Potential and limitations of BIOLOG for microbial community analysis

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ABSTRACT

Characterization of community-level substrate utilization via incubation of environmental samples in BIOLOG microplates has been increasingly applied in many areas of microbial ecology. The easily-produced multivariate profile allows for intensive spatial and temporal analysis of microbial communities, and it is a sensitive and reproducible tool for characterizing microbial communities. This work addresses several procedural and ecological issues with the assay which have been raised in the literature.

Introduction

A rapid method for characterizing microbial communities based on inoculation of whole community samples into Biolog microtiter plates was introduced over 7 years ago [3]. The approach, termed community-level physiological profiling (CLPP) [12], is based on the multivariate profile of color production caused by utilization of sole carbon sources and concomitant reduction of tetrazolium dye in the 95 separate wells. The ability to easily produce a large data set has led to wide application of the method (see 7 or 12 for literature review). An international workshop on the approach was held in 1996, and the presentations from the over 150 participants are summarized in Insam and Rangger [10] and a special issue (30:1) of *the Journal of Microbiological Methods*. The workshop summaries as well as two recent review articles [6,11] provide thorough overviews of the present state of knowledge regarding this approach. The overviews include many examples where the approach has been able to detect subtle shifts within microbial communities. In an effort to limit redundancy in the literature, this article will address the potential and limitations of the approach in the context of procedural and ecological issues raised in the most recent review article by Konopka et al. [11].

Development of Kinetic Analysis

Various procedural approaches have been proposed to address the non-linearity and inoculum density dependence of color development in the plates with incubation [4,6,8-9,14]. The most effective, straightforward approach appears to be approximate standardization of inoculum density (i.e., within an order of magnitude), and analysis of samples at similar overall amount of color development, typically quantified as the average well color development (AWCD). Since differences in inoculum density may alter the rate of AWCD, comparison of samples at a single incubation time without consideration of potential differences in AWCD among samples may yield a classification of samples based on the overall amount rather than relative pattern of carbon source utilization. The overall

Microbial Biosystems: New Frontiers
Proceedings of the 8th International Symposium on Microbial Ecology
Bell CR, Brylinsky M, Johnson-Green P (ed)
Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.

amount of carbon source utilization may be useful for approximate estimation of the density of active cells [6], but confounds pattern shifts indicative of changes within the community independent of overall density. Repeated readings of the plates and subsequent selection of readings closest to a given AWCD for analysis, or normalization of the data to account for differences in AWCD prior to analysis, is recommended to eliminate the confounding effects of overall rate of color formation [6].

Since the color development in the individual wells typically follows a sigmoidal trend with time, it has been suggested that analysis of the kinetic profile of color development may increase the analytical power of the method [3, 9]. Two approaches for kinetic analysis have been proposed: 1) analysis of the integral of color development [8], and 2) non-linear curve fitting with subsequent analysis of coefficients of the curve. Color development curves have been successfully fit to the modified Gompertz equation [17], which provides terms describing lag (λ), maximum rate (μ), and threshold (A) [14,16].

The potential value of the integral approach is that it detects differences among samples in both fast and slow responding wells. The single point plate reading approach, conversely, detects differences in either fast or slow responding wells depending on whether samples are compared at a low (0.25-0.50 abs. units) or high (0.75-1.00 abs. units) AWCD, respectively. Therefore, the integral approach captures a more complete picture of differences in carbon source utilization in a single data set than is feasible with the single point plate reading approach.

The non-linear curve fitting approach could provide additional information since it describes three different aspects of the color response (the lag, maximum rate, and threshold) that are indistinguishable with either the single point or integral approaches. The ecological meaning of these different terms has not been established, and such definition is warranted before the effort to collect additional information can be justified. Defining the degree of correlation among the different parameters is also necessary to determine if they contain unique or redundant information.

One important potential limitation of the integral and curve-fitting approaches is the confounding effects of inoculum density. This effect can be clearly seen from the example in Fig. 1. A ten-fold dilution of a suspension of rhizosphere organisms from hydroponically-grown wheat as described in Garland [5] results in a significant increase in lag time prior to color development (λ) and a significant decrease in the maximum rate of color development (μ). The threshold of color development (A) appears unchanged. The increased lag and decreased maximum rate results in a clear reduction in the integral. Therefore, classification of samples based on the multivariate profile of either integral, λ, or μ would be confounded by inoculum density differences. The most effective single point plate reading approach, as outlined above, involved normalization for differences in the rate of overall color formation either by analyzing plates at different incubation times or normalizing the data prior to analysis. Since the first approach is not feasible with kinetic analysis, special attention must be paid to standardization of inoculum density and/or normalization of the data with either the integral or curve fitting approaches.

The most immediate research priority in this area is a thorough comparison of these different analytical approaches. To date, alternative approaches have been published without a side-by-side comparison of the new method with existing approaches [8,14]. Comparison

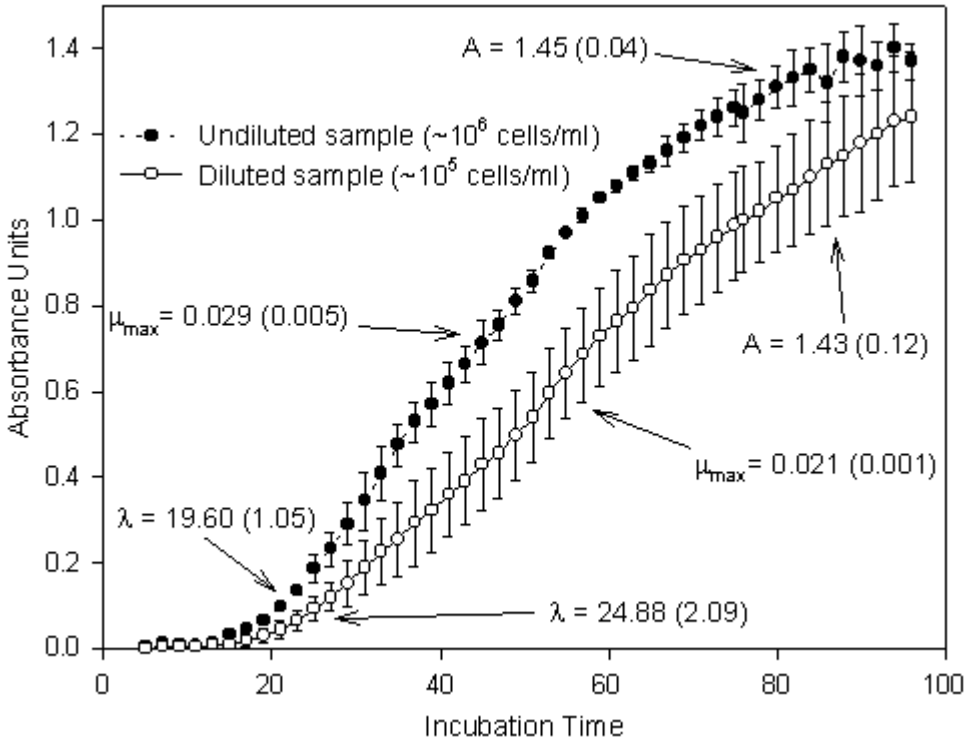

Fig. 1. Kinetic profile of an undiluted and diluted sample of rhizosphere extract from hydroponically-grown wheat. Values represent mean and standard deviation of the average well color development of three replicates. Parameters (λ, μ_{max}, and A) represent parameters from the modified Gompertz equation fit to the curve of AWCD versus time.

of samples of varying levels of similarity and inoculum densities using single point, integral, and curve-fitting approaches is necessary before their relative effectiveness can be defined.

Culture bias of the method

The requirement for growth in the wells results in enrichment for a subset of the original community, particularly those organisms which can respond rapidly to high substrate concentrations (i,e., copiotrophs). This fact was demonstrated earlier by Winding and Hendrickson [16] who reported an increase in percentage of culturable cells within the wells (15-78%) relative to original inoculum (0.3%). Smalla et al. [13] found that the diversity of 16sRNA fragments was reduced in the wells relative to the inoculum, and the dominant organisms within the wells were members of the γ sublass of Proteobacteria which are largely fast-growing copiotrophs.

Since a response is the result of a selectively-enriched subset of the community, extrapolation of the profiles to differences in *in situ* carbon source utilization or even phenotypic potential of the actual community should be done with caution [6]. Experiments with bioreactors manipulated with specific carbon source amendments provided empirical evidence that changes in CLPP do not necessarily reflect changes in the functional abilities of communities even under copiotrophic conditions [7].

Konopka et al. [11] conclude that this inability to measure all members of a community without bias indicates that CLPP will not provide fundamental insights into the nature of

microbial communities. This conclusion seems to rely on too strict an interpretation of the potential use of CLPP. As mentioned above, the functional relevance of profiles should be interpreted with caution. However, the assay is very sensitive in detecting change in microbial communities. For example, while the CLPP did not show analogous changes to carbon source amendments to bioreactors (i.e., increased utilization of compound x in the reactor did not correlate with increased response to compound x in the Biolog plate), the overall profile was very responsive to perturbations within the bioreactor [7]. Significant insight into microbial communities can be provided by understanding the relative amount of change caused by different biotic (e.g., invasions) and abiotic stresses. A partial indirect view of a community may be as useful as a complete, direct view to detect relative change, just as a person's shadow is almost as effective at detecting movement as a full body view.

The key to the utility of CLPP as an indicator of temporal dynamics is the degree of linkage between the fraction of the community detected by CLPP (i.e., active copiotrophs) and the overall community. It seems reasonable to classify the types of microorganisms in a community into two major groups:1) culturable types comprising fast-growing copiotrophs able to grow on solid agar and respond rapidly in the Biolog plates, and 2) nonculturable or slow-growing types which have specific growth requirements which limit growth on normal solid media and the Biolog plates. A great deal is known about the physiological shifts in

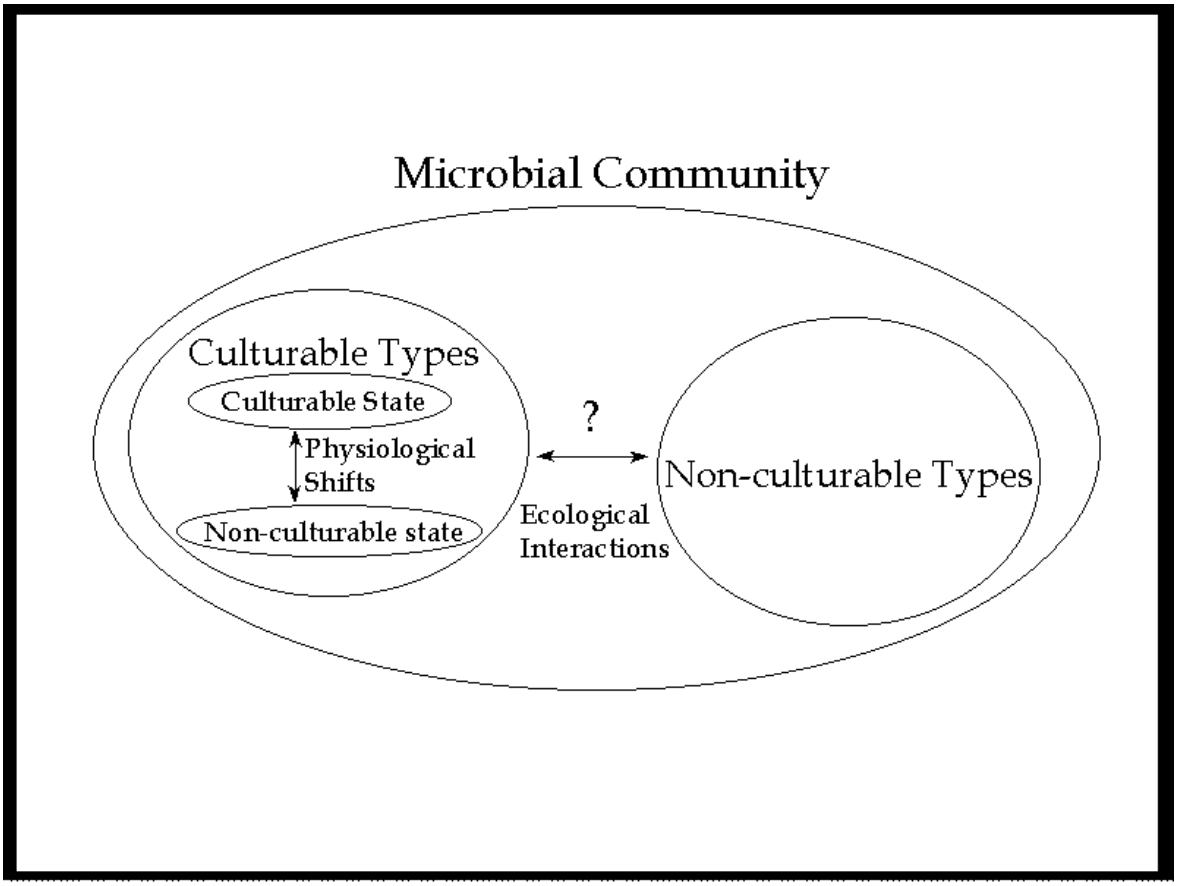

Fig. 2. Conceptual model of microbial community indicating culturable and non-culturable types, and the shift in the physiological state of culturable types from a culturable and non-culturable state.

culturable types in response to starvation which result in the transition to a viable but non-culturable state, but very little is known about the ecological interactions between culturable and non-culturable types within a community (Fig. 2). The view of a community as an interactive group of populations would suggest that changes in one fraction of community would lead to changes in the other, just as reduction in the density of tree species in a forest would lead to changes in understory vegetation. Further study is needed to quantify the degree of linkage between copiotrophs and non-culturable types in order to define the utility of CLPP for detecting community dynamics.

Ecological Relevance of Substrates

The 95 substrates in the Biolog GN plate are comprised of simple, common substrates selected on their ability to discriminate among bacterial isolates [1]. They do not represent, as Konopka et al. [11] indicate, recalcitrant or unusual substrates which may be of particular importance in the habitat of interest (e.g., cellulose in terrestrial ecosystems, halogenated phenolics in wastewater treatment). However, the overall question of ecological relevancy of the substrates needs to be placed in the context of appropriate questions that should be addressed by this approach. As discussed above, previous studies have indicated that the substrate responses do not necessarily reflect *in situ* function, or even the relative abundance of different phenotypic potentials. The cultural bias of the approach limits the proportion of the community which can respond in the assay, and concomitantly, the functional diversity which this assay can detect. Given these limitations, the emphasis on the use of ecologically relevant substrates may be misplaced, actually leading to misapplication of the approach.

The more appropriate use of CLPP as a rapid detector of community change suggests that substrates should be selected based on their discriminatory power, rather than ecological relevance. Konopka et al [11] indicate that the use of less common substrates may provide more discrimination, but they do not use the most appropriate example for illustration. Victorio et al. [15] found that samples from wastewater treatment plants showed a positive response to 85% of the substrates in the GN plates, but only 65% of the wells in customized plates containing a large number of compounds associated with bleached kraft mill effluent . A lower number of positive responses does not necessarily indicate, as Konopka et al. [11] suggest, a more “distinctive pattern”. The distinctiveness in the CLPP depends on variation in responses among samples, not the number of responses. Differences in the number of positive tests are more important when data is analyzed in a binary fashion, but this severely limits the discriminatory power of the assay. A study by Campbell et al. [2] represents a better verification of the potential increase in discriminatory power when using a customized set of substrates. They found more distinctive CLPP of soil samples when using a set of carbon sources containing many known root exudates compared to the GN substrates. They hypothesized that the exudate substrates may have selected for a greater diversity of organisms, including more slow growing types.

In summary, the GN set of substrates appears very useful for discrimination in a variety of systems, although individual researchers may want to use alternative substrates to increase discrimination and/or evaluate *potential* utilization of more relevant substrates. However, the value of this approach is related to multivariate discrimination, not

measurement of substrate utilization. Research questions related to shifts in substrate utilization should use more appropriate techniques (e.g., isotope labeling, enzyme activity, etc.).

Effects of Metabolic Redundancy

Konopka et al. [11] argue that the CLPP are insensitive to changes in population structure due to metabolic redundancy. They correctly indicate that it is possible that species composition could change without a shift in the profile of positive responses since a positive community-level response to a substrate does not allow the researcher to define how many or what types of organisms are present which can utilize that compound. The majority of samples from natural habitats show positive responses to most of the GN substrates, and therefore, they would be indistinguishable from one another if binary responses were analyzed. However, the real discriminatory power of the assay depends on analysis of continuous data, and the empirical evidence indicates that these profiles are sensitive to shifts in community structure [5,7,9].

Conclusions

CLPP is a rapid means of detecting relative change in microbial communities. Since shifts in CLPP can not be related to changes in the abundance of specific phylogenetic or functional groups, results can not be used as the sole basis for defining mechanistic models of the factors affecting community stability. CLPP has considerable utility when used intelligently as a means for conducting preliminary, hypothesis-generating studies because it is fast and relatively inexpensive. Intelligent use includes considering the procedural and ecological issues discussed in this paper and other review articles [6,11).

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