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Classification and Characterization of Heterotrophic Microbial Communities on the Basis of Patterns of Community-Level Sole-Carbon-Source Utilization

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The BLOLOG redox technology based on tetrazolium dye reduction as an indicator of sole-carbon-source utilization was evaluated as a rapid, community-level method to characterize and classify heterotrophic microbial communities. Direct incubation of whole environmental samples (aquatic, soil, and rhizosphere) in BIOLOG plates containing 95 separate carbon sources produced community-dependent patterns of sole-carbon-source utilization. Principal-component analysis of color responses quantified from digitized images of plates revealed distinctive patterns among microbial habitats and spatial gradients within soil and estuarine sites. Correlation of the original carbon source variables to the principal components gives a functional basis to distinctions among communities. Intensive spatial and temporal analysis of microbial communities with this technique can produce ecologically relevant classifications of heterotrophic microbial communities.

The classification and characterization of animal and plant communities have helped define the factors that regulate both the structure and the function of such communities. Microbial ecologists have been less successful at identifying and classifying microbial communities due to the small size and morphological similarity of constituent members, particularly bacteria. Highly structured communities have been reported for communities composed of microorganisms of distinct morphology or nutritional strategy or both (10, 14). Delineation of heterotrophic communities has been very limited, yet such communities dominate most aquatic and terrestrial habitats.

Most studies of heterotrophic microbial community structure have involved isolate-based methods. Numerical taxonomic studies use either profiles of cellular constituents (12, 13) or phenetic characteristics (11) of isolates to define operational taxonomic units as defined by Sneath and Sokal (20). Analysis of the presence and distribution of different operational taxonomic units within a community (e.g., diversity) can provide insight into the ecological functioning of communities (1). The analysis of taxonomic structure of communities alone, however, limits insight into the ecological relevance of community structure. The exclusion or addition of different bacterial types does not necessarily change the resultant function of the community (24). Analysis of phenetic characteristics which directly relate to important processes in the environment under investigation (15) and correlation of such characteristics to environmental parameters (2, 17) allow for greater insight into the factors which regulate community structure. Isolate-based methods, however, depend on cultural methods, which can exclude the majority of endogenous microbes from study due to the selective nature of the media (22). Furthermore, the time-consuming nature of isolate-based methods severely limits the spatial and temporal intensity of sampling. For example, using isolate-based methods, Bell et al. (2) were restricted to 12 samples in an evaluation of seasonal trends in microbial community structure in two rivers.

White and Findlay have developed a community-level approach to characterize microbial community structure by evaluating shifts in fatty acid methyl esters from whole environmental samples (25). This method has successfully detected changes in biomass of total bacteria (19), diatoms (19), anaerobic sulfate-reducing bacteria (7), and microeukaryotes (8) within various microbial habitats. While this approach eliminates the bias associated with cultural methods and increases the potential scope of sampling, structural markers for shifts within the heterotrophic bacterial community are still being developed.

The application of the community-level approach to assays of microbial function would provide a more sensitive and ecologically meaningful measure of heterotrophic microbial community structure. Rather than relying on determination of changes in individual abundances which may not equate to meaningful shifts in community function, this approach would provide measures of the metabolic abilities of the community. Sole-carbon-source utilization exerts a strong influence on the classification of isolates (13) and may be a useful, community-level, functional measure in the characterization and classification of heterotrophic communities.

A community-level assay of microbial community structure based on carbon source characters requires rapid multiple assays of carbon source utilization. A redox-based technique has been developed recently by BIOLOG, Inc., for carbon source utilization testing of bacterial isolates for strain identification. Color produced from the reduction of tetrazolium violet is used as an indicator of respiration of sole carbon sources. Commercially available microplates allow for simultaneous testing of 95 separate carbon sources. Direct incubation of whole environmental samples in BIOLOG plates, therefore, may produce patterns of metabolic response suitable for the rapid classification of heterotrophic microbial communities.

This research examined the potential of the patterns of color response produced from direct incubation of whole environmental samples in BIOLOG plates to resolve microbial community structure. Color response in wells was quantified from digitized images of plates after selected

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TABLE 1. Sole carbon sources in BIOLOG GN microplates

C sources		
Carbohydrates	Carboxylic acids	Amino acids
N-Acetyl-D-galactosamine	Acetic acid	D-Alanine
N-Acetyl-D-glucosamine	*cis*-Aconitic acid	L-Alanine
Adonitol	Citric acid	L-Alanyl-glycine
L-Arabinose	Formic acid	L-Asparagine
D-Arabitol	D-Galactonic acid lactone	L-Aspartic acid
Cellobiose	D-Galacturonic acid	L-Glutamic acid
i-Erythritol	D-Gluconic acid	Glycyl-L-aspartic acid
D-Fructose	D-Glucosaminic acid	Glycyl-L-glutamic acid
L-Fucose	D-Glucoronic acid	L-Histidine
D-Galactose	α-Hydroxybutyric acid	Hydroxy-L-proline
Gentiobiose	β-Hydroxybutyric acid	L-Leucine
α-D-Glucose	γ-Hydroxybutyric acid	L-Ornithine
m-Inositol	*p*-Hydroxyphenylacetic acid	L-Phenylalanine
α-Lactose	Itaconic acid	L-Proline
Lactulose	α-Ketobutyric acid	L-Pyroglutamic acid
Maltose	α-Ketoglutaric acid	D-Serine
D-Mannitol	α-Ketovaleric acid	L-Serine
D-Mannose	D,L-Lactic acid	L-Threonine
D-Melibiose	Malonic acid	D,L-Carnitine
β-Methylglucoside	Propionic acid	γ-Aminobutyric acid
Psicose	Quinic acid	
D-Raffinose	D-Saccharic acid	Aromatic chemicals
L-Rhamnose	Sebacic acid	
D-Sorbitol	Succinic acid	Inosine
Sucrose		Urocanic acid
D-Trehalose	Alcohols	Thymidine
Turanose		Uridine
Xylitol	2,3-Butanediol	
	Glycerol	Brominated chemicals
Esters		
	Amides	Bromosuccinic acid
Mono-methylsuccinate		
Methylpyruvate	Succinamic acid	
	Glucuronamide	Amines
Polymers	Alaninamide	
		Phenylethylamine
Glycogen	Phosphorylated chemicals	2-Aminoethanol
α-Cyclodextrin		Putrescine
Dextrin	D,L-α-Glycerol phosphate	
Tween 80	Glucose-1-phosphate	
Tween 40	Glucose-6-phosphate	

lengths of incubation. Principal-component analysis (PCA) of the multivariate data set (95 color responses) differentiated samples on the relatively gross scale of microbial habitat type and the finer scale of spatial gradients within habitats.

MATERIALS AND METHODS

BIOLOG plates. The BIOLOG GN microplates (BIOLOG, Inc.) used in this research rely on the redox dye tetrazolium violet to detect respiration (actually, NADH formation) of sole carbon sources. The 96-well GN microplate comprises 95 substrate-containing wells and a control well without a carbon source (Table 1). Substrate, dye, and nutrients are supplied in each well in a dried-film form which is reconstituted upon addition of sample (4).

Data collection. Portions of samples (prepared as discussed below) were inoculated into plates with a multipipettor. Approximately 17 ml of sample was required to inoculate one plate (0.15 ml/well plus residual sample in pipette tips).

Digitized images of plates were obtained by using a digitizing video scanner (Eikonix Corp.), and the images were analyzed with ERDAS software (ERDAS, Inc.). This software is intended for use as a geographical information system, but its flexibility in reading digitized images made it ideal for the present application. The image consisted of a 1,024-by-1,024 matrix of pixels (the smallest unit of resolution). Reflectance in the green scanning band for each pixel was converted to a gray scale value indicative of relative intensity. Gray scale values from approximately 160 for colorless wells to 30 for highly colored wells were observed. A Turbo Pascal computer program was used to sample a 3-by-3 matrix of pixels within each well. The mean pixel value for each matrix was used as the measure of color response in each well.

Data analysis. Overall color development in BIOLOG plates was expressed as average well color development (AWCD). AWCD was derived from the mean difference among gray scale values of the 95 response wells (containing sole carbon sources), R, and the gray scale value of the control well (without a carbon source), C: $[\Sigma(C - R)]/95$. Color production in the control well, which occurred upon

incubation of certain samples, yielded color values for individual wells (termed raw difference) that were 30 to 40 color units lower than values obtained against a colorless control well. When color development was observed in the control well, raw-difference values were calculated on the basis of a reference position on the plate outside of any well. This procedure resulted in values that were only 4 or 5 color units different from those obtained with a colorless control well (when divided by 95 to obtain the AWCD, this small difference was trivial).

The pattern of sole-carbon-source utilization of each plate was expressed in two ways: (i) raw-difference data obtained by subtracting the color response of each of the response wells from the color response of the control well (or reference position) $[C - R]$, and (ii) transformed data calculated by dividing the raw difference value for each well by the AWCD of the plate: $(C - R)/\{[\Sigma(C - R)]/95\}$. Both expressions of the pattern of sole-carbon-source utilization generate a set of 95 variables for each sample at each reading. Values of the raw-difference data ranged from 0 to approximately 130 and were generally higher for most carbon sources at later incubations, as color production increased with time. Values of the transformed data ranged from 0 to approximately 4, with values of <1 indicating a color response less than the AWCD and values of >1 indicating a color response greater than the AWCD.

The relationships among different samples on the basis of either the raw-difference data or the transformed data were determined by PCA, using the PC-ORD software package (Holcomb Research Institute, Butler University). This technique projects the original data onto new axes (principal components [PCs]) which reflect any intrinsic pattern in the multidimensional data swarm (16). Each PC extracts a portion of the variance in the original data, with the greatest amount of variance extracted by the first axis. Relationships among samples are readily observed by plotting samples in two dimensions on the basis of their scores for the first two PCs. Such relationships are difficult to observe when examining the original variables in multidimensional space (in this case, 95-dimensional space). PCA also calculates the proportion of variance in each variable explained by a given PC. By correlating PCs with the original variables, the axes can be "labeled" with a subset of the original variables. In summary, PCA of the BIOLOG color responses allows for comparison of microbial samples on the basis of differences in the pattern of sole-carbon-source utilization. The comparisons are a posteriori in nature; PCA determines how the samples are different, but does not test among samples for specific differences selected a priori.

Sample sources and preparation. Three disparate types of communities (sample types) were tested in BIOLOG plates: (i) water (including samples of freshwater, estuarine, seawater, and hydroponic solutions used for growing wheat), (ii) rhizosphere samples from hydroponically grown wheat (*Triticum aestivum* cv. *Yecora roja*), and (iii) soil samples.

All water samples were pipetted directly into BIOLOG GN microplates. Freshwater samples were obtained during mid-March of 1990 from ponds and streams in a watershed near Shenandoah National Park in Green County, Virginia. Samples were similar in pH (6.3 to 7.0), temperature (7 to 12°C), and conductivity (40 to 60 $\mu S\ cm^{-1}$). Estuarine and seawater samples were collected from a salt marsh-ocean inlet transect at the Virginia Coast Reserve Long Term Ecological Research site (Table 2) during mid-July 1990. Separate plates were inoculated with two replicate water samples at each sample site. Replicate plates for three sample sites were lost due to accidental spillage during transport.

TABLE 2. Site descriptions for coastal marine samples

Station[a]	Water depth (ft)[b]	pH	Water temp (°C)	Salinity (‰)	Dissolved oxygen (mg/liter)
UPC	3	7.89	24.2	32.1	5.5
LPC	3	7.92	26.5	32.0	6.0
M3	5	8.06	27.0	31.8	6.4
176	21	8.05	25.0	32.5	7.2
H1	30	8.02	24.0	33.0	7.4
UCC	3	ND[c]	ND	ND	ND
LCC	5	7.92	25.5	34.5	6.2
SC	18	7.93	25.0	33.5	7.2
PI	3	7.87	26.0	35.0	6.8
QI	62	7.97	25.0	33.5	7.1

[a] UPC, upper reaches of mainland salt marsh creek; LPC, lower reaches of mainland salt marsh creek; M3, mud flat area adjacent to mainland salt marsh creek; 176, open channel; H1, open channel; UCC, upper reaches of barrier island salt marsh creek; LCC, lower reaches of barrier island salt marsh creek; SC, open channel through perched marsh; PI, swash zone behind aggrading spit on barrier island; QI, ocean inlet.
[b] 1 ft = 30.48 cm.
[c] ND, not determined.

Rhizosphere samples were obtained by shaking roots in sterile saline solution (0.85% NaCl) containing glass beads (5-mm diameter). The resulting suspension was filtered through a 10-μm-pore-diameter polycarbonate filter to remove plant cells prior to inoculation.

Air-dried samples of each of three soil samples (Table 3) were incubated in dilute media to prepare a cell suspension without the interfering color of soil extracts. To determine the potential effects of selective enrichment, suspensions were prepared with three separate dilute media: (i) 0.0025% yeast extract (YE medium), (ii) 0.0025% yeast extract and 0.025% Bacto-Peptone (YP medium), and (iii) 0.0025% yeast extract and 0.005% each fructose, xylose, dextrose, lactose, and mannitol (YS medium). All prepared medium components were purchased from Difco Laboratories. Portions of the suspension from the shaken flask cultures were withdrawn after incubation for 18 h at 25°C on a rotary shaker (model G2; New Brunswick Scientific) operated at 100 rpm, thus avoiding the soil material, and were inoculated directly into BIOLOG plates. Two replicate suspensions were prepared for each soil-medium combination.

TABLE 3. Physiochemical characteristics of soils[a]

Factor	Soil no.		
	22	23	35
Organic matter (%)	9.65	15.81	6.28
pH	6.1	5.4	7.0
Sand (%)	35.8	56.4	8.0
Silt (%)	56.2	26.1	15.0
Clay (%)	8.0	54.2	63
Moisture (% of dry wt)	119	80	37
P (mg/kg)	19	7	13
Mg (meq/g)	1.16	0.63	3.10
K (meq/g)	0.33	0.51	0.85
Ca (meq/g)	8.26	1.05	11.60
Fe (mg/kg)	106	174	122
Cu (mg/kg)	6	8	19
Acidity (meq/g)	0.3	0.5	0.2

[a] Data obtained from Soto and Blum (21).

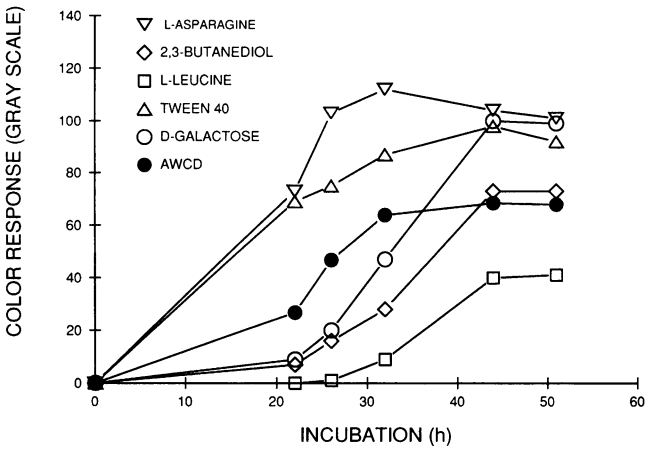

FIG. 1. Color development with incubation. Plots present the color response in five individual response wells (gray scale value of control well minus gray scale value of response well). The plot of AWCD represents the mean color response for all 95 response wells.

All plates were incubated at 25°C without agitation. An initial scan was performed when significant color production was present in a number of wells, usually after 20 to 40 h, depending on the sample. A second scan typically was performed on the plates 16 to 24 h later.

RESULTS

Behavior of control wells. Color development in the control well was minimal (gray scale values of >140 after 50 h of incubation) for all freshwater, estuarine water, and soil samples analyzed in this study. Reduction of tetrazolium dye in the control well was more pronounced (gray scale values as low as 110 after 50 h of incubation) in samples from the rhizosphere and hydroponic solutions that contained high concentrations of organic material. Respiratory activity in the control well, i.e., color production, appeared to be a result of carbon added with the inoculum; dilution of samples prior to inoculation always reduced color production in the control well.

Rate of color development. (i). AWCD. Color development (expressed as AWCD) followed a sigmoidal curve with incubation time. In the sample shown in Fig. 1, no color production occurred in the control well, so similar AWCD values were calculated by using either the reference point or the control well. The simplest estimate of overall rate of color development in a plate is obtained by dividing the AWCD value for an early incubation during the linear increase in color development by the length of incubation (e.g., between 20 and 26 h in Fig. 1). Rate estimates using AWCD values for later incubations would be less accurate because of the asymptotic nature of color development.

To determine the relative importance of inoculum size versus community activity on the rate and extent of color development, cell numbers were determined in 23 hydroponic solution samples, using the acridine orange method with epifluorescence microscopy (9). The activity of the same samples was determined as the turnover of a ^{14}C-labeled amino acid mixture (0.5 ng/ml or 0.2 nCi/ml), using the specific methods of Williams and Askew (26). For the 23 samples, color development rates estimated using AWCD values calculated from reference points on the plate as opposed to from the control well were more highly correlated with the inoculum cell density ($\log_{10}$ of the AODC) (r = 0.677) than with the amino acid turnover rate constant of the community (r = −0.470). These results suggest that color production in the BIOLOG community-level assay is caused by growth of bacteria within wells after inoculation rather than respiration of the inoculated community. Observation of dense mats of stained bacteria in highly colored wells also indicates the importance of bacterial growth in color formation.

Correlation of AWCD values, using the control well for the same set of 23 hydroponic solutions, were much more poorly correlated to either inoculum density (0.184) or activity (−0.173) than the AWCD values calculated by using a reference cell. Significant color production in the control well was recorded for only about half of the hydroponic solution samples. Color production in the control well did not appear to be a background color present in all wells. Rapid growth of specific guilds in the response wells at high substrate concentrations may overwhelm the respiration observed in the control well at the expense of the lower concentrations of organics present in the inoculum.

Clearly, further work is required to address better the relationship between control well and response well color formation. At present, elimination of the control well color response by diluting samples containing high concentrations of organics prior to inoculation is advisable. In our experiments the degree of dilution necessary to minimize color development in the control well never diluted the cell numbers to a level unacceptable for color production in the response wells.

(ii) Individual wells. Color development varied greatly among response wells (Fig. 1). In some of the response wells color developed rapidly and reached a maximum value within 20 to 30 h. Color development in other wells showed a lag phase for the first 24 h followed by a linear increase during the second 24 h of incubation. The lag phase in AWCD is shorter than that observed for the less responsive wells because of the immediate color production in the more responsive wells. In all of the samples analyzed, only a few wells showed no color response after 50 h of incubation.

Patterns of sole carbon source utilization. (i) Influence of rate of color development. The observed relationship between inoculum density and AWCD indicates that differences in the multivariate pattern of color response among samples as determined by PCA also may be strongly influenced by the relative abundance of bacteria in samples. This relationship was found for the raw-difference color response of plates from early incubations of freshwater samples, as indicated by the strong correlation (r^2 = 0.89) between coordinates of samples for the first PC and AWCD values (Fig. 2). In other words, PCA extracted a pattern in the structure of the data which can be explained, in large part, by differences in the rate of color development among plates. Transformation of the data by dividing each raw difference by the AWCD of the plate significantly reduced the influence of rate of color development on classification of samples. Correlation of coordinates of samples for the first PC with AWCD values was much lower (r^2 = 0.28) than found with the raw-difference data (Fig. 2). The amount of variance in the data set extracted by the first PC was similar for analysis of the raw-difference and transformed data (25 and 21.5%, respectively). This result indicates that PCA of transformed data explained a significant structure in the data set as a result of differences in the relative utilization of sole carbon sources among samples.

It was concluded that transformed data sets would be

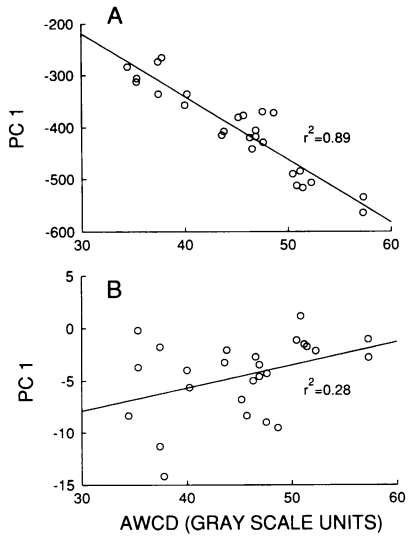

FIG. 2. Comparison of AWCD with scores for the first PC on the basis of PCA of (A) raw-difference and (B) transformed data for 40-h incubation of freshwater samples in BIOLOG plates. Lines represent the least-square regressions (n = 26). r^2 values are the squares of correlation coefficients.

more useful than raw-difference data for classifying samples among and within microbial habitats by PCA. Furthermore, analyses were performed on plates from later incubations so that differences among samples in color production in less responsive wells (which may show no color response in early readings) could be evaluated.

(ii) Differences among microbial habitats. Samples from three different microbial habitats (soil, freshwater, and hydroponic systems) had distinctive patterns of sole-carbon-source utilization on the basis of PCA of transformed color response data (Fig. 3). PCA was performed with the data from all samples from each habitat (i.e., all three soil types, lake and stream samples, hydroponic solution, and rhizosphere). Soil samples had much lower coordinate values (PC scores) for the first PC, which explained 34% of the variance in the data, than either the freshwater or hydroponic samples. Samples from the latter two microbial habitats had similar values for PC 1, but had different PC scores along the second PC, with the freshwater samples possessing higher coordinate values (with the exception of a single hydroponic system sample). The second PC explained 15% of the variance in the data.

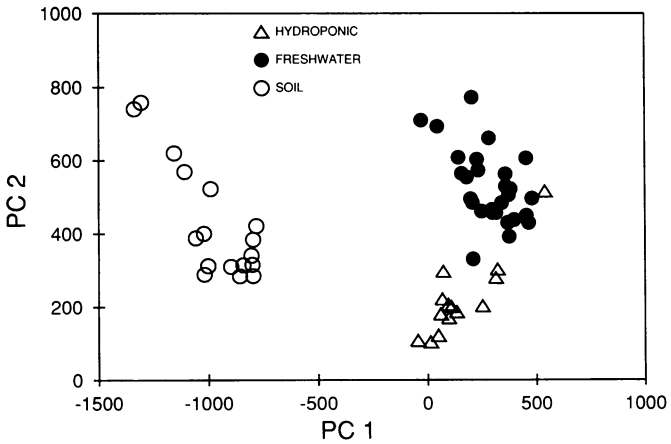

FIG. 3. Ordination produced from PCA of all soil, freshwater, and hydroponic samples. Scores of each sample for the first and second PCs are plotted.

The separation of samples in PC space can be related to differences in carbon source utilization by examining the correlation of the original variables to the PCs. The most important carbon sources in differentiating among the communities were defined as those which had at least half of their variance explained by PC 1 or PC 2 (Table 4). The degree to which a PC explains the variance in the color response of an individual carbon source is related to differences in the response in samples with different coordinate values for that PC. For example, samples with higher coordinate values for PC 1 showed a greater response for carbon sources positively correlated to PC 1 as compared with samples with low PC scores. Poor correlation of a carbon source to a PC (the majority of carbon sources in most cases) does not necessarily mean that it was poorly utilized by samples, but rather that its utilization was not different among samples with higher and lower coordinate values for that PC (i.e., it could not help differentiate samples).

Analysis of PC 1 (Fig. 3) indicates that soil microbial communities utilized a number of carbohydrates (glycogen, *N*-acetyl-D-galactosamine, D-arabitol, gentiobiose, D-melibiose, and β-methylglucoside) and phenylethylamine, to a relatively lesser degree, and several carboxylic acids (γ-hydroxybutyric acid, quinic acid, and sebacic acid), glucoronamide, γ-aminobutyric acid, and mono-methylsuccinate, to a relatively greater degree, than microbial communities associated with either the freshwater or the hydroponic samples examined. On the basis of PC 2 analysis, microbial communities associated with freshwater sites showed a greater relative utilization of three carbohydrates (arabinose, galactose, and trehalose), four carboxylic acids (citric acid, formic acid, D-glucosaminic acid, and α-hydroxybutyric acid), and three amino acids (glutamic acid, glycyl-L-aspartic acid, and glycyl-L-glutamic acid) and a lesser relative utilization of β-hydroxybutyric acid compared with microbial community samples from hydroponic systems.

(iii) Differences among samples from coastal lagoon transect. PCA of the coastal lagoon-ocean inlet gradient revealed a separation of samples from open-water and marsh creek sites along the first PC, which explained 21% of the variance in the data (Fig. 4). Open-water samples had higher responses for wells containing glycogen, several amino acids (L-alanyl-glycine, glycyl-L-aspartic acid, and glycyl-L-glutamic acid), two carboxylic acids (*cis*-aconitic acid and gluconic acid), and glucose-6-phosphate (Table 5). Included in the marsh creek grouping are sites from both the inland- and barrier island-associated marshes and a mud flat station. Replicate plates of one of the barrier island-associated marsh creek sites showed wide divergence, with one of the replicates falling in the open-water grouping. The variability among these replicates may have been due to cross-well contamination caused by vibration of plates during the boat ride across rough water. In general, replicate plates (as denoted by letter) possessed similar data patterns, as represented by similar coordinate values. The second PC, which explained 13.6% of the variance in the data, distinguished the ocean inlet site from all lagoon or marsh creek sites. This site showed greater relative response in wells containing *m*-inositol, α-lactose, asparagine, and L-phenylalanine (Table 5).

TABLE 4. Correlation of carbon source variables to PCs for analysis of soil, freshwater, and hydroponic samples

PC 1		PC 2	
Carbon source	r^a	Carbon source	r
Carbohydrates		Carbohydrates	
Glycogen	0.796	Arabinose	0.747
N-Acetyl-D-galactosamine	0.894	Galactose	0.676
D-Arabitol	0.916	Trehalose	0.676
β-Methylglucoside	0.943		
D-Melibiose	0.884	Carboxylic acids	
Gentiobiose	0.770	Citric acid	0.702
D-Melibiose	0.884	Formic acid	0.699
β-Methylglucoside	0.943	D-Glucosaminic acid	0.702
		α-Hydroxybutyric acid	0.743
Methyl esters		γ-Hydroxybutyric acid	−0.803
Mono-methylsuccinate	−0.755		
		Amino acids	
Carboxylic acids		Glycyl-L-glutamic acid	0.673
γ-Hydroxybutyric acid	−0.740	Glycyl-L-aspartic acid	0.664
Quinic acid	−0.719	Glutamic acid	0.662
Sebacic acid	−0.739		
Amides			
Glucuronamide	−0.810		
Amino acids			
γ-Aminobutyric acid	−0.708		
Aromatics			
Uridine	−0.726		
Amines			
Phenylethylamine	0.887		

[a] r, Pearson's regression coefficient.

(iv) Differences among soil samples. PCA of the preincubated soil samples revealed distinctive patterns among both soil types and incubation media (Fig. 5). Differences among soil types, however, were always greater than differences within soil types due to incubation media. The first PC, which explained 27.9% of the variance, separated samples from all incubation media of soil 23 from samples of soils 22 and 35. Separation was largely a result of greater utilization of a number of carbohydrates (*m*-inositol, D-mannitol, D-raffinose, L-rhamnose, and D-sorbitol) and lesser utilization of a number of amino acids (D-alanine, L-alanine, L-ornithine, L-proline, and L-serine), succinamic acid, and phenylethylamine by samples of soil 23 (Table 6). The second PC, which explained 26.7% of the variance, separated samples of soil 23 incubated in YE medium from those incubated in YS and YP media. The second PC also separated soil 22 samples (particularly those incubated in YE and YP media) from samples of soil 35. PC 2 was positively correlated to four amino acids (D,L-aspartic acid, L-asparagine, L-aspartic acid, and L-glutamic acid) and three carboxylic acids (*cis*-aconitic acid, citric acid, and D-gluconic acid) and negatively correlated to six carbohydrates (adonitol, α-D-glucose, α-D-melibiose, β-methylglucoside, and psicose). AWCD of samples was highly correlated ($r^2 = 0.775$) to the coordinates of PCA 2, but was uncorrelated ($r^2 = 0.044$) to coordinates for axis 1. Replicate plates of samples had very similar coordinate values.

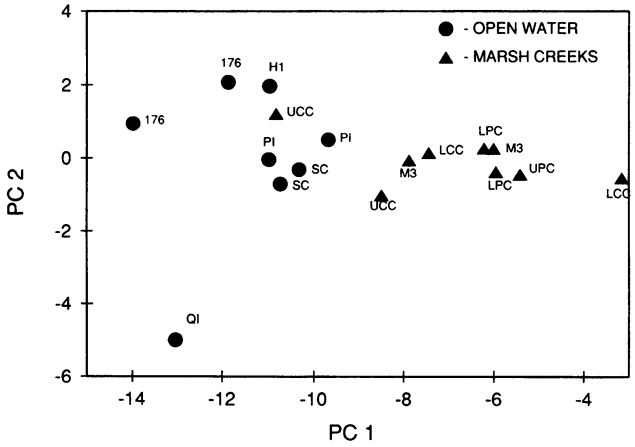

FIG. 4. Ordination produced from PCA of coastal marine water samples. Scores of each sample for the first and second PCs are plotted. Symbol type distinguishes sample sites associated with inland or barrier island marshes (marsh creek) from those located in channels or the ocean inlet (open water). Letters denote sampling site (see Table 2 for site descriptions). Identical letters represent scores for plates taken of replicate water samples at a site.

DISCUSSION

The inoculation of samples containing intact microbial communities into BIOLOG plates produced response patterns that distinguished among widely disparate samples and among dissimilar sample types within larger categories. Differentiation of samples from different habitats was a useful preliminary test of the assay. The ability to distinguish among samples within similar habitats, however, was a much more powerful test of the resolving power of the assay.

TABLE 5. Correlation of carbon source variables to PCs for analysis of salt marsh-ocean inlet transect samples

PC 1		PC 2	
Carbon source	r^a	Carbon source	r
Polymers		Carbohydrates	
Glycogen	−0.811	*m*-Inositol	−0.806
Carbohydrates		α-Lactose	−0.727
Turanose	0.770	Amino acids	
Xylitol	0.743	Asparagine	−0.745
Carboxylic acids		L-Phenylalanine	−0.846
cis-Aconitic acid	−0.751		
Gluconic acid	−0.917		
Glucosamic acid	0.738		
β-Hydroxybutyric acid	0.714		
Amino acids			
L-Alanyl-glycine	−0.808		
Glycyl-L-aspartic acid	−0.802		
Glycyl-L-glutamic acid	−0.853		
Serine	−0.777		
Phosphorylated chemicals			
Glucose-6-phosphate	−0.721		

[a] *r*, Pearson's regression coefficient.

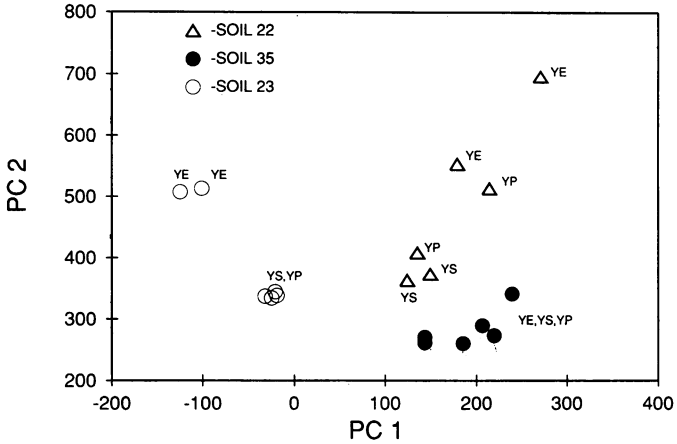

FIG. 5. Ordination produced from PCA of soil samples. Scores of each samples for the first and second PCs are plotted. Symbol type indicates soil sample (see Table 3 for physiochemical descriptions). Letters refer to the medium in which soils were incubated prior to inoculation into BIOLOG plates. Identical symbol-letter combination represent replicate shake flask cultures.

This level of resolution allows for the examination of the structuring agents within specific types of microbial communities (soil, planktonic, etc.).

The resolution of all three soil samples analyzed on the basis of patterns of sole-carbon-source utilization indicates that the BIOLOG community assay can be a useful tool for classifying soil microbial communities. It would be particularly interesting to study the relationship between classification of soils based on microbial community structure and the well-established classification based on physiochemical factors. The intensive sampling required for such a study is feasible given the rapid processing of samples by the BIOLOG community assay. Recent research in our laboratory suggests that preincubation of soils may be unnecessary, further decreasing the time required for sample processing. Blending soils, with subsequent flocculation of soil particles (5), produces colorless suspensions suitable for direct inoculation into BIOLOG plates, and this procedure is now our method of choice. The differences observed among replicate soil samples inoculated in different media suggest that relatively short-term (18-h) responses of soil microbial communities to changes in carbon sources are discernible with the BIOLOG assay. This level of resolution would allow for the examination of changes over time (succession) in soil microbial communities exposed to both natural (root exudates) and anthropogenic (industrial wastes) sources of carbon.

Three types of suspended aquatic microbial communities within an estuarine system were identified: open water, marsh creek, and ocean inlet. The open-water and marsh creek sites included samples from spatially distinct sites. Such spatial homogeneity is not surprising for suspended microbial communities in a tidally mixed system. In fact, tidal influx of ocean water appears to be a major influence on the structure of the suspended microbial community; the relatively shallow water sites close to the ocean inlet but removed from the marsh (open channel through perched marsh and swash zone behind aggrading spit on barrier island) were similar to the open-water sites. The BIOLOG community assay is rapid and, it appears, sensitive enough to conduct the intensive sampling required to examine the mechanisms coupling sources of bacterial substrates to dynamics in the bacterioplanktonic community in aquatic systems. Ducklow (6) suggests that the collection of water samples every 15 min for 5 to 7 days in a ship averaging 8 knots would be necessary to monitor changes effectively in marine bacterioplanktonic communities. A single worker could use the BIOLOG community assay at this rate (1 min to inoculate plates, 2 to 3 min to read plates) to produce estimates of community structure and approximate overall

TABLE 6. Correlation of carbon source variables to PCs for analysis of soil samples

PC 1		PC 2	
Carbon source	r^a	Carbon source	r
Carbohydrates		Carbohydrates	
Gentiobiose	0.741	Adonitol	−0.804
m-Inositol	−0.812	α-D-Glucose	0.932
D-Mannitol	−0.765	α-Lactose	−0.864
D-Raffinose	−0.719	D-Melibiose	−0.855
L-Rhamnose	−0.724	β-Methylglucoside	−0.769
D-Sorbitol	−0.977	Psicose	−0.850
Carboxylic acids		Carboxylic acids	
α-Ketovaleric acid	−0.746	*cis*-Aconitic acid	0.854
Succinamic acid	0.894	Citric acid	0.840
		D-Gluconic acid	0.827
Amino acids		D,L-Lactic acid	0.811
D-Alanine	0.904		
L-Alanine	0.861	Amino acids	
L-Alanyl-glycine	−0.704	L-Asparagine	0.837
L-Leucine	−0.705	L-Aspartic acid	0.851
L-Ornithine	0.869	L-Glutamic acid	0.810
L-Proline	0.817		
L-Serine	0.764		
Amine			
Phenylethylamine	0.909		

[a] *r*, Pearson's regression coefficient.

density. Similarly, intensive sampling regimens would be useful in the continuous monitoring of the effects of polluted effluents on aquatic microbial communities.

While the ability to distinguish among samples may be of importance in itself, an understanding of the basis for classification of samples is necessary to understand the factors which regulate microbial community structure. The use of functional characteristics (i.e., sole-carbon-source utilization) gives ecological meaning to classification of samples with the BIOLOG community-level assay. Separation of samples along PC axes can be directly related to differences in utilization of sole carbon sources among samples. Discussion of the potential causes for the differences in carbon source utilization among samples observed in this research is beyond the scope of the present study, but should be of primary importance in subsequent studies that use this technique. Several approaches may aid in the interpretation of functional differences among communities: (i) replication of studies to observe recurring patterns of carbon source utilization, (ii) reduction of the number of carbon source variables by summing the color responses for different types of substrates (e.g., hexoses, pentoses, aromatics, etc.), (iii) use of carbon sources not found in the GN microplate to fit specific research questions, and (iv) rotation of axes to maximize high correlations and minimize low ones (e.g., via the varimax technique [16]).

The specific meaning of differences in carbon source utilization is related to the mechanism of color development. The color response in a given well appears to be related to the number of microorganisms which are able to use the substrate within the well as a sole carbon source and are concomitantly stained with tetrazolium violet. This conclusion is based on microscopic examination of colored wells and on the observed correlation of AWCD with inoculum density rather than activity. The number of stained cells at a given incubation appears to be a function of the number of cells in the original inoculum able to utilize the substrate as a sole carbon source and the growth of these cells during incubation. The immediate development of color in certain response wells (Fig. 1) suggests that the inoculum contained a large fraction of microorganisms able to utilize the substrate and that staining of these cells was sufficient to produce detectable color. The lag phase in color development in other wells indicates that a longer period of growth was necessary to produce a sufficient density of stained cells, reflecting a smaller percentage of microorganisms in the inoculum able to utilize the substrate as a sole carbon source. Despite these strong suggestions, we cannot currently discount the possibility that rapid color development may be due, in some cases, to a few cells with high specific activities and growth rates on the carbon source included in an individual well.

The importance of growth indicates that the color responses produced in the BIOLOG community assay are a reflection of functional potential rather than in situ functional ability. In a strict sense, the role of growth also suggests that the assay is a cultural method (i.e., it requires the growth of microorganisms on media). This method, however, does not contain the bias associated with isolate-based methods because functional tests are performed on the original community rather than on some subset of the community.

Given the correlation between inoculum density and AWCD, among-sample comparisons of the color production in a given well are dependent on both the density and the composition of the inoculum. A sample with a greater density of microorganisms would produce a different pattern of color response data than a sample of identical composition but lower density of microorganisms for similar incubation times. Differences caused by density may be of interest when comparing samples on a unit basis (e.g., differences between response per milliliter of water or milligram soil). However, the influence of density is a confounding factor when the research questions the relative composition of communities or comparison of samples from different habitats.

Several methods may be used to overcome this potential influence of inoculum density on color response data: (i) dilution or concentration of samples to achieve equivalent inoculum densities, (ii) use of multiple readings over a time course of incubation, or (iii) transformation of the data to account for different inoculum densities. The last method normalized the raw color response data to the AWCD and greatly reduced the correlation of PC scores to the overall rate of color development. Inoculum density may still influence the structure of transformed data sets, as indicated by the high correlation between AWCD and the second PC produced from PCA of the soil samples. While the correlation does not necessarily reflect causation, it indicates that the structure in the data set extracted by PCA may reflect differences in the rate of color production. The nonlinear nature of color production complicates attempts to use a single plate reading to compare multiple samples of different densities. For optimal characterization of environmental samples using this method, samples of approximately equivalent density should be used and color production should be closely monitored. Our research has indicated that the most efficient methodology involves two plate readings: an initial reading upon color development used for the calculation of AWCD as an indicator of microbial density and a subsequent reading 12 to 24 h later used for multivariate analysis. When more intensive sampling is feasible, a time course of color development could be produced for each well. This approach, while time-consuming and data intensive, would allow for the precise definition of, in effect, 95 separate growth curves for each sample.

The structure in the transformed data set more likely reflects differences among samples in the fraction, rather than absolute density of microorganisms in the inoculum able to utilize substrates as sole carbon sources. In this way, the transformed data set reflects the evenness in distribution of functional abilities within the community. An index of functional evenness similar to that of Troussellier and Legendre (23) could be calculated from the color response data. We believe that the approach utilized in this study, which emphasizes differences among samples in specific functional tests rather than a general index of functional evenness, maximizes (i) the resolving power of the assay and (ii) the identification of ecologically relevant differences among communities. However, evaluation of an overall index of functional evenness may be useful for certain research questions.

Quantitative scores of color response produced from digitized images were considered superior to qualitative scores (presence or absence of color) for the resolution of communities due to our initial observation of color production in almost all wells with incubation of most environmental samples. Alternative approaches may be feasible for researchers without access to microplate readers that will generate color values. Samples could be classified on the basis of dominant functional tests (e.g., the first 20 or 30 wells to show the presence of color). While the resolving power of the method might be expected to decline with the

use of a reduced number of binary test scores, several workers have found that a limited number (20 to 30) of tests were sufficient to describe the diversity of microbial communities using the numerical taxonomic approach (3, 15). Since microbial communities may be dominated by only a few types of individuals (12), evaluation of dominant functional characteristics may be sufficient to classify and characterize microbial community structure. Furthermore, by emphasizing wells with very rapid color production, classifications based on dominant functional characteristics more accurately represent in situ functional abilities compared with classifications described above.

The results of this research indicate that the direct incubation of environmental samples in BIOLOG plates produces patterns of metabolic response useful in the classification and characterization of microbial communities. The ability to rapidly visualize community structure as a composite of functional abilities (or potentials) enables both the intensive comparison of microbial communities across a wide range of spatial and temporal scales and the identification of ecologically relevant functional differences among communities. Widespread use of this technique should provide a more robust and ecologically relevant classification of heterotrophic microbial communities than presently exists.

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