

The Biolog Plates Technique as a Tool in Ecological Studies of Microbial Communities

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Abstract

The Biolog technique was introduced into ecological studies to estimate metabolic potential of microbial communities. While utilizing carbon substrates (95 or 31x3, depending on the plate type), microbes reduce a colourless dye to violet formazan. The colour is measured spectrophotometrically. This is a rapid and quite convenient method but it has many drawbacks – for example, only microbes that are cultivable and able to grow in high-nutrient conditions contribute to substrate utilization. The method was used mainly to investigate microbial communities from bulk and rhizosphere soil and to estimate the impact of stressors on soil microbial communities.

Keywords: Biolog, physiological profile, AWCD, microbial community

Microorganisms as an Important Component of Ecosystems and Objects of Environmental Studies

Soil microorganisms play a crucial role in one of the most important ecological processes – the turnover of chemical elements in an ecosystem. Soil microbes break down complex organic molecules and release inorganic nutrients which then can be used by plants. The rate of dead organic matter decay depends on the biomass and metabolic activity of microorganisms. These parameters are in turn controlled by numerous natural factors such as temperature and humidity [1], chemical composition of organic matter [1, 2], vegetation type [3] and soil properties [4]. Disturbances to the process of organic matter decomposition may lead to abnormalities in a turnover of elements in an ecosystem, restriction of availability of nutrients to plants and reduction in ecosystem productivity, which sometimes can cause even forest decline [5]. Microorganisms react quickly to changes in their environment because of the high surface-to-volume ratio and low homeostasis [6], and changes in their biomass, metabolic activity or community structure may be an early signal

of alterations (also adverse alterations) in the whole ecosystem.

In recent years, methods for studying community structure, such as the analysis of phospholipid fatty acids (PLFA) [7, 8] or denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) [9-11], are getting popular. The above-mentioned methods are based on molecular assays. Measurements of physiological activity of microorganisms represent another approach, allowing the study of different characteristics of microbial communities. The Biolog technique is one of the methods which rely on measurements of utilizing different carbon substrates by microorganisms. Measurements of substrate use enables qualifying microbial metabolic capabilities and hence functional diversity of a microbial community. The Biolog method has undoubted advantages but also many weaknesses. The aim of this article is to present both the advantages and the drawbacks of the method when applied towards studying soil microbial communities.

The Principle of the Biolog Method

The Biolog plates method was first used to compare metabolic activity of heterotrophic microbial communities

from different habitats – water, soil and wheat rhizosphere [12]. The technique is a redox system. Various types of plates are used, but Biolog GN plates for gram-negative bacteria are the most popular ones. These are plastic microtiter plates containing 95 different carbon substrates in wells, and no substrate in one well which is used as control. Among the 95 substrates one can distinguish a few groups of chemical compounds, for example, carbohydrates, amino acids, carboxylic acids, amines, amides and polymers.

Additionally, each well contains a colourless tetrazolium dye.

Soil samples are shaken in a suitable solution and then the soil solution is inoculated into the plate wells. During incubation at a constant temperature, soil microorganisms oxidize substrates in the plate wells and, simultaneously, reduce colourless tetrazolium dye to a violet formazan. Colour development is measured spectrophotometrically (absorbance or so-called optical density – OD) [13]. The rate of utilization of different substrates by different groups of microorganisms varies, so one can observe high variability in the rate of colour development and its intensity depending on the composition of a microbial community. The characteristic pattern, called microbial “metabolic fingerprint” [14] or “community-level physiological profile (CLPP)” [15, 16], is obtained in this way.

Types of Biolog Plates

The Biolog GN plates have been designed for identification of gram negative bacteria and contain substrates appropriate for this group of microorganisms. Analogically, the GP plates are adapted for identification of gram positive bacteria. The Biolog database enables quick and easy identification of 1449 species of yeast and bacteria, mainly of medical importance [17]. In recent years the GN and the GP plates were used also for ecological research of soil microorganisms, and the GN plates are the most popular. Preston-Mafham et al. [17] reported that about 75% of scientific publications from the last 10 years, related to microbial communities analyses on the Biolog plates, deal with the use of GN plates. Recently, the Biolog GN2 plates, which are a modification of the GN plates, have been developed. They have partly different substrates than original GN plates. These changes have been introduced to restrain colour development, which is not connected with microbial activity [18].

Microbial response to GN2 substrates is weaker than to GN substrates, so using GN2 plates (as well as ECO plates; see below) for investigating very active microorganisms is recommended [18, 19]. The above-mentioned Biolog ECO plates have been typically designed for ecological study of whole microbial communities, not for identification of microbial strains. Preston-Mafham et al. [17] have informed us that substrates on ECO plates are selected especially for ecological research. Plates contain 31 substrates (and a control well without a substrate) in 3

replicates. Nine of the 31 substrates are known as components of exudates of plants roots [20]. Classen et al. [21] claim that the biggest advantage of the ECO plates is that they contain 3 replicates of each substrate, which increases the probability that a physiological profile developed on a plate indeed represents the microbial community studied. Tetrazolium dye used in the GN, GP and ECO plates wells is not metabolized by fungi, so fungi do not contribute to CLPP on these plates [17].

For the rapid identification of fungi another plate type is used – the Biolog FF plates. However, they have been developed especially for studies of plant and human pathogens (Biolog Inc.). Not only the substrates on FF plates differ from those on the GN and GP plates but also the tetrazolium dye is modified so that it can be metabolized by fungi [17]. In research with the use of FF plates both the formazan production (connected with the amount of utilized substrate) and the turbidity of a solution in plate wells (connected with the hyphae growth) are measured [22].

The Biolog MT plates containing redox dye and no substrates are also available. These plates enable the choice of any substrate according to particular needs [17, 23].

Statistical Analyses

Before statistical analyses are done, the absorbance value of a control well (no substrate) is subtracted from the absorbance value of each well containing a substrate. In that way one receives the so-called *net absorbance value* [13]. Because the absorbance value of the control well can be slightly higher than the absorbance values of some substrates, Hitzl et al. [24] subtracted the absorbance value of the least utilized substrate. In that way negative absorbance values in results can be avoided.

Statistical analyses of results can be carried out in many ways. The choice depends in the first instance on how many measurements of absorbance were done. Because of the necessity of choosing an optimal incubation time, which may be different for different soil communities, a single measurement is not a good solution. While in some plates the production of colour is not yet observed (for the least active communities), in others the colour development may reach the saturation point. Smalla et al. [11] also points out that during incubation the community structure changes. Due to this, a single measurement which is taken too late reflects tetrazolium dye reduction performed by only a few dominant microbial species [25]. Garland [26] suggests doing a number of measurements during the incubation and then choosing for statistical analyses those readings that exhibit approximately the same average well colour development (AWCD) (mean of the absorbance values for a whole plate), for example 0.25, 0.5, 0.75, 1.00, irrespective of the actual incubation time that is needed to reach this AWCD for particular plates. Choosing measurements of AWCD equal to 0.75 is regarded to be the best solution because at this value

the response of a microbial community can be seen in most wells and the wells with the most active microbial communities reach the asymptote of colour development [27].

Kinetic analysis of colour development on plates has been commonly used as well [28-30]. This kind of analysis considerably increases the amount of information which can be obtained from a physiological profile [31]. In most cases the rate of substrates utilization (colour development) can be illustrated with a sigmoidal curve [20]. By taking a number of consecutive measurements one can obtain a curve which is described with three parameters of the reaction kinetics. These are:

1. λ – lag time, i.e. time from the inoculation of a soil solution into wells to the beginning of colour development;
2. μ_m or r – maximum rate of colour development, which is reflected by the slope of a curve;
3. A or K – threshold or asymptote, i.e. the maximum absorbance in a well.

Graphs of this type (absorbance versus time) can illustrate colour development in individual wells (separately for different substrates) or mean colour development for a whole plate (all substrates) (Fig. 1). The parameters are obtained by fitting the Gompertz equation to the data:

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

where y is the absorbance in time t .

The parameters of the Gompertz model are weakly correlated with each other, so each of them provides a different type of information about microbial communities [32]. Regardless of what kind of absorbance measurement is chosen (net OD of individual substrates, AWCD or parameters received from the kinetic analysis: λ , μ_m , A), the use of one of multidimensional statistical techniques is usually necessary. The most popular one is Principal Component Analysis (PCA). The absorbance of each substrate on a plate (95 substrates in the case of GN and GP plates) or each parameter of each kinetic

curve is a variable, so the number of variables can be higher than the number of samples [25]. The PCA usually reduces the number of variables to a few or just over a dozen significant principal components (PCs). Each PC is a different combination of initial variables and explains a portion of total variability. One can check then which original variables built particular PCs with correlation analysis of initial variables with PCs [31]. Besides PCA such methods as cluster analysis or canonical variate analysis (CVA) are used relatively often to compare results from the Biolog plates [17].

As a measure of the number of substrates utilized (substrate richness) and diversity of the extent of utilization of particular substrates (substrate evenness), the Shannon index is used [33]:

$$H = - \sum_{i=1}^N p_i \ln p_i$$

where p_i is the proportion of a microbial activity on substrate i in total microbial activity, and N is the number of substrates on a plate (95 for the GN and GP plates, 31 for ECO plates).

Harch et al. [34] suggest using the Lorenz curve for estimating the number and extent of utilization of the substrates. On the basis of the curve the Gini coefficient can be calculated, which is a measure of inequality in a population [35]:

$$G = \frac{\sum_{i=1}^N \sum_{j=1}^N |x_i - x_j|}{2N^2 \bar{x}}$$

where x_i and x_j are the absorbance values of each well, N – the number of substrates, and \bar{x} – AWCD.

The Lorenz curve and the Gini coefficient enable estimating general activity (AWCD) and inequality in substrate utilization (richness and evenness) and allow comparing functional diversity of microbial communities [34].

Advantages of the Biolog Method

The Biolog plates enable characterization of a community on the basis of so-called *individual physiological profiles* which are obtained as a result of inoculation of bacterial isolates onto a plate [31]. Garland and Mills [12] were the first who omitted the isolation of microorganisms from soil and inoculated soil solutions directly onto the Biolog GN plates. The inoculation of soil solutions directly onto plates allows obtaining physiological profiles of the whole microbial communities. Omission of the isolation phase caused a huge interest in the Biolog method as a rapid and convenient tool for studying and comparing functional diversity of whole microbial communities [33].

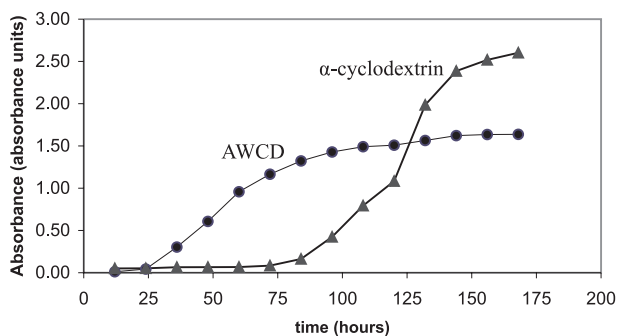


Fig 1. Sigmoidal curve of colour development during 168h of incubation for the substrate α -cyclodextrine and AWCD (the mean for all substrates on a GN2 plate) (data not published).

Analysis of physiological profiles with multidimensional statistical methods allows estimation of similarity between microbial communities from various environments and habitats. The rate of colour development in wells provides information about density and/or metabolic activity of bacterial cells in an inoculum, while the diversity of colour development in wells about microbial diversity in soil solution [31]. It is important that the Biolog method enables observation of even small shifts in microbial communities; for example, a response of rhizosphere microorganisms to changing plant age [36]. The Biolog plates technique provides vast possibilities in environmental protection. The Biolog plates can be used as a rapid method for estimating changes in soil microbial communities in response to short or long-term exposure of soil to contamination. The method enables evaluation of adverse changes in microbial communities on the basis of differences in metabolic response between communities from contaminated and control areas. Such measures as AWCD give information about the overall changes in total microbial abundance and/or activity in response to pollution, while CLPP allows us to disclose effects on microbial functional diversity.

Reduction of biodiversity often indicates extinction of sensitive species as a result of contamination [37, 38]. Such results can point out the necessity of reclamation of contaminated soils. Effects of reclamation management on soil microbial communities may also be estimated with the Biolog plates technique [13].

What Exactly Does the Biolog Technique Investigate?

One question still remains unanswered – is the Biolog method indicator of a function or a structure of a microbial community? Garland and Mills [27] claim that techniques connected with community analysis are good indicators of both of these parameters. In the case of the Biolog plates the information about the community structure comes from the characteristic pattern of substrate utilization, while analysis of microbial growth rate and substrate utilization in particular wells informs about microbial community function. On the other hand, Preston-Mafham et al. [17] stressed that “community function” implies actual catabolic activity. In fact, the Biolog method indicates potential, but not actual, catabolic activity of a community [11]. Positive response in wells on a plate indicates the ability of a community to adapt metabolism to an artificial (*in vitro*, not *in situ*) environment [17, 39].

It is important to notice that the problems and ambiguities connected with the use of the Biolog technique are numerous. Because of the huge microbial species diversity, different nutrient request and complicated between-species interactions it is still not known precisely which microorganisms contribute to substrates utilization on a plate. Probably mainly fast-growing bacteria, which are adapted to high substrate concentrations, participate in

tetrazolium dye reduction on bacterial plates, especially if low density solution is inoculated, for example 10^4 CFU/ml (CFU – colony forming units) [28, 40]. Slow-growing bacteria, incapable of growing in such conditions, are not usually included in the analysis [40].

There is also a problem of different ability of bacteria to utilize the substrates that a plate contains. Glimm et al. [41] noticed that an assortment of substrates does not necessarily reflect substrates which are available to bacteria in the soil environment, so one can suspect that some microbial species are incapable of growing on plates because of lack of proper substrates.

In turn Heuer and Smalla [25] inform that *Clavibacter michiganensis* grows in plate wells but reduces tetrazolium dye only in the case of high substrate concentration (excess of energy). Additionally, not all bacteria are able to reduce tetrazolium dye [42]. It seems that a number of studies have confirmed the hypothesis that physiological profile obtained on a plate is a result of activity of only a part of microbial community and therefore one should be very careful when drawing conclusions about the structure or function of a whole community.

The most accurate statement is that the Biolog method investigates functional diversity [33] or metabolic potential [43] of that part of a community which is capable of being metabolically active and growing in plate conditions.

Problems Concerning the Use of the Biolog Method

Extraction of Microorganisms

In order to extract microorganisms soil samples are shaken in suitable solutions. Extractants like phosphate buffer, Tris buffer and 0.9% NaCl solution are the most often used [13, 14, 24]. Kelly and Tate [13] claim that commonly used phosphate buffer is not a suitable solution for extraction of soil with high zinc content because phosphate reacts with zinc and precipitation hampers absorbance measurements and gives false readings. They suggest using the Tris buffer, which does not react with zinc. Physiological profiles of microbial communities can differ from each other because of the use of different buffers, probably due to the fact that phosphate buffer is also a source of nutrients for microorganisms [13]. Thus it is important to pay attention to the extraction method when comparing physiological profiles. Another question is if the extraction of microbes from soil is effective enough and enables preparing inoculum representative for the original microbial community [44]. Probably the extraction is biased towards organisms that are readily extractable from soil [45].

Impact of an Inoculum Density

Standardization of the amount of microorganisms in inoculum before inoculation onto a plate is not necessary

when general metabolic response of microorganisms, for example, after exposure to contaminants is studied, or when communities from different types of soil are compared. But if understanding of the dynamics of a community composition is the goal, standardization of inoculum density becomes a necessity [31]. Inoculum density affects AWCD and kinetic analysis parameters (λ , μ_m , A) [32]. Estimates of the number of microorganisms in a solution can be done by counting CFU on agar plates, with the use of dyes and epifluorescence microscopy and with the use of redox dyes. The degree of dye reduction is useful in evaluating the number of active cells which reduce the dye. The amount of active cells is better correlated with overall colour development on plates than the total amount of cells [31].

The number of microorganisms is fixed on a certain level (for example 3×10^8 cells/ml) by suitable dilution of soil solution and then such standardized inoculum is inoculated into plate wells [23]. However, diluting soil solution can lead to the loss of rare members of a community [32]. Therefore, dilution should be limited to a needful minimum. Estimating the number of microbes before inoculation makes the Biolog method a time-consuming technique. Another way to cope with different solution densities is normalization of data obtained from absorbance measurements or of parameters received from kinetic analysis. Effective kinetic analysis demands normalization of the data (or inoculum density standardization) because, for example, an increase in the amount of microorganisms in inoculum causes a decrease of λ value and an increase of μ_m and A values [31]. Normalization is done by dividing kinetic parameters (λ , μ_m , A) for individual wells by the mean values of respective parameters for a whole plate. In turn, absorbance values of particular wells can be normalized by dividing them by the AWCD [32]. Dividing by the mean is useful only for samples of similar AWCD, because plates of different AWCD usually show different numbers of positive responses. This is what limits the normalization of the data by dividing by the mean [32]. Normalization of data does not eliminate the impact of inoculum density completely, but reduces it significantly [41].

Garland [32] claims that analysis of measurements of chosen, reference AWCD, is the least sensitive to impact of inoculum density. On the other hand, analysis of the data of reference AWCD is significantly influenced by greater than 10-fold dilution of inoculum.

Although Winding and Hendriksen [40] and Lawley and Bell [46] did not notice the impact of inoculum density on kinetic parameters, this may be due to the fact that the amount of only one bacterial species (*Escherichia coli* or *Pseudomonas fluorescens*), not the amount of a whole heterotrophic community, was changing in their studies. This implies that physiological profiles are insensitive to changes in an inoculum density caused by a single species. Additionally, Haack et al. [23] found that dependence of the rate of colour development on the amount of bacterial cells in inoculum is not clear because of the complicated

character of metabolic response. In other words, metabolic response is influenced by effects of both the cooperation and the competition between microorganisms.

Application of the Biolog Plates Technique in Environmental Studies

The Biolog plates method is used inter alia for studying metabolic response of microbial communities from rhizosphere and non-rhizosphere soil [47], communities from different parts of the rhizosphere (along the root) [14], and from the rhizosphere of different plant species [48]. It is also applied for estimating the influence of different land use [15] or application of sewage sludge [49], and for estimating compost maturity [30]. The Biolog plates technique is more and more frequently used for evaluating impact of stressing factors such as heavy metals [50] or hydrocarbon contamination [51], high salinity and high soil pH [52] or heating [53].

Because of the problems connected with the use of the Biolog plates, this method probably poorly characterizes soil microbial communities. For this reason the Biolog method should not be applied to microbial community characterization but rather to comparative research, e.g. for comparing functional diversity of microbial communities from contaminated and non-contaminated soils. In this way the Biolog plates may be used in studies on pollution-induced community tolerance (PICT) of polluted soils. This kind of research is useful in environmental risk assessment (ERA) [54].

Alternative Methods for Investigating Physiological Profiles of Microbial Communities

In the response to drawbacks of the Biolog method a few new techniques for investigating microbial community physiological profiles were proposed. Degens and Harris [55] suggested studying of the catabolic response of the microbial community to the addition of several dozen carbon substrates directly to separate soil samples. The response was measured as CO₂ efflux with a gas chromatography. Direct and rapid measurements of CO₂ production or O₂ consumption instead of indirect (tetrazolium dye reduction) assessment of respiration enable reduction of incubation time of soil [56]. This method does not require extraction of soil, so there is no problem with inefficient extraction and inoculum density. The problem of culturability of microorganisms disappears, because culturing of microbes is not necessary when adding substrates directly to soil. The response of the microbial community is usually measured after 0-6 h and relies rather on indigenous microbial activity than on microbial growth, unlike the Biolog plates [45, 55]. Furthermore, Degens [39] claims that utilization of a particular substrate often demands the presence of multiple organic compounds (soil organic matter). This requirement is not implemented in

case of the Biolog method because every substrate is in a separate well on a plate. Thus, catabolic profiling proposed by Degens avoids many problems related to the use of the Biolog plates, but it still requires high substrate concentrations which can inhibit some species or cause substrate-accelerated death [44, 45]. Additionally, the necessity of manipulating a lot of containers with soil makes the method laborious and inconvenient [45].

Another way to measure catabolic potential of microbial community and avoid the limits of the Biolog technique is using of the deep-well microtiter plates method (MicroResp method) proposed by Campbell et al. [45]. The depth of 96 wells on a plate made possible using whole soil samples, unlike in the Biolog method. Two methods to detect the evolved CO₂ were used. The first one relies on a colorimetric reaction in absorbent alkali and the second one is connected with radioactive carbon sources and scintillation counting. The merit of the radioisotope method is that it is possible to ascribe CO₂ evolved to decay of the substrate. The MicroResp method has the advantages of the Degens' catabolic profiling technique [55] but does not require handling numerous soil containers or jars. On the other hand, costs may constrain the use of the method.

Garland et al. [56] proposed community-level physiological profiling on microplates with the use of fluorometric detection of oxygen consumption as a measure of microbial activity. The method has the advantage that 10 to 100 times lower concentrations of substrates are needed to obtain detectable responses of microbes. The required incubation time (24h) is shorter than incubation time with the Biolog plates but longer than in CO₂ monitoring approach. The drawback is that soil suspensions were inoculated into plate wells instead of a whole soil. Additionally, the method seems to be expensive because it requires specialistic equipment to detect O₂.

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