrates than did the community at Stn 6. The substrates that correlated with PC2 with a variance of −0.5 > r > 0.5 included several carboxylic acids (α-ketovaleric acid, acetic acid, α-ketoglutaric acid, and propionic acid), 2 aromatic compounds (thymidine and uridine), carbohydrates (lactulose, xylitol, and adonitol), and 2 amino acids (L-proline, L-threonine). There were also 3 carboxylic acids (quinic acid, D-saccharic acid, and *p*-hydroxy-phenylacetic acid) and 2 carbohydrates (α-D-glucose and sucrose) that exhibited a negative correlation with PC2. This indicates that the bacterial community at Stn 1 used relatively *less* of these substrates than did the community at Stn 6.

Changes in plate color development over time

Plate color development was measured every 24 h. The change in color development is similar in appearance to a growth curve. To obtain the overall rate of color development, the total color development ($\sum R$ = TCD) was exchanged for bacterial cell number in the growth equation: $\ln(N_t) - \ln(N_o) = kt$ to obtain: $\ln(\text{TCD}_t) - \ln(\text{TCD}_o) = kt$, where N_t = cell number at time t, N_o = cell number of original population, and k =
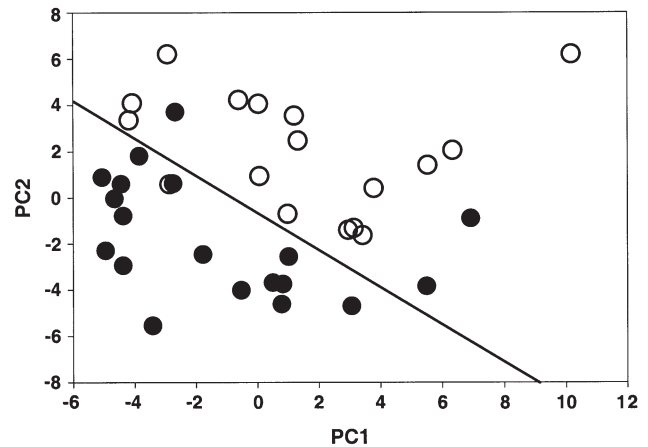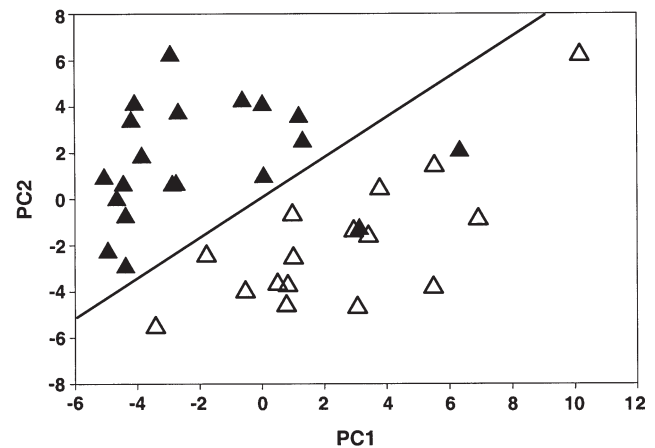

Fig. 7. Score plot of PC1 against PC2 of Biolog data obtained from water samples collected bi-monthly along the length of the York River, July 1997 to May 1998. Samples are labeled by salinity. (△) Samples collected when salinity <12‰. (▲) Samples collected when salinity >12‰. The line was drawn by eye to suggest a general separation of data

specific growth rate constant. This gives a rate of color change analogous to specific growth rate. This equation was applied to all the samples for the York River cruises. Rates for total color development were compared to growth rates obtained from measurements of bacterial cell abundance and incorporation of ^{3}H-TDR (Table 2). This comparison was not meant to show that the change in TCD was equal to growth rates, but that the rate of color development showed the same pattern as growth rates along the river. Indeed, as seen in Table 2, growth rates typically follow the same pattern as rate of color development over every sampling

Table 1. Correlation coefficient for scores of the first 2 principal components (PC1 and PC2) from the York River data collected at Stns 1 to 6 and the transformed measured absorbance for each substrate. All substrates with an r > 0.5 are shown (p < 0.005). r: Pearson's correlation coefficient

PC1		PC2	
Substrate name	r	Substrate name	r
D,L-carnitine	0.763	Alpha-keto valeric acid	0.663
D-galacturonic acid	0.720	Lactulose	0.654
β-hydroxybutyric acid	0.718	Acetic acid	0.581
L-pyroglutamic acid	0.692	Thymidine	0.565
2-amino ethanol	0.683	Alpha-keto glutanic acid	0.557
Putrescine	0.665	L-proline	0.556
Urocanic acid	0.636	Propionic acid	0.529
m-inositol	0.629	Uridine	0.522
γ-hydroxybutyric acid	0.579	Xylitol	0.521
L-phenylalanine	0.556	Adonitol	0.521
L-leucine	0.534	L-threonine	0.506
D-saccharic acid	0.528	Quinic acid	−0.671
D-psicose	0.518	α-D-glucose	−0.624
D-melibiose	0.509	Sucrose	−0.571
Hydroxy L-proline	0.500	D-saccharic acid	−0.572
Glycogen	−0.697	p-hydroxy phenylacetic acid	−0.559
Glycyl-L-glutamic acid	−0.665		
α-cyclodextrin	−0.633		
D-gluconic acid	−0.619		
Cellobiose	−0.610		
L-alanyl-glycine	−0.563		
D,L-lactic acid	−0.557		
D-mannitol	−0.515		
L-threonine	−0.514		

period. Growth rate (as measured by ^{3}H-TdR incorporation) and rate of color development were positively and significantly correlated in every month sampled (r = 0.85, p < 0.01 in July 1997; r = 0.95, p < 0.001 in September 1997; r = 0.67, p < 0.1 in November 1997; r = 0.71, p < 0.05 in January 1998; r = 0.90, p < 0.05 in March 1998; r = 0.94, p < 0.002 in May 1998). They were both relatively low at Stn 1 and they increased with distance upstream to Stn 6. Thus, it appears as though general conclusions on the relative magnitude of growth rates are consistent with the rate of change of color development in Biolog plates. However, due to the unrealistic substrate concentrations (Konopka 1998) and other concerns, care should be used when making these types of comparisons.

DISCUSSION

There are several concerns regarding the use of Biolog plates with natural samples. Haack et al. (1995) found that cell density in each well must reach 10^8 ml^{-1} before color development is observed. Most natural environments, including estuaries, have cell densities far below 10^8 ml^{-1}. Thus, Biolog plates are an enrichment culture and the inoculum will grow a selective subset of the entire population. Also, as stated earlier, the substrates included in the Biolog plates are not necessarily representative of the substrates available in the natural environment (Konopka et al. 1998). Konopka et al. (1998) clearly summarized these and other concerns regarding the use of Biolog with natural samples. Therefore, attempting to use Biolog plates to characterize the 'functional diversity' of microbial communities (Zak et al. 1994) may not be prudent.

Biolog plates, however, may still be useful for use with environmental samples. Konopka et al. (1998) conceded that Biolog plates can distinguish between different communities in natural samples. Several investigators have successfully used Biolog plates to differentiate between microbial communities (Garland & Mills 1991, Zak et al. 1994, Bossio & Scow 1995, Garland 1996, Grayson & Campbell 1996, Guckert et al. 1996, Harvey & Miller 1996, Insam et al. 1996, and Victorio et al. 1996). Carbon substrate utilization patterns may also be used to identify ecologically relevant bacteria isolated from environmental samples. McCarthy & Murray (1996) used Biolog plates on 103 Gram-negative isolates cultured from a contaminated aquifer. They were able to tentatively identify 55 of the isolates and kept records of the carbon source utilization pattern of the remaining 48 isolates for future characterization.

Another potentially important use for Biolog plates is in substrate addition studies or other studies requiring long incubations. Bacterial population composition may shift during long incubations (Suzuki 1998) causing misleading results. Biolog plates may be quickly and cheaply used to determine if bacterial communities have changed over the course of incubation.

Finally, by separating different communities, Biolog plates can be used to gain insight into bulk measurements of bacterial properties. Knowing that there are different microbial assemblages in space and/or time may allow the investigator to stratify data such that more information may be extracted.

Shiah & Ducklow (1995) found that temperature plays a strong role in controlling estuarine bacterial dynamics. Therefore, to ensure that bacterial activity is not stimulated or retarded relative to their natural environment, our samples were incubated at *in situ*

Table 2. Temperature, salinity, chl *a*, cell abundance, specific growth rate and rate of change in total color development (ΔTCD) for each station over the course of the sampling period (July 1997 to May 1998). Growth rates are derived from incorporation of ^{3}H-thymidine measurements and abundances. Change in TCD is the maximum 24 h change over the length of the incubation

Date	Stn	Temp. (°C)	Salinity (‰)	Chl *a* (µg l^{-1})	Abundance (× 10^9 cells l^{-1})	Growth rate (d^{-1})	ΔTCD (d^{-1})
Jul 1997	1	26.3	18.0	34.2	6.8	0.6	2.1
Jul 1997	2	26.5	16.2	63.7	5.1	1.3	1.4
Jul 1997	3	27.5	14.9	45.2	4.8	1.5	2.1
Jul 1997	4	27.8	13.9	85.8	4.2	2.8	2.3
Jul 1997	5	28.0	10.4	75.4	7.9	2.2	3.7
Jul 1997	6	28.7	1.0	46.2	2.4	5.4	5.0
Sep 1997	1	24.0	22.6	47.6	5.2	1.5	0.4
Sep 1997	2	24.3	22.1	51.4	2.6	3.5	0.4
Sep 1997	3	24.2	20.5	47.6	2.1	4.7	0.5
Sep 1997	4	24.2	17.5	86.2	2.6	4.9	0.6
Sep 1997	5	24.2	14.8	85.8	2.0	7.1	0.8
Sep 1997	6	24.6	3.7	91.6	2.5	8.2	0.9
Nov 1997	1	8.9	20.1	3.2	2.3	0.6	0.2
Nov 1997	2	8.3	18.2	4.7	1.6	1.0	0.3
Nov 1997	3	8.2	16.0	6.2	1.4	1.4	0.3
Nov 1997	4	8.0	13.0	8.0	1.4	1.7	0.5
Nov 1997	5	8.3	7.7	13.2	2.3	1.6	0.7
Nov 1997	6	8.2	0.1	3.6	1.3	7.0	0.7
Jan 1998	1	6.1	18.8	12.0	2.6	0.4	0.1
Jan 1998	2	6.4	17.2	14.9	2.2	0.6	0.2
Jan 1998	3	6.5	15.2	29.3	2.5	0.5	0.1
Jan 1998	4	6.4	11.7	17.8	1.6	1.1	0.3
Jan 1998	5	6.6	9.7	11.7	1.7	0.3	0.4
Jan 1998	6	6.6	0.1	3.4	2.4	1.6	0.6
Mar 1998	1	13.5	12.2	23.7	3.3	3.3	0.8
Mar 1998	2	15.0	7.5	10.5	3.7	3.7	0.7
Mar 1998	3	15.7	3.9	7.4	2.6	4.2	0.9
Mar 1998	4	16.9	2.2	6.4	2.5	5.7	0.9
Mar 1998	5	17.1	0.1	6.9	2.4	10.3	1.0
Mar 1998	6	17.5	0.0	6.4	2.5	11.4	1.1
May 1998	1	22.1	12.1	9.1	6.9	0.8	0.6
May 1998	2	21.6	9.9	15.7	6.7	1.8	0.6
May 1998	3	22.3	8.0	36.5	5.5	2.6	0.6
May 1998	4	21.7	5.1	33.7	4.0	4.4	1.0
May 1998	5	21.2	3.8	17.7	3.7	4.6	1.2
May 1998	6	20.7	0.0	13.9	1.9	19.9	1.9

temperatures. PCA and HCA of the patterns of carbon substrate utilization separated the microbial assemblage of the York River estuary into 4 communities (Fig. 8). These analyses showed that communities within the York River differed both by temperature and by salinity. Two points should be emphasized here. One is that the 4 communities may or may not be separated by assemblages of species. The purple color quantified in analysis of Biolog plates is due to bacterial oxidation of the substrate. If a species of bacteria is unable to utilize the substrate, due to environmental factors such as extreme temperature or salinity, that species will not reduce the tetrazolium dye and will not add to the measured absorbance of the well. Thus, depending on the environment at the time of collection, a species may be present but not accounted for in the pattern seen on plates. The second point to be made is that, although temperature and salinity separate these communities, these factors may not directly control the bacterial community. Instead, temperature and salinity may foster conditions that indirectly influence the structure of the bacterial community (i.e. primary production).

Perhaps the most valuable use for Biolog plates is to use the groupings identified by the plates to stratify data sets. Stratification of bulk data as suggested by analysis of Biolog plates may also allow investigators to focus on relationships between bacteria and environmental factors that might otherwise be missed. Correlations run on the entire data set collected by Schultz & Ducklow (unpubl.) show no significant relationships between bacterial abundance and temperature, TdR incorporation, or chl *a*. Significant relationships are seen, however, when the data are stratified (Table 3).
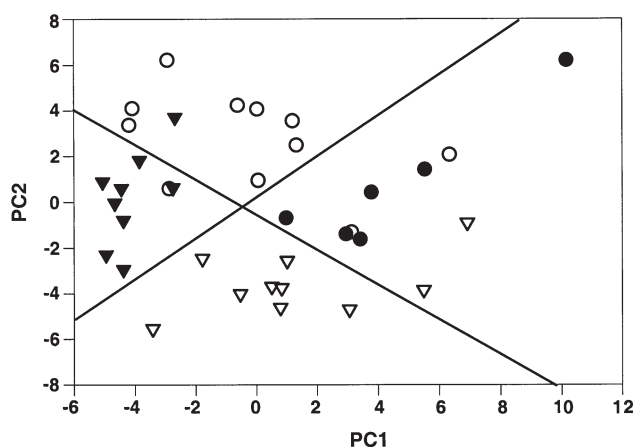
Fig. 8. Score plot of PC1 against PC2 of Biolog data obtained from water samples collected bi-monthly along the length of the York River, May 1997 to May 1998. (○) Samples collected when temperature >21°C and salinity >12‰. (●) Samples collected when temperature >21°C and salinity <12‰. (△) Samples collected when temperature <21°C and salinity <12‰. (▲) Samples collected when temperature <21°C and salinity >12‰. Lines were drawn by eye to suggest a general separation of data

Table 3. Results of Model I regressions (r) between bacterial abundance, bacterial activity (^{3}H-TdR incorporation), temperature, and chl *a* in the 4 bacterial communities of the York River as identified by Biolog plates. Community I has temperature <21°C and salinity <12‰, n = 42; Community II has temperature >21°C and salinity <12‰, n = 21; Community III has temperature <21°C and salinity >12‰, n = 25; Community IV has temperature >21°C and salinity >12‰, n = 20. Data from all 18 cruises were included (Schultz & Ducklow unpubl.). Numbers in **bold** identify significant relationships (p < 0.01)

	Abundance	Activity	Temp.	Chl *a*
Community I				
Abundance	–	0.03	0.36	0.32
Activity	0.03	–	**0.55**	−0.16
Temp.	0.36	**0.55**	–	0.08
Chl *a*	0.32	−0.10	0.08	–
Community II				
Abundance	–	−0.20	0.15	−0.18
Activity	−0.20	–	−0.02	**0.68**
Temp.	0.15	−0.02	–	0.22
Chl *a*	−0.18	**0.68**	0.22	–
Community III				
Abundance	–	0.10	**0.77**	0.16
Activity	0.10	–	0.38	0.17
Temp.	**0.77**	0.38	–	0.00
Chl *a*	0.16	0.17	0.00	–
Community IV				
Abundance	–	**−0.62**	−0.14	**−0.67**
Activity	**−0.62**	–	0.13	**0.69**
Temp.	−0.14	0.13	–	0.33
Chl *a*	**−0.67**	**0.69**	0.33	–

Stratifying data based on carbon substrate utilization also reduces the risk of auto-correlation with the factors that most strongly influence the separation of communities. In this study, salinity and temperature were the only 2 environmental factors found to separate the bacterial communities. Since many of the environmental parameters may be strongly influenced by salinity and temperature, the risk of auto-correlation is great. Examining the data by community reduces the influence of temperature and salinity and allows determination of the environmental parameters that affect bacterial dynamics within each community. For example, using all the unstratified data collected, bacterial abundance is not significantly related to chl *a*, but is significantly related to temperature. If the data are stratified as indicated by the PCA, bacterial abundance is significantly related to temperature only in Community III when salinity is greater than 12‰ and temperature is less than 21°C (Table 3). Bacterial abundance and chl *a* are significantly and negatively related only where salinity is greater than 12‰ and temperature is greater than 21°C (Community IV) (Table 3). Since abundance and temperature are not significantly related in this community, this cannot be an auto-correlation. This may indicate that bacteria in the higher salinity region of the York River may be more dependent upon phytoplankton as their carbon source than the bacteria in low salinity region of the York River. This suggestion agrees with the conclusions generated by Schultz & Ducklow (unpubl.).

Biolog plates are not capable of determining the species composition of a natural sample and cannot identify the types of substrates utilized by a bacterial community in their natural environment (Konopka et al. 1998), but as has been demonstrated here, they are useful in differentiating between microbial communities and in determining the factors that most influence the separation of these communities. Knowing where these separations occur in natural systems may prove to be invaluable in determining how best to stratify measurements of bulk bacterial properties and environmental parameters for analysis. The relative low cost (US $5.44 per plate as of June 2000) and ease of use makes these sole carbon source utilization plates an attractive addition to any study of bacterial dynamics conducted in a natural system over space and/or time.

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