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Relative effectiveness of kinetic analysis vs single point readings for classifying environmental samples based on community-level physiological profiles (CLPP)

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Abstract

The relative effectiveness of average-well-color-development-normalized single-point absorbance readings (AWCD) vs the kinetic parameters μ_m, λ, A, and integral (AREA) of the modified Gompertz equation fit to the color development curve resulting from reduction of a redox sensitive dye from microbial respiration of 95 separate sole carbon sources in microplate wells was compared for a dilution series of rhizosphere samples from hydroponically grown wheat and potato ranging in inoculum densities of $1 \times 10^4 - 4 \times 10^6$ cells ml^{-1}. Patterns generated with each parameter were analyzed using principal component analysis (PCA) and discriminant function analysis (DFA) to test relative resolving power. Samples of equivalent cell density (undiluted samples) were correctly classified by rhizosphere type for all parameters based on DFA analysis of the first five PC scores. Analysis of undiluted and 1:4 diluted samples resulted in misclassification of at least two of the wheat samples for all parameters except the AWCD normalized (0.50 abs. units) data, and analysis of undiluted, 1:4, and 1:16 diluted samples resulted in misclassification for all parameter types. Ordination of samples along the first principal component (PC) was correlated to inoculum density in analyses performed on all of the kinetic parameters, but no such influence was seen for AWCD-derived results. The carbon sources responsible for classification differed among the variable types with the exception of AREA and A, which were strongly correlated. These results indicate that the use of kinetic parameters for pattern analysis in CLPP may provide some additional information, but only if the influence of inoculum density is carefully considered. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Community level physiological profiles; Gompertz equation; Carbon sources

1. Introduction

A rapid method for studying microbial communities based on the direct inoculation of mixed microbial samples into BIOLOG® microtiter plates has been increasingly used in microbial ecology (Garland and Mills, 1991; Garland, 1997). The approach uses the patterns of potential carbon source utilization generated from respiration of the different sole carbon sources and concomitant reduction of a redox sensitive tetrazolium dye within the 95 separate wells. The patterns of potential carbon source utilization, or community-level physiological profiles (CLPP) (Lehman et al., 1995) are analyzed using multivariate statistical techniques such as principal components analysis (PCA) to evaluate the relative degree of similarity among environmental samples. The multivariate analysis can be strongly influenced by differences in the overall amount of color formed among samples, resulting in classifications strongly confounded by the inoculum density of BIOLOG® responsive cells (Garland, 1996a, 1997). Differences in the overall rate of color development can be accounted for by: (1) standardizing inoculum density; (2) dividing each individual well response by the average well color development (AWCD); or (3) monitoring color development over time and selecting plate readings with similar AWCD for multivariate analysis. The first approach is limited by the time needed for estimating cell density (especially since a major advantage of the CLPP approach is use as a rapid screening tool) and the difficulty in selecting an enumeration technique that specifically targets only those cells which will respond in the BIOLOG® plate (Garland, 1997). The second approach is useful only for samples with relatively similar AWCD, because the unequal numbers of positive wells typically present in samples with very different AWCD cannot be normalized through division by the mean. The third

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approach when used with samples of roughly equivalent inoculum density (i.e. within an order of magnitude) has been successfully used (Garland, 1996b, and others). However, a thorough evaluation of the effects of inoculum density on classification of samples using this approach is lacking.

The third approach, while based on the analysis of a single plate reading, involves temporal monitoring of color development. The potential use of general descriptors of the kinetic profile of color development rather than absorbance values at an individual plate reading for multivariate analysis was conceptually discussed by Garland and Mills (1991); Haack et al. (1995). Guckert et al. (1996) first used a kinetic parameter, the integral of color development, for subsequent statistical analysis. Mayr et al. (1999) used a modification of the integral approach by calculating a 'Riemann's sum' for each well. Verschuere et al. (1997) used the parameters of the Gompertz equation (maximum rate of color development [μ_m], lag phase [λ], and maximum extent of color development [A]) fit to the color development curve to classify model communities. Similar curve-fitting approaches have been used to classify microbial communities from rivers using the Gompertz model (Lawley and Bell, 1998) and soils using a modified logistic model (Lindstrom et al., 1998) to obtain the same kinetic parameters.

While the kinetic approaches, by definition, are independent of incubation time, effects of inoculum density are unclear. Reduction of the tetrazolium dye does not occur until the cell numbers in the well reach about 10^5 cells ml^{-1} (Mills and Bouma, 1997). The lag phase in color development should be inversely correlated to inoculum density (Lindstrom et al., 1998), leading to a similar relationship between inoculum density and estimates of the integral of color development (unless μ_m or A are inversely related to inoculum density and balance the effect of a large value of λ). Lindstrom et al. (1998) concluded that two of the kinetic parameters (maximum rate and maximal extent of color development) were invariant with respect to inoculum density, but based their conclusions on dilutions and analysis of *E. coli* suspensions, i.e. dilution of cells with a uniform distribution of functional abilities. Dilutions of mixed microbial communities will result in the extinction of rarer types of organisms. To the extent that those organisms might be responsible for color development for specific carbon sources, not only will the resulting overall pattern of carbon source utilization change (Garland and Lehman, 1999), but the kinetics associated with the wells in which the rare organisms play a predominant role will also change. While use of the various kinetic parameters might potentially increase the analytical power of CLPP, a better understanding of the information content of the parameters and of their correlation to one another and to inoculum density is needed.

The objectives of the present work were to evaluate the effects of inoculum density on the effectiveness of the kinetic and single point analytical approaches for classifying mixed microbial communities and to examine the degree of correlation among the parameters to determine the suitability of each parameter for CLPP interpretation.

2. Materials and methods

2.1. Sample sources and preparation

Rhizosphere communities were obtained from dwarf wheat (*Triticum aestivum* L. cv. USU-Apogee) and white potato (*Solanum tuberosum* L. cv. Norland) grown in recirculating nutrient film technique (NFT) hydroponic culture (Wheeler et al., 1990). Wheat seeds were sown at a density of 800 seeds m^{-2} into rectangular 0.08-m^2 plastic trays fitted with inserts to support wheat growth. Two pairs of trays were connected to separate 10-l nutrient solution reservoirs. Two nodal cuttings of potato were planted in trapezoidal 0.25-m^2 plastic trays covered with plastic sheeting for plant support. Two pairs of trays were connected to 20-l nutrient solution tanks. Wheat was grown in a reach-in environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA, Model M-12). A 20-h light/4-h dark photoperiod was maintained using high pressure sodium lamps (400-W GE Lucalox, General Electric Corp., Cleveland, OH, USA) providing a photosynthetic photon flux (PPF) of ~500 μmole m^{-2} s^{-1}. Air temperature was maintained at 22°C, and nutrient solution temperature averaged 24°C. Potato was grown in a walk-in environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA, Model M-48). A 12-h light/12-h dark photoperiod was maintained using cool white fluorescent lamps (215-W F96712/CW/FHO, Sylvania Corp., Danvers, MA, USA) providing a photosynthetic photon flux (PPF) of ~500 μmole m^{-2} s^{-1}. Air temperature was maintained at 20°C during the light cycle, and 16°C during the dark cycle. Nutrient solution temperature ranged between 22–23°C. Relative humidity was maintained at 65% in both experiments.

The liquid level and nutrient content of the hydroponic solution were maintained by daily addition of deionized water and nutrient replenishment solutions, respectively. Nutrient levels were maintained according to a modified half-strength Hoagland's solution recipe (Wheeler et al., 1999). In the potato study and the control Hoagland's treatment in the wheat study, nutrient replenishment solutions consisted of stock solution made exclusively from reagent grade chemicals (Wheeler et al., 1999). In the compost leachate treatment in the wheat study, nutrient replenishment solutions consisted of aqueous leachate from composted inedible wheat biomass supplemented with reagent grade chemicals (Yorio et al., 1999). The compost leachate treatment was part of experiments evaluating nutrient recycling in closed systems. pH was continuously maintained at 5.8 by the addition of dilute acid (0.4 M HNO_3).

Rhizosphere samples were obtained by excising small portions (~1 × 1 cm) of the root mat at 21 days (wheat) or 84 days (potato) after planting. Rhizosphere suspensions were obtained by shaking the root tissue by hand in deionized water and glass beads (3 mm diameter) for 2 min. The resulting suspension was fixed for later estimation of total cell density using acridine orange staining and epifluorescence microscopy (Hobbie et al., 1977). A Biolog plate inoculum was generated by adding 2 ml of suspension to 18 ml of DI water. These full-strength samples were further diluted (1:4, 1:16, 1:64, 1:256) in DI water prior to inoculation in Biolog GN microplates (Biolog, Inc., Hayward, CA, USA).

2.2. Plate reading

Color development (absorbance at 590 nm) in the wells was determined every 2 h for 96 h in a microtiter-plate reader equipped with an automated stacker/loader (Model EL320, Biotek Instruments Inc., Winooski, VT, USA).

2.3. Parameter estimation

The average well-color development for the entire plate was computed at each time that the plates were read. Profiles for each plate were examined for the time at which the AWCD was closest to the value chosen as the target (viz., 0.5).

The modified Gompertz equation (Zweitering et al., 1990)

$$y = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\}$$

was used to estimate the parameters μ_m, λ, and A, where t is time, μ_m is the maximum specific growth rate, λ is the lag, defined as the x-intercept of the tangent line through the inflection point of the color development curve (the slope of this line is μ_m), A represents the maximum absorbance, and y represents the absorbance at time t. Note that y and A are scaled values: $y = \ln(A_t/A_0)$ and $A = \ln(A_\infty/A_0)$, where A_t represents the absorbance at time $= t$, and A_∞ and A_0 represent the absorbance at $t = \infty$ and $t = 0$, respectively. The equation was fit to each data set using a Levinberg–Marquardt, least squares algorithm. If no significant color development occurred within the 96 h (i.e. the absorbance at 96 h was less than 0.50), then the λ, μ_m, and A were set to 99, 0, and 0, respectively.

The area under each color development curve (AREA) was determined by integrating the curve for the raw data from $t = 0$ to $t = t_{max}$ by the trapezoid method.

We developed the Visual Basic program, CLPP, and used it for all curve fitting analyses, integration of the color development curves and determination of color intensity in each well at the time when the AWCD for each plate was closest to the specified set point. This program is available from the authors.

2.4. Multivariate statistical comparison of communities using the different parameters

The distinguishing power of the different parameters (AWCD, μ_m, λ, A, and AREA) was assessed using a combination of principal component analysis (PCA) and discriminant function analysis (DFA). PCA is useful for visualizing trends in the data such as the relative similarity among samples, and produces a reduced number of variables (i.e. principal components or PCs) suitable for further statistical testing. DFA is a means for predicting community membership (e.g. plant rhizosphere type) based on certain predictor scores (e.g. PCs). The initial analytical step involved separate PCA of each parameter type for undiluted samples. Results were graphed to visualize differences, and the first five PCs were subsequently used to tests for plant rhizopshere differences using DFA. The effect of dilution on classification was evaluated using two statistical approaches. First, separate PCA was performed with each parameter type on all samples which showed a minimal threshold of color response (AWCD of 0.50 abs. units at the end of 96 h incubation). This data set consisted of 33 separate samples (FS, 1:4, 1:16, and 1:64 dilutions from both types of wheat rhizosphere treatments and FS, 1:4, and 1:16 dilutions of potato rhizosphere). None of the 1:256 diluted samples reached this threshold of response and these data are not presented. PCA results were plotted to visualize differences, and the relationship between inoculum density and the first PC was assessed by linear regression. Finally, a separate analysis of each parameter type was conducted for data sets containing increasing levels of dilution. Undiluted and 1:4 diluted samples from each rhizosphere type ($n = 18$) were analyzed with PCA, and the first five PC were used for DFA. An identical analysis was conducted with undiluted, 1:4, and 1:16 samples of each rhizosphere type ($n = 27$).

3. Results

Correlations among the three kinetic parameters (λ, μ_m, A) resulting from fitting the color development data to the Gompertz equation were evaluated by graphing the scores for all the individual responsive wells from all the wheat samples, nearly 1900 data points or each parameter (Fig. 1), and by computing the Pearson's product moment correlation coefficient for each pair of variables. All three kinetic parameters were correlated to one another, but the correlations were weak; r-values ranged from −0.096 to 0.386 [Fig. 1(a)–(c)]. Correlation of the parameters with AREA was, however, much stronger than the correlations among the kinetic parameters. AREA was positively correlated to A and μ_m, and negatively correlated to λ [Fig. 1(d)–(f)]. The correlation was much stronger for A ($r = 0.845$), than either μ_m ($r = 0.574$) or λ ($r = -0.616$).

PCA on all five of the parameters determined for full

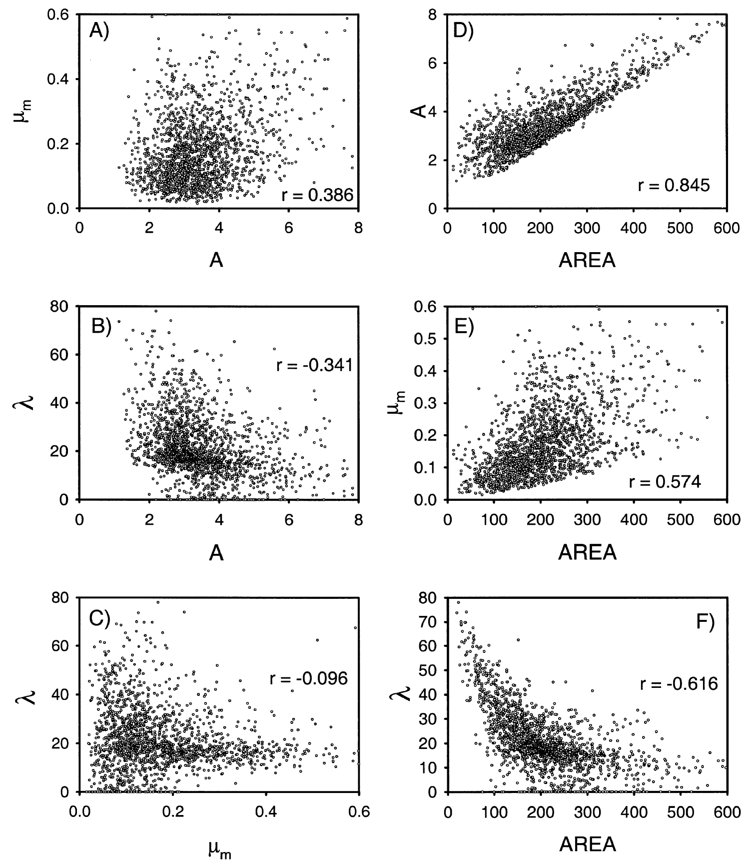

Fig. 1. Correlation of parameters generated from fitting the color development in individual wells within Biolog GN plates to the modified Gompertz equation. Data are presented for all responsive wells from the wheat rhizosphere samples ($n = 1887$).

strength rhizosphere samples discriminated between different plant types (wheat vs potato). On average, potato samples had higher scores along the first PC axis for the analyses conducted with each of the parameters, although the separation was greater for μ_m, AREA, and AWCD compared to λ or A (Fig. 2). Separation of the nutrient solution treatment within the wheat rhizosphere samples was evident along the second PC for analyses on the AWCD and A, but not the other parameters. DFA of the first five PCs assigned samples to the correct rhizosphere type except in the case of μ_m (Table 1), in which one sample of each wheat rhizosphere type was misclassified.

While PC 1 separated wheat and potato rhizosphere samples for all the parameters, the specific carbon source responsible for this separation differed substantially among the parameters (Table 2). The carbon sources responsible for the separation along the first PC were most distinctive for λ and AWCD for which no more than 10 of the 30 most influential carbon source were shared with each other or with any of the other parameters. μ_m shared more influential substrates (18 out of 30) with A and AREA. A and AREA shared a high percentage (25 out of 30) of influential substrates, an observation consistent with the strong correlation between these parameters [Fig. 1(d)].

Inclusion of the diluted samples altered the classification of the rhizosphere samples based on PCA for all of the parameters (Fig. 3). Variation within rather than between plant type became the primary effect along PC 1 for analyses conducted on all the kinetic parameters [Fig. 3(a)–(d)]. Separation of plant type was still consistent along PC1 for the AWCD data, although the distinction was less clear than observed when only undiluted samples were used for the analysis. Furthermore, the secondary effect of nutrient solution type observed with the undiluted AWCD samples was no longer apparent [Fig. 3(e) vs 2(e)]. The apparent reason for the distortion in the PCA among the kinetic parameters was variation in inoculum density and concomitant rates of color production, as evidenced by the correlation between the first PC scores and inoculum density for the kinetic parameters (Fig. 4(a)–(d)], but not AWCD [Fig. 4(e)].

Inclusion of diluted samples in the analysis resulted in misclassification of samples, although the effect varied among parameter type. Analysis of undiluted and 1:4 diluted samples resulted in misclassification of at least two of the wheat rhizosphere samples for each parameter type except AWCD for which classification remained 100% correct (Table 1). Analysis of undiluted, 1:4 diluted, and 1:16 diluted samples resulted in misclassification of at least two wheat rhizosphere samples for all parameters types. Classification of potato rhizosphere samples was

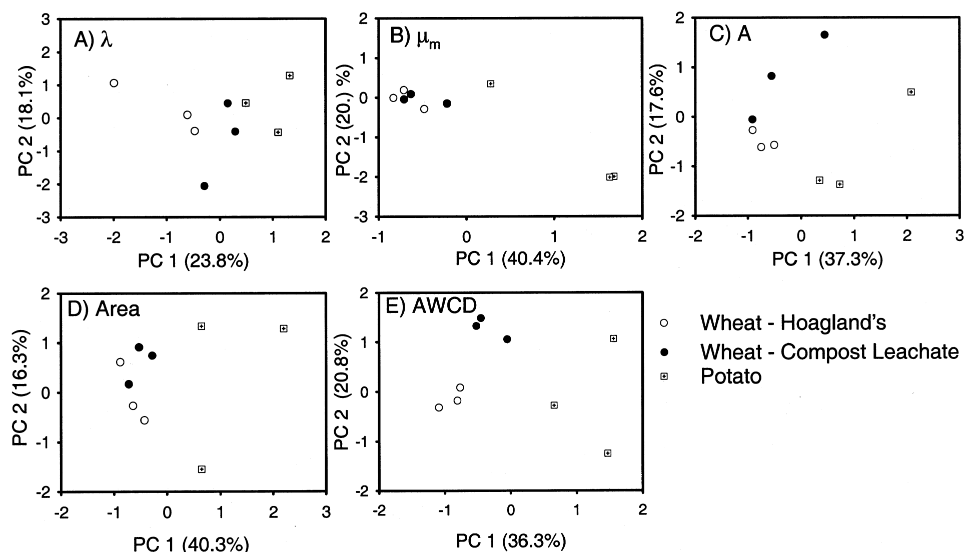

Fig. 2. Classification of undiluted rhizosphere samples based on Principal Component Analysis (PCA) of different parameters from the 95 individual wells in Biolog GN microplates: (a) the lag in color formation or λ; (b) the maximum rate of color development or μ_m; (c) the asymptote of color development or A; (d) the area under the curve of color development, or AREA; and (e) the absorbance value when the plate was at an average well color development (AWCD) of 0.50. Numbers in parentheses represent the amount of total variance explained by that principal component.

correct for all analyses, except for one misclassificaton in the μ_m data set including undiluted and 1:4 diluted samples, and the λ data set containing all samples (Table 1).

4. Discussion

The data collected in this study indicate that the use of kinetic parameters for community discrimination represents a potential augmentation of the CLPP approach, but that the approach is not as robust with respect to variable inoculum density as analysis using AWCD information. The utility of a kinetic analysis is supported by both the ability to discriminate communities from the rhizosphere of two plant types and the weak correlation among the parameters of the Gompertz model. This finding is consistent with previous evaluation of kinetic-type analyses (Verschuere et al., 1997; Lawley and Bell, 1998; Lindstrom et al., 1998). The present work adds further support for the kinetic approach by demonstrating that the different kinetic parameters (λ, μ_m,

Table 1

Results of discriminant function analysis to predict plant rhizopshere type based on the first five prinicpal component scores. Results reported for separate principal component analysis of undiluted samples only ($n = 9$), undiluted and 1/4 diluted samples ($n = 18$), and undiluted, 1/4, and 1/16 diluted samples ($n = 27$) for each data type (AWCD, Area, λ, $\mu_{max,}$ and A. Data represent the percentage of each rhizosphere type which was correctly identified

Predictor variable	Rhizosphere type	Undiluted (UD)	UD, 1/4	UD, 1/4, 1/16
A	Wheat	3/3	6/6	8/9
	Wheat–Compost	3/3	4/6	7/9
	Potato	3/3	6/6	9/9
μ_{max}	Wheat	2/3	4/6	7/9
	Wheat–Compost	2/3	2/6	5/9
	Potato	3/3	5/6	9/9
λ	Wheat	3/3	4/6	8/9
	Wheat–Compost	3/3	5/6	9/9
	Potato	3/3	6/6	8/9
Area	Wheat	3/3	6/6	9/9
	Wheat–Compost	3/3	4/6	7/9
	Potato	3/3	6/6	9/9
AWCD	Wheat	3/3	6/6	8/9
	Wheat–Compost	3/3	6/6	8/9
	Potato	3/3	6/6	9/9

Table 2
Degree of similarity in carbon source loadings from principal component analysis of rhizosphere samples using different response variables. Data represent the number of matches among the 30 carbon sources with the highest degree of correlation to the first principal component. Data presented for positive and (negative) matches

Response variable	λ	μ_{max}	A	AWCD	Area
λ		6 (2)	2 (8)	3 (7)	0 (8)
μ_{max}			18 (0)	4 (5)	18 (0)
A				4 (5)	25 (0)
AWCD					8 (2)

and A) are not correlated to a substantial degree, and, therefore, each parameter yields a classification based on largely different information about microbial communities. However, at this point it is not clear what information each parameter contains.

Individuals who wish to develop CLPP patterns from curve fitting to time-dependent color development values with the modified Gompertz equation (Zweitering et al., 1990) are cautioned to examine the equation carefully. The y-axis value (y) and the asymptote (A) are not absorbances at time $= t$ and time $= \infty$, respectively, rather they are log-transformed, scaled values, namely $y = \ln(A_t/A_0)$ and $A = \ln(A_\infty/A_0)$. Fitting the curve as published to untransformed, unscaled data will result in serious errors.

Our results also indicate limitations to the kinetic approach; most important is the confounding effect of inoculum density. Variation in inoculum densities of 4–64-fold introduced sufficient noise to eliminate the ability to observe primary effects on the data, as evidenced by the loss of the plant-type discrimination along the first PC when diluted rhizosphere samples were added to the data set, and the misclassification of samples by DFA. This conclusion is counter to the finding by Lindstrom et al. (1998) that μ_m and A were invariant with respect to inoculum density. Lindstrom et al.'s conclusion is not surprising given the fact that variation in the inoculum densities of mixed microbial communities such as used here may lead to the loss of the rare types resulting in concomitant changes in functional responses, as opposed to the dilution of a homogeneous distribution of function among members of a monoculture as tested by Lindstrom et al. (1998).

Dilution was used in the present study to experimentally assess the effects of variation in inoculum density on sample classification. In other words, samples were diluted to produce a quantifiable range in inoculum densities consistent with what a researcher may find within a given habitat across spatial, temporal, or experimental gradients. This is distinctive from the proposed use of multiple dilutions of a single sample as a tool to assess relative structural diversity, either through the examination of the rate of extinction of functional richness (Garland and Lehman, 1999) or the rate in pattern change (Lindstrom et al., 1999). In these applications, sensitivity to changes upon dilution is useful for discrimination. In the context of the present study, however, the variation in the multivariate pattern of response produces noise within the signal for classification.

The value of the area under the curve (AREA) as a kinetic parameter appears limited by both inoculum density dependence and strong correlation with the individual kinetic parameters. Not only was classification of samples based on AREA clearly affected by variation in inoculum density, the value of the parameter for samples of equivalent

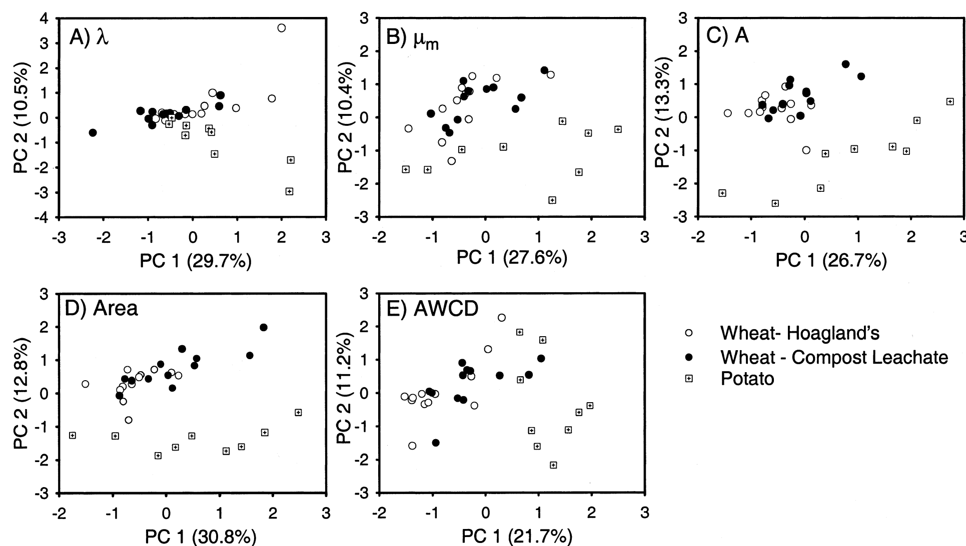

Fig. 3. Classification of undiluted and diluted rhizosphere samples based on Principal Component Analysis (PCA) of different parameters from the 95 individual wells in Biolog GN microplates: (a) the lag in color formation or λ; (b) the maximum rate of color development or μ_m; (c) the asymptote of color development or A; (d) the area under the curve of color development, or AREA; and (e) the absorbance value when the plate was at an average well color development (AWCD) of 0.50. Analysis conducted for all samples which reached AWCD of 0.50; 12 wheat rhizosphere (three reps each of undiluted, 1:4, 1:16, 1:64), 12 compost wheat rhizosphere (three reps each undiluted, 1:4, 1:16, 1:64), and nine potato rhizosphere (three reps each of undiluted, 1:4, 1:16). Numbers in parentheses represent the amount of total variance explained by that principal component.

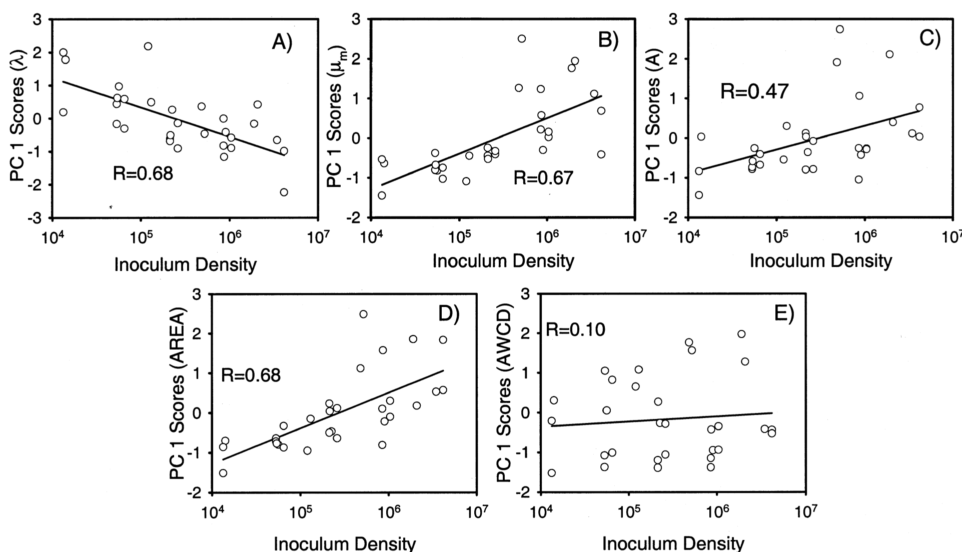

Fig. 4. Correlation of the scores for the first principal component resulting from PCA of the diluted and undiluted samples with inoculum density (based on acridine orange counts). Correlations are presented for PC1 scores from analysis of (a) the lag in color formation or λ; (b) the maximum rate of color development or μ_m; (c) the asymptote of color development or A; (d) the area under the curve of color development, or AREA; and (e) the absorbance value when the plate was at an average well color development (AWCD) of 0.50. Data represent all samples which reached AWCD of 0.50; 12 wheat rhizosphere (three reps each of undiluted, 1:4, 1:16, 1:64), 12 compost wheat rhizosphere (three reps each undiluted, 1:4, 1:16, 1:64), and nine potato rhizosphere (three reps each of undiluted, 1:4, 1:16).

densities is unclear. Since it was highly correlated with the individual kinetic parameters, the AREA parameter yielded little additional information. Given that AREA is an integration of all the information contained in the kinetic variables, correlation between AREA and each of the kinetic parameters, is expected. The strong correlation between AREA and A is likely a result of the relatively long incubation time used in this study (96 h), resulting in extended asymptotic periods for more rapidly producing wells. This time was selected to allow color development in wells with larger λ values to reach asymptotic levels. Selection of a shorter incubation period might reduce the degree of correlation between AREA and A, but it would also result in an increased number of wells classified as unresponsive (i.e. wells with large λ) and still result in an AREA measure correlated to varying degrees to all of the individual parameters. Use of integrated parameters, whether direct estimates of the area under the curve or Riemann's sum (Mayr et al., 1999), seems to be at odds with any potential strength of the kinetic approach. Such use eliminates the ability to distinguish differences in the non-linear shape of color development, and confounds any potential interpretation of the basis of classification using the kinetic parameters.

Despite the potential utility of the kinetic approach to analysis of CLPP results, kinetic analysis does not appear to be the best approach for rapid classification of samples. Our data indicate that the use of single point plate readings at a setpoint in AWCD is a better tool for classification given less dependence on inoculum density. However, dilutions greater than 10-fold did affect classification with AWCD. These studies clearly show, as has been previously argued (Garland, 1997), that the AWCD approach is limited to samples with relatively equivalent levels of inoculum density. Temporal monitoring of color development with subsequent selection of plate readings at a set point in AWCD appears to be the best approach when using CLPP for rapid, preliminary assessments of temporal or spatial variation in microbial communities. The multivariate pattern is less sensitive to variation in inoculum, requires less intensive monitoring of the plates (i.e. plates need to be read repeatedly but not for as long a time period necessary to capture the entire kinetic profile in all responsive wells), and has reduced analytical requirements.

The potential of the kinetic approach is that a more detailed understanding of the nature of the color response may be feasible. However, this concept should be approached with caution given the general lack of understanding concerning the physiological or ecological basis for differences in the kinetic parameters (λ, μ_m, and A), and the known selective bias due to growth in the plates (Smalla et al., 1998). Experimental evidence suggest that the overall rate of color development in the Biolog plate is not necessarily related to in situ carbon source utilization (Garland et al., 1997; Winding and Hendriksen, 1997), so the potential for overinterpretation of changes in the nature of the color response using the kinetic parameters exists and should be assiduously avoided. Specific, hypothesis-driven experiments are needed to define and validate the information content in the kinetic parameters. Linking kinetic analysis to more sensitive methods for detecting carbon source utilization which do not rely on selective enrichment may also prove valuable.

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