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MICROBIAL COMMUNITY ANALYSIS: A KINETIC APPROACH TO CONSTRUCTING POTENTIAL C SOURCE UTILIZATION PATTERNS

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Summary—The analysis of multiple substrate metabolism by assemblages of bacterial strains may be used to differentiate inocula from environmental samples. Biolog plates, 96-well microtiter plates containing nutrients, a single carbon test substrate in each well and a tetrazolium redox dye to monitor substrate oxidation, have been used for this purpose. One of the difficulties faced by users of this technique is determining which substrates have been metabolized. Reliance on single-time-point absorbance data for each well is problematic due to variably non-linear rates of color development for each well. Previous efforts to use color-normalized single plate readings have been successful in discriminating between environmental sample types, but substrate-use contributions to sample classifications vary depending on the duration of the plate incubation. We present a model based on the logistic equation for density-dependent population growth providing a good (low χ^2) fit to the sigmoidal kinetics of color development data. The kinetic parameters generated by the model can be used as surrogates for single-time-point data in constructing carbon source utilization patterns, and contribution of substrate use to sample classification does not depend on incubation time. This technique obviates the need to choose the time following inoculation to read the plate absorbance data and also provides two kinetic parameters that are invariant with respect to inoculum density. We provide a comparison of community potential substrate use analyses using single-time-point microplate data and parameters from our kinetic model.

INTRODUCTION

Patterns of carbon substrate metabolism have been used to characterize microbial communities from environmental samples (e.g. Garland and Mills, 1991; Winding, 1994; Zak *et al*., 1994; Bossio and Scow, 1995; Garland, 1996). Most of these efforts have involved the use of commercially available microtiter plates containing 95 carbon test substrates and a tetrazolium redox dye (e.g. Biolog GN Microplates; Biolog, Inc., Hayward, CA). While these plates were originally designed to classify isolates based on their pattern of substrate use, community-level analysis entails inoculating the plates with whole environmental samples (e.g. soil suspensions, hydroponic solutions, etc). Substrate use patterns are then quantified by comparing tetrazolium dye color development in each of the 95 wells of the plate with a control well containing no test substrate. Substrate metabolism is typically reported either by establishing minimum threshold values of color density to generate binary data (presence or absence of metabolism in a given well; Zak *et al*., 1994) or by comparing color densities of wells after subtracting the measured density of the control well (Zak *et al*., 1994; Bossio and Scow, 1995). Whichever method is used, the data are then subjected to multivariate analysis (e.g. principal components analysis) to both discriminate communities based on their substrate use patterns and reduce the dimensionality of the data set generated from the 95 variables (substrates) measured.

Analysis of community level metabolic diversity, as reflected by microplate C source use patterns, is restricted by the ability of microbes to metabolize substrates in the microplate environment. It cannot, therefore, be seen as a tool to directly assess total microbial functional diversity in environmental samples. However, functional diversity of the organisms able to metabolize microplate substrates *in vitro* can be a useful tool to differentiate environmental samples if care is taken to protect against biases introduced in the analysis of microplate responses. When these biases are removed, it may be possible to associate *in vitro* metabolic diversity to phenomena *in situ*.

One problem faced by researchers using this technique is that the rate of color development in wells

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is a non-linear process depending on both time and inoculum density. As a result, color densities or binary data regarding substrate use patterns vary depending on the time of incubation following inoculation (Haack *et al.*, 1995). One way around this problem has been to read optical density (OD) of the microplate wells at a set time following inoculation or onset of color development in the microplate (Garland and Mills, 1991; Bossio and Scow, 1995). However, this is problematic as inoculum density variably affects the lag time from inoculation to onset of color development in any given well. Garland (1996) showed that the time to achieve a given average microplate well color development (AWCD) value was correlated negatively with inoculum density. Among the data analysis options he suggested were comparing substrate use patterns of samples when the plates had equivalent AWCD values, and normalizing each substrate's color density datum at a given time by dividing by that sample's AWCD at that time. While each technique was successful in discriminating his rhizosphere samples, he pointed out that the specific substrates contributing to the separation of sample types varied depending on duration of the incubation prior to reading the plate. Faster developing wells were shown to be more influential in classifications based on plate readings after short incubations, and more slowly developing wells contributed more to sample separations after longer incubations. Haack *et al.* (1995) suggested that the kinetics of color development, rather than degree of development at a given time, could be used as data to assess the functional diversity of the organisms growing in the microplate.

We used Biolog GN plates to detect differences in whole environmental samples from different field treatments, and in axenic and mixed laboratory cultures. Our approach avoided some of the pitfalls associated with single-time-point determinations by repeatedly measuring color density in each well over several days. Our technique also allowed us to compare samples with different initial populations by relying on analysis of kinetic parameters which are invariant with respect to inoculum density. We modeled the course of color development using a modified logistic equation and fit the data to the three parameters in this model. Patterns of potential C substrate metabolism were then analyzed using the kinetic parameters of the model, rather than the single-time-point color absorbance data commonly used. Each parameter provides an alternative to single-time-point optical density data for analysis by multivariate techniques. To allow comparison of our approach to previous methods, we analyzed our microplate data using the kinetic approach, AWCD-normalized single-time-point OD, and the method of comparing ODs of samples at equal AWCDs.

MATERIALS AND METHODS

We used both well-characterized isolate cultures and soil suspensions as microplate inocula in our experiments. The first experiments used soil suspensions in time course measurements of test well OD to produce the color development curves for our kinetic model. A second set of experiments also used a soil suspension to determine cell growth in the Biolog microplate during color development. A third group of experiments used laboratory-grown isolates axenically or as a mixture to evaluate the effect of mixed cultures and inoculum density on the parameters of our kinetic model.

Microplate data

All experiments used Biolog GN microplates (Biolog, Inc., Hayward, CA) incubated at 21°C to assess multiple substrate metabolism. Absorbance data were collected at 595 nm using a Bio-Rad Model 3550-UV microplate reader (Bio-Rad Laboratories, Hercules, CA). This wavelength was selected because it provides maximum absorbance for tetrazolium violet; however, turbidity from cell growth may also contribute to OD at this wavelength. To resuspend any cells or precipitate which had settled to the bottom of the well, each plate was shaken for 5 s three times prior to reading. Plates were read within 1 h following inoculation and again repeatedly over the course of a week or two (in 8–24 h intervals depending on the experiment) to generate color development curves.

Environmental sampling

The soil samples used in the development of our kinetic model were collected from the Caribou-Poker Creeks Research Watershed (referred to as the 'field site') located 48 km north-east of Fairbanks, Alaska. The sampling area, an open black spruce forest, is the site of an experimental crude oil spill which took place in 1976 (Collins *et al.*, 1994). Samples were collected as soil cores in both the oiled area (OIL) and an adjacent, oil-free area (OF) and placed in coolers immediately following collection. The O horizon was separated from the A horizon (Brady, 1990), homogenized by sieving (2 mm) twice, and placed in sterile plastic bags and refrigerated until further processing. Microplates were inoculated with these O horizon samples within 48 h of sampling.

Microplate inocula

Ten 5 g organic horizon soil samples from the study area were diluted 1000-fold in sterile saline solution (Ringer solution; Collins *et al.*, 1989). Thus tetrazolium dye reduction (color development) could be monitored with minimal interference from suspended and dissolved soil components. The original soil samples used as inocula had initial total bacterial populations (determined by direct count at

Oregon State University's Soil Microbial Biomass Services Laboratory) ranging from 7×10^7 to 7×10^8 cells g^{-1} dry soil. Final inoculum density was *ca.* 10^5–10^6 cells ml^{-1}. These samples were used as microplate inocula in the first time course studies to produce the kinetic curves used to generate our model. To determine cell growth during color development in the microplates, we used a soil suspension from a single OF field sample (9.05×10^7 cells ml^{-1} inoculum) in the second set of experiments.

Cultures of *Escherichia coli* (ATCC #25922; Difco, Detroit, MI) and *Enterobacter aerogenes* (ATCC #13048; Difco, Detroit, MI) were used as Biolog plate inocula to evaluate single isolate contributions to microplate well OD kinetics in the third group of experiments. These organisms were chosen for their rapid growth rates and because both enteric organisms would be likely to use many of the same substrates on the microplates. This would allow us to compare the kinetic parameters for each substrate metabolized by both isolates. Overnight flask cultures (nutrient broth; Difco, Detroit, MI) were harvested by centrifugation and resuspended in saline twice to wash residual nutrients from the cultures. These suspensions were used either as pure cultures or as a mixture of the two cultures as inocula in the Biolog plates. The inoculum density of the axenic cultures and of the mixture was adjusted so that the total cell numbers were approximately equal for all treatments (*ca.* 4×10^7 cells ml^{-1}).

To assess the effect of inoculum density on the parameters of the kinetic model, we used four densities of *E. coli* as inocula. After washing an overnight culture of this organism as described above, three dilutions of the final suspension were made. This provided inocula representing four orders of magnitude (*ca.* 2×10^4–10^7 cells ml^{-1}) of cell densities.

Kinetic analysis and model development

To evaluate dye reduction and color development following inoculation of the microplates, we used suspensions from five samples from each field site (OIL and OF) for the first set of experiments. These plates were read at approximately every 8 h for 1 wk following inoculation. From the time course change of each used substrate's OD at 595 nm, kinetic curves were determined to be sigmoidal in shape, suggesting a density-dependent logistic growth curve. Using these data, a model based on a modified form of the logistic equation was developed (see Results).

Cell growth and color development

To determine the relationship between changing OD values in microplate wells and microbial densities, we enumerated populations of microorganisms growing on two of the substrates (β-hydroxy butyrate and L-glutamate) in the GN microplates over time in a second experiment. These substrates were selected as representing two groups of compounds, a common microbial storage product and an amino acid. Glutamate has been shown to be a good representative substrate for measuring heterotrophic microbial activity (Griffiths *et al.*, 1977). Using a single OF soil suspension from the study site diluted 1000-fold, we inoculated 15 GN microplates. The plates were read every 24 h for 5 d following inoculation. After each reading, 100 μl of culture was removed from the β-hydroxy butyrate and L-glutamate wells in three of the plates not yet sampled, and placed in 900 μl of filtered (0.45 μm) formalin (1.8% v/v; Sigma Chemical Co., St. Louis, MO). Three 100 μl aliquots of the inoculum were collected and preserved in a similar manner prior to inoculating the plates to determine initial cell density.

The formalin-preserved samples were enumerated by acridine orange direct count epifluorescent microscopy (Hobbie *et al.*, 1977) as modified by Braddock *et al.* (1984). A minimum of 10 fields per filter were counted unless fewer than 30 cells per field were observed, in which case more fields were counted until 300 cells were enumerated.

Optical density of every well in each of the plates in this experiment was determined every 24 h with the exception of the two wells in those plates which had already been sampled for microbial enumeration. Due to this destructive sampling, the mean OD for these two substrates was calculated using three fewer data for each successive sampling period (i.e. $n = 15$ for initial and 24 h readings, $n = 12$ for 48 h reading, etc).

Evaluation of kinetic model

The reproducibility of the kinetic model parameters fit to the color development time course data was evaluated using laboratory-grown isolates in a third group of experiments. First, Biolog GN plates were inoculated in triplicate using equivalent cell densities of *E. coli, E. aerogenes* or mixtures of the two organisms in a 1:1 ratio. Inocula were prepared as described above. The plates were read immediately following inoculation and on eight occasions over 120 h. Production of polysaccharides by *Enterobacter* species that may yield false positive microplate responses was checked by observing that the control well did not develop color (as recommended by the manufacturer; Biolog, 1992).

Another experiment used *E. coli* at several cell concentrations to evaluate the effect of inoculum density on the model parameters. We inoculated triplicate Biolog plates with four concentrations of inoculum and read the 12 plates immediately after inoculation and repeatedly during the next 330 h.

Data analysis

Raw OD data at a given reading time for every test well were corrected by subtracting that plate's blank well OD. Initial OD values for all wells in the plate ranged from *ca.* 0.250 to 0.450. Substrates with a final corrected OD less than 0.200 were omitted from the data set to be fit with the model. The corrected data for each substrate showing color development were fit to our kinetic model and its parameters were estimated with the personal computer-based data analysis program, Origin (version 3.5; Microcal Software, Inc., Northampton, MA). This software uses the Levenberg–Marquardt algorithm and the simplex method for nonlinear least-squares curve fitting. A simple macro written in Origin script language was used to fit the time course OD data set for each well in the plate, and produced estimates of the model parameters and their standard errors along with an estimate of the goodness of fit (χ^2). Using a Pentium processor-based personal computer, data for 95 test wells from a given microplate could quickly be fit to our model (*ca.* 3 min per plate). Curve parameters with standard errors larger than the parameter value were taken as evidence of a bad fit and the kinetic data for these substrates were not used. Only about 5% of the curves generated for the environmental samples (45 of 824) were excluded from further analysis. All parameters used in the analysis came from fit curves with χ^2 values less than 0.01. Each kinetic parameter was then used to construct a data matrix for calculation of principal components (PCs). Thus a kinetic parameter from the model fit to the time course data for each substrate metabolized was used in place of the single-time-point datum commonly used in evaluating substrate use patterns. Separate sample analyses were performed using each of the three model parameters as data.

To compare our kinetic data analysis results with the single-time-point approach typically used in community-level substrate metabolism studies, we used two other methods to prepare our microplate data for analysis. Average well color development (AWCD; Garland, 1996) data were calculated for each sample at each reading time, and time points providing equivalent microplate AWCDs for each sample were selected from the data sets for laboratory culture and environmental inocula. The first comparative analysis used blank-corrected OD data from sample microplates measured at equivalent AWCDs. Negative corrected OD data were set to zero for all single-time-point data analyses. Sample AWCDs at the selected reading times were determined to be not different by testing for significant differences among samples with a one-way analysis of variance using each plate's 95 substrates' OD data as input. The second comparative data treatment involved normalizing each sample's single-time-point OD datum by dividing it by the sample's AWCD at that time. Both OD data types were then subjected to the same multivariate analysis as the kinetic parameter data.

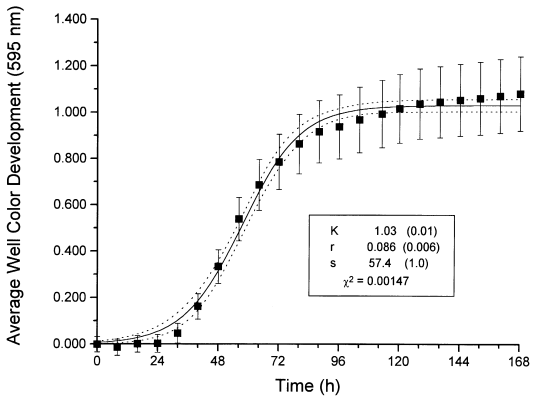

Fig. 1. Kinetics of average well color development for an oil-free (OF) environmental sample. Absorbance values (■) are the mean of 95 substrate well absorbances corrected by subtracting reference well absorbance at each time point. Error bars are standard errors. The solid line (—) is a plot of the equation fit to the mean absorbance data and the dotted lines (· · ·) represent upper and lower 95% confidence limits for the fit equation. Kinetic model K, r and s parameter data for the fit equation are presented (standard errors in parentheses) along with the χ^2 value of the fit to the absorbance data.

Principal components analysis (PCA) of both the single-time-point OD and kinetic correlation matrices data and other statistical analyses were performed using Systat software (version 5.05; SPSS, Inc., Chicago, IL).

RESULTS

Kinetic model

The first set of experiments showed test well dye reduction was non-linear in microplates inoculated with the soil samples, and the shape of the color development curve was generally sigmoidal (Fig. 1). Test well OD values over time suggested a kinetic model based on the density-dependent logistic growth equation of the general form

$$N(t) = \frac{K}{1 + be^{-rt}} \quad (1)$$

(Ricklefs, 1990) to describe a population of individuals, $N(t)$, at time t exhibiting density-dependent growth under conditions of environmental constraint. In this equation, the exponential rate of population change is determined by the exponent r and is expressed in units of reciprocal time. K represents the 'carrying capacity' of the system, or asymptote approached by the curve. The unitless coefficient b affects the horizontal displacement of the curve.

We modified equation (1) to a form providing parameters which could be interpreted more readily

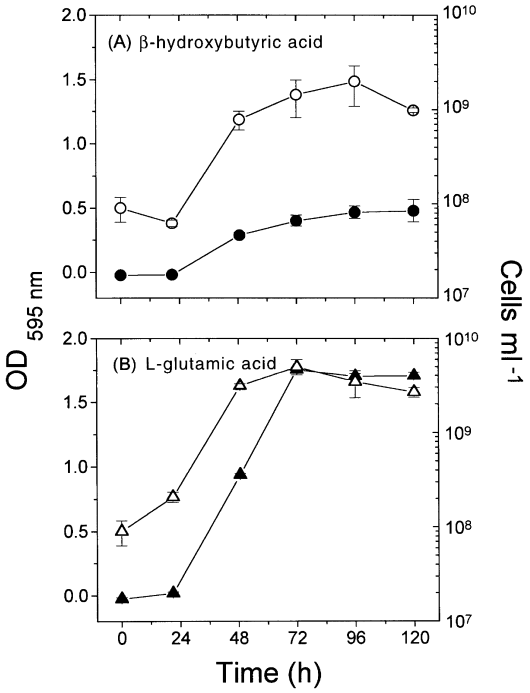

Fig. 2. Kinetics of color change (OD_{595}) and population increase (cells ml^{-1}) in two Biolog GN microplate substrate wells. Inoculum biomass was 9.05×10^7 cells ml^{-1}. OD data for (A) β-hydroxybutyric acid (●) and (B) L-glutamic acid (▲) are presented as mean corrected absorbance of replicate plates not yet sampled for cell enumeration (n = 15 at 0 and 24 h, n = 12 at 48 h, n = 9 at 72 h, n = 6 at 96 h, and n = 3 at 120 h) in this experiment. Cell number data (○-β-hydroxybutyric acid; △-L-glutamic acid) are presented as the mean of replicate samples (n = 3) removed from the plates at each time indicated. Error bars are standard errors.

with respect to the shape of the OD kinetic curve and to the underlying microbiological behavior driving its shape (equation (2)).

$$y = \mathrm{OD}_{595\mathrm{nm}} = \frac{K}{(1 + e^{-r(t-s)})} \qquad (2)$$

In this equation, K represents the asymptote ($y = K$) that the test well OD curve approaches, r determines the exponential rate of OD change, and t is the time following inoculation of the microplate. We have included equation (1)'s coefficient b as the exponential parameter s in the denominator of equation (2). The parameter s is the time to the midpoint of the exponential portion of the curve (when $y = K/2$). The value of s is expressed in units of time and is related to b in equation (1) as

$$s = \frac{\ln b}{r}, \quad \text{or} \quad b = e^{rs} \qquad (3)$$

Cell growth and color development

Microbial growth during OD change in two microplate substrate wells (β-hydroxybutyric acid and L-glutamic acid) for the second experiment is presented in Fig. 2. Cell numbers changed linearly with OD ($r^2 = 0.70$ for β-hydroxybutyric acid and $r^2 = 0.90$ for L-glutamic acid; n = 12) from the initial values at inoculation ($9.0^5 \times 10^7$ cells ml^{-1}) through the third sampling (i.e. prior to decrease in rate of color change). Beyond the time the OD change began to level off, little change in microbial population was observed. The kinetic model fit to the OD data for the two substrate wells yielded r parameter values of 0.135 and 0.172 for β-hydroxybutyric acid and L-glutamic acid respectively. When we fit our kinetic model to the direct count data, the r parameter values were lower than those derived from OD data (r = 0.069 and 0.139 for β-hydroxybutyric acid and L-glutamic acid, respectively).

Effect of inoculum density on kinetic model parameters

The triplicate plates inoculated with washed *E. coli* cells at concentrations ranging over four orders of magnitude demonstrated the stability of the model's K and r parameters with changing inoculum density in the third experiment. The values for the s parameter generally decreased with increasing inoculum density. The general behavior of the kinetic model's parameters with changing inoculum density can be seen in plots of average well color development (AWCD) over time for the four sets of plates (Fig. 3). Each plot in Fig. 3 presents the mean OD change over time in the 285 blank-corrected test wells of the three replicate plates. Similar results were seen in the OD kinetic data for each individual substrate used. The values of K and r for each metabolized substrate and their coefficients of variation (CV) were calculated for all 12 plates in the experiment to assess the invariance of these parameters with changing inoculum density. The mean CV (±S.E.) for all substrates across four orders of magnitude of inoculum density was 0.11 (±0.01) for K data and 0.20 (±0.02) for r data.

Spearman rank correlation coefficients (r_s; Zar, 1984) were calculated to determine the relationship between inoculum density and the value of the kinetic parameters for each metabolized substrate. Correlation coefficients (r_s) for K values appeared to be randomly distributed among the substrate variables, ranging from −0.605 to 0.777 with a mean r_s (±S.E.) of 0.217 (±0.054); P-value > 0.5. The r parameter correlation values ranged from −0.648 to 0.820 with a mean r_s (±S.E.) of 0.000 (±0.068; P-value > 0.5), also indicating no correlation with inoculum density. The value of the model's s parameter, however, was negatively correlated with the initial cell number in the inoculum. All but two substrates had s values negatively correlated with initial cell density. The range of correlations for s with inoculum density was from 0.259 to

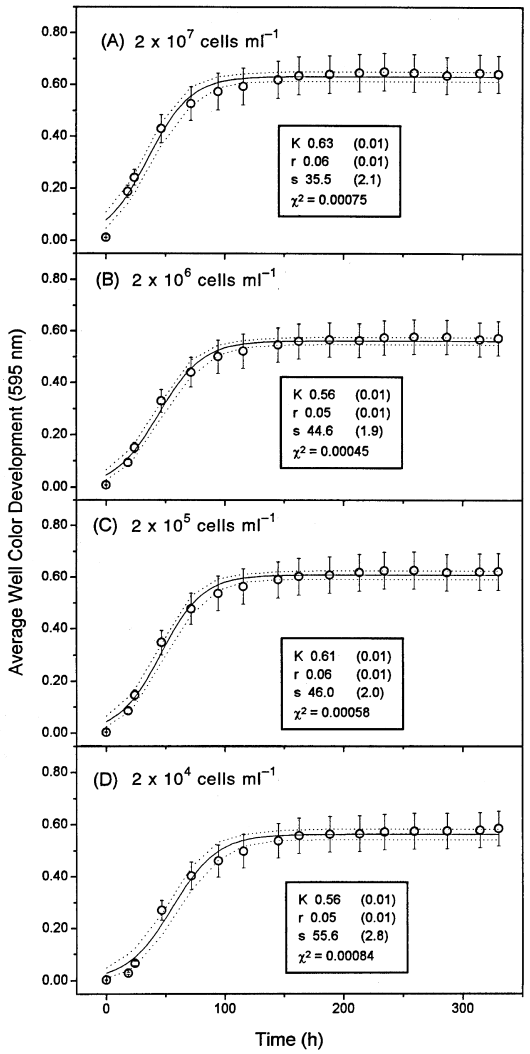

Fig. 3. Kinetics of average well color development (○) for suspensions of *E. coli* at four inoculum densities: (A) 2×10^7 cell ml^{-1}, (B) 2×10^6 cells ml^{-1}, (C) 2×10^5 cells ml^{-1}, and (D) 2×10^4 cells ml^{-1}. The solid line (—) is a plot of the equation fit to the mean absorbance data and the dotted lines (· · ·) represent upper and lower 95% confidence limits for the fit equation. Kinetic model *K*, *r* and *s* parameter data for the fit equation are presented (standard errors in parentheses) along with the χ^2 value of the fit to the absorbance data for each curve.

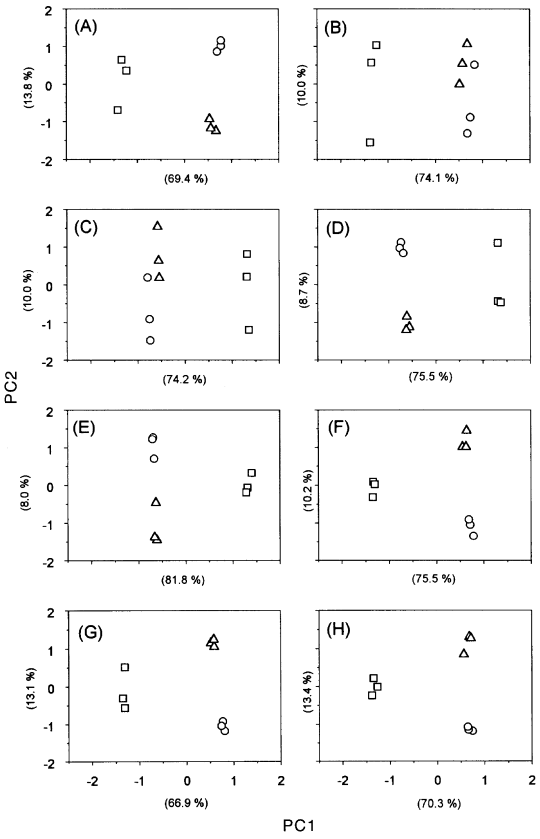

Fig. 4. Ordination produced from laboratory culture microplate OD data using single-time-point OD values or kinetic parameters from time course OD data fit to the logistic model. Component scores for *E. coli* (□), *E. aerogenes* (○), and a 1:1 mix of the two cultures (△) are plotted for first two principle components calculated using correlation matrices constructed from each metabolized substrate's datum. Percent variance explained by each PC axis is given in parentheses. (A) Principal component (PC) scores determined for OD data when microplate AWCD = 0.2. (B) Scores for OD data when AWCD = 0.3. (C) Scores for 24 h AWCD-normalized OD data. (D) Scores for 48 h AWCD-normalized OD data. (E) Scores for 72 h AWCD-normalized OD data. (F) Principal component scores calculated from logistic model *K* parameter values. (G) Scores calculated from model *r* parameter values. (H) Scores calculated from model *s* parameter values.

−0.972, with a mean Spearman rank correlation coefficient (±S.E.) of −0.747 (±0.043); *P*-value < 0.001.

Data ordination using single-time-point OD data or kinetic parameters

Principal components analysis (PCA) was used to detect differences between sample types in the pure culture experiments as well as with the field site samples. Single-time-point OD data from plates at equivalent AWCD values, AWCD-normalized OD data at various incubation times, and kinetic parameter values from our model fit to the OD time-course data were used in this analysis. Each substrate (variable) from a microplate generated three kinetic variables (*K*, *r* and *s*; see equation (2) and equation (3)) which were used individually to calculate principal components.

Principal component plots derived from the pure culture experiment OD data are shown in Fig. 4. The component scores calculated using single-time-point OD data from plates with equivalent AWCDs (0.2 and 0.3), using AWCD-normalized OD data at 24, 48 and 72 h, and using each kinetic parameter (*K*, *r* and *s*) for the laboratory-grown cultures are plotted for the first and second PCs in this figure.

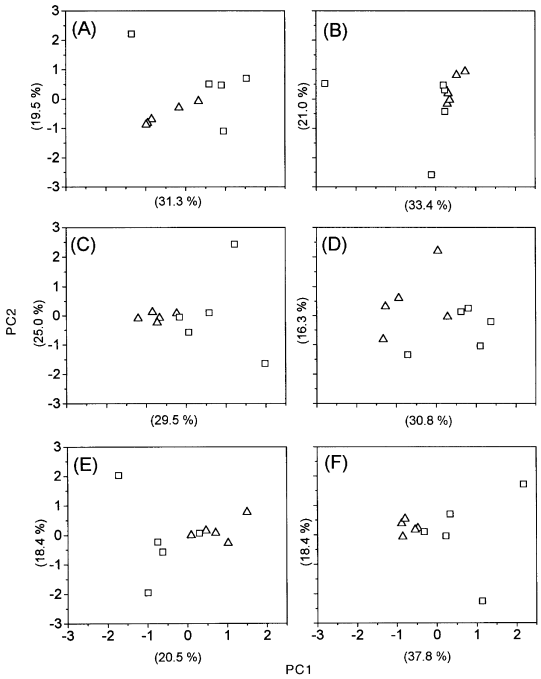

Fig. 5. Ordination produced from field site soil suspension microplate OD data using single-time-point OD values or kinetic parameters from time course OD data fit to the logistic model. Component scores for oiled (OIL,□) and oil-free (OF,△) environmental sample soil suspensions are plotted for first two principal components calculated from correlation matrices constructed from each metabolized substrate's datum. Percent variance explained by each PC axis is given in parentheses. (A) Component scores determined for OD data when microplate AWCD = 0.8. (B) Scores for 72 h AWCD-normalized OD data. (C) Scores for 96 h AWCD-normalized data. (D) Scores calculated from logistic model *K* parameter values. (E) Scores calculated from model *r* parameter values. (F) Scores calculated from model *s* parameter values.

In all analyses using the laboratory cultures, the first two PCs explain at least 80% of the data variance. The percentage of variance explained by each PC plotted is displayed on each plot's axes. Plates with AWCD values of 0.2 and 0.3 generated component scores providing separation of pure cultures along the first principal axis (Fig. 4A,B). At an AWCD of 0.2, separation of the *E. aerogenes* data from the data for the 1:1 mix of cultures was achieved along the second component axis (Fig. 4A), while this did not occur at an AWCD of 0.3 (Fig. 4B). Ordination of the AWCD-normalized single-time-point OD data (Fig. 4C–E) also separated the pure cultures along the first principal component axis, though only the 48 and 72 h normalized data sets discriminated the pure culture samples from the mixed cultures. The *K*, *r* and *s* kinetic parameter data (Fig. 4F–H) all resulted in separation of the axenic cultures of *E. coli* and *E. aerogenes* from each other as well as from the 1:1 mix.

A similar analysis was applied to the microplate data generated by soil suspensions from the field site. Single-time-point OD data at a microplate AWCD value of 0.8, AWCD-normalized data at 48, 72 and 96 h, and the three kinetic model parameters were used to compute PCs for the environmental samples. In general, the first two PCs explained much less of the variance in these data than for the laboratory culture experiments presented in Fig. 4. Data ordination plots of the first two principal components for the various data treatments are presented in Fig. 5 along with the percent variance explained by each PC. Separation of oiled (OIL) and oil-free, reference (OF) samples along the first PC axis was generally successful for the OD data set taken at an AWCD of 0.8, with the exception of a single OIL sample (Fig. 5A). Normalizing the OD data through division by AWCD failed to discriminate OIL and OF samples at 48 h and 72 h [72 h AWCD-normalized OD data shown in (Fig. 5B)]. Principal component analysis of 96 h AWCD-normalized data resulted in separation of OIL and OF samples along the first PC axis (Fig. 5C). Principal component analysis of kinetic parameter *K* and *r* data was generally successful at separating oiled and oil-free, reference samples with the exception of a single OIL sample in each case (Fig. 5D,E). Use of the *s* parameter data separated OIL and OF samples along the first PC (Fig. 5F).

DISCUSSION

The use of multiple substrate microtiter plates to evaluate community functional diversity has relied until now upon the determination of substrate well color development at a single time point. The decision regarding the proper time to make this measurement in various studies has been based on differing criteria. These include achievement of a given color development threshold relative to the well with maximum absorbance on the plate (Haack *et al.*, 1995) and reading at a set time following onset of color development (Bossio and Scow, 1995). Efforts to normalize the single-time-point OD data using AWCD values appear to succeed in correcting for biomass differences to the extent that the rate of reaching a given AWCD correlates with biomass (Garland, 1996). However, the relative contribution of any substrate to a pattern of potential C source use characteristic of a given sample depends on the time chosen to evaluate substrate use. Differences among rapidly metabolized substrates dominate principal component scores generated after short incubation times, while their contribution to principal components is eclipsed by differences in more slowly metabolized substrates after prolonged incubation. For example, at 48 and 72 h, AWCD-normalized data failed to discriminate

OIL and OF samples, while 96 h normalized data succeeded (see Fig. 5), suggesting that the AWCD-normalized substrate uses patterns of the sample types diverged after 72 h.

Concerns over the effect of inoculum density on the rate of color development are founded on the observation that a denser inoculum typically produces a positive well response more quickly than a less dense culture. Garland (1996) demonstrated that the time to achieve a given AWCD value following inoculation was inversely proportional to the inoculum density. Using our kinetic technique to fit the time course of the OD change showed that this is an artifact of a shorter 'lag' period in test well color development for denser inocula. The average rate of color change from inoculation to reading time includes this lag period, causing less dense cultures to exhibit an apparently slower rate of dye reduction. Thus, the rate of OD change from the time of inoculation to a given AWCD (as opposed to the rate from an OD of zero to AWCD), and therefore the AWCD-normalized substrate use pattern, depends on the inoculum biomass and not its functional metabolic diversity or 'taxonomic richness'.

Fitting the kinetic model to the color development data removed the effect of the lag period and the *actual* rate of color change (from zero absorbance to K) was determined. This technique yielded color development rates that were insensitive to inoculum density in a range up to four orders of magnitude lower than called for in the microplate manufacturer's standard protocol (Biolog, 1992) and that were sensitive to the community composition in the well. Two of the kinetic parameters (K and r) do not vary with inoculum density and, subject to the limitations discussed below, reflect the composition of the microbial assemblage in each test well. Because the kinetic approach uses all the time course OD data, rather than individual sampling times (either equivalent AWCD set points or AWCD-normalized data), the relative contributions of substrates (variables) to characteristic potential C source utilization patterns do not vary with sampling time or inoculum density. The model parameters provide at least three data options (parameters K, r and s) for inclusion in substrate-use analysis schemes.

The parameter K in our model is most similar to the data currently used in multiple substrate metabolism analysis as both are measures of optical density. It represents the asymptote that the modeled absorbance curve approaches and may be viewed as an estimate of the maximum potential extent of dye reduction seen in a given test well for a particular inoculum. The parameter r, an estimate of the exponential rate of color development, provides information about a different aspect of the same community in the test well, i.e. how rapidly reducing power is generated in the well. The inverse relationship of s with inoculum density suggests that, in general, this may not be a useful descriptive parameter for environmental samples since the inoculum biomass is likely to vary among samples. The fact that s increases with decreasing inoculum density while the actual rate of color change (from zero absorbance to K) remains constant (see Fig. 3) is consistent with the observation by Garland (1996) that time to a midpoint in the AWCD for his rhizosphere samples was negatively correlated to biomass. This implies there is a minimum active biomass threshold necessary for dye reduction to be detected. Thus growth rate and induction of the necessary catabolic enzymes are likely to play roles in the lag period prior to onset of color development in the wells.

K and r appear to be constant over a range of inoculum densities, but their values for a metabolized substrate may depend both on abiotic and biological factors. One abiotic factor that could affect K is complete reduction of the tetrazolium redox dye prior to nutrient limitation. In the absence of abiotic limits to K, the traditional biological interpretation of K as 'carrying capacity' is possible. Considering each microplate well as a 150 μl batch culture, K represents a biologically imposed limit on dye reduction in the wells. Biological factors such as efficiency of reducing power production (affecting reduced tetrazolium yield per cell) or cell growth, exhaustion of substrate or mineral nutrients, temperature, oxygen limitation, accumulation of waste products or production of secondary metabolites may affect the values of K and r. Metabolite production may allow growth of organisms unable to grow on the well's designated C source, or may inhibit organisms otherwise able to grow. Thus, K and r will be affected by synergistic or antagonistic effects due to the mix and relative abundance of organisms that can be cultured in the test wells. In other words, at a fixed temperature K and r are constants that depend on the taxonomic richness (metabolic diversity) of microplate-culturable organisms and their relative abundance, and not on the inoculum density *per se*. Evidence of this is seen in the separation of laboratory-grown cultures (Fig. 4) and, to a lesser extent, environmental samples based on K and r parameter data (Fig. 5).

Our curve fitting technique fits the time course OD data for microplate test wells closely and is useful for extracting kinetic parameter data that reflect the response of culturable organisms in the microplate inoculum. The resemblance of the model to a batch culture growth curve, however, does not imply that strict physiological interpretations may be ascribed to its parameters. For example, Fig. 2 displays field sample OD and population kinetics for two microplate substrates whose microbial

growth rate constants were substantially lower than the r values calculated for the OD change in the wells. This may be due to differences in cell yields of the dominant organisms selected by the substrate in each test well. Also, since color change depends on production of reducing power in the microplate well, microbial energy spilling reactions not tied to ATP production, cell growth or maintenance (Tempest and Neijssel, 1987) may provide dye reduction rates (r) or extents (K) above that directly attributable to microbial growth.

Our experience with environmental samples and laboratory cultures suggests that ODs of plates should be read repeatedly for as long as 1 wk following inoculation. This allows more slowly developing test wells the opportunity to achieve their ultimate OD (K value). Longer incubation periods may be useful for inocula that are less dense than those used in this study (i.e. less than *ca.* 10^4 cells ml^{-1}), but care should be taken to inhibit the evaporation of liquid from the wells causing increased OD values. Frequent plate readings (i.e. at intervals of less than 24 h) tend to provide data yielding better fits (lower χ^2 values) when model parameters are estimated.

The primary drawback of our approach is that the construction and analysis of data matrices based on our model's kinetic parameters entails more data collection work than the single-time-point data approach. Frequent readings over the course of several days need to be compiled to provide time course OD data for each substrate. The kinetic model needs to be fitted to the data and the model parameters estimated. The kinetic parameter data then need to be screened for the quality of their fit to the time course OD data (based on standard errors of estimates and χ^2 values). Only after these steps are the data ready to be analyzed by multivariate techniques. Despite the extra work involved, however, we believe that the benefits of this approach outweigh the drawbacks. This method removes concerns regarding variable inoculum densities in environmental samples. Our technique provides objective data parameters for community analysis and frees investigators from the need to choose the AWCD values for comparing microplates or the time at which single-time-point data are normalized. Thus, the contribution of test substrates to a sample's C source metabolism pattern is no longer an artifact of the incubation period or inoculum density. The kinetic approach provides a method for gathering data from multiple substrate microtiter plates which is reproducible and amenable to data analysis techniques currently used in multiple substrate metabolism studies. Future work using kinetic data analysis may further illuminate the precise relationship between population dynamics, microbial physiology and dye reduction in these microplates.

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